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Table of Contents

Session 1:
Food Safety and Monitoring of Safety Aspects **1**

Influence of antimicrobials on starter cultures in milk – model trials 3
G. Suhren

Impact of bacterial spores on food safety 10
R.G.K. Leuschner, A. Darke, P.J. Lillford

Halotolerant histamine-forming bacteria isolated during the ripening of salted anchovies 16
M. Hernandez-Herrero, A.X. Roig-Sagues, E.I. Lopez-Sabater, J.J. Rodríguez-Jerez, M. Teresa Mora-Ventura

Results of a feeding study concerning the carry over of toxaphene into laying hens and eggs 22
K.H. Schwind ,M. Kaltenecker ,K.H. Ueberschär

Carry-over of diethylhexylphthalate and aromatic nitro compounds into the milk of lactating cows 25
A. Blüthgen, U.Ruoff

Carry-over of polychlorinated dibenzo-para-dioxins into milk and prevalence in other food of animal origin in Germany 33
U. Ruoff, A. Blüthgen

Toxin production of *Bacillus cereus* isolates from a milk powder plant 40
C. Wiebe, P. Hammer

Chiral amino acid analysis related to food quality and food authenticity 45
T. Erbe, K. Thummes, H. Brückner

Application of two novel PCR assays for the rapid detection and discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group 52
F. von Stetten, K.P. Francis, S. Scherer

Multiplication of Staphylococcus (S.) aureus and production of enterotoxins during the experimental manufacturing of Camembert cheese 57
G. Hahn, M. Müller

Antibody capture ELISA for the sensitive direct detection of heat-stable toxin of Escherichia coli from soft cheese 65
G. Hahn, B. Bonniksen

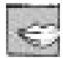
Influence of sample sizes and processing of milk powder for the isolation of Salmonellae 69
G. Hahn, U. Schleert

A post-modern food control labs network in the Argentine Northwest 74
C.A. Andrada, R. Sánchez

Detection of residues of quinolones in milk 77
G. Suhren and P. Hammer

Proficiency testing of microbial inhibitor tests 82
G. Suhren, J. Reichmuth

Characterization of microbial transglutaminase 88
W. Drewes, F. Schwägele



Session 2: Food, Nutrition and Well Being 91

Biogenic amines and the quality of food 93
J. Kirschbaum, I. Busch, S. Flassig, A. Meier, H. Brückner

Determination of amino acids and biogenic amines in oriental fermented food and beverages for quality assessment 98
I. Baek, C. Weitkamp, T. Erbe, A. Meier, J. Kirschbaum, H. Brückner

Reducing sugars, vitamin C and phenol compounds in potato tubers of various cultivars in Poland 103
M. Mikos-Bielak, R. Czeczko, F. Kluza, B. Rudzinska, W.E.L. Spiess

Reducing sugars, vitamin C and phenol compounds in potato tubers cultivated with new synthetic growth regulators application 107
M. Mikos-Bielak, R. Czeczko, F. Kluza, B. Rudzinska, W.E.L. Spiess*

The effect of industrial emissions in the Stalowa Wola region on heavy metals content in a potato tuber 113
M. Mikos-Bielak, R. Czeczko, R. Karwacinski

Oxidative stability of ω3-rich oil from *Camelina sativa* 121
D. Ní Eidhin, D. O'Beirne

Selected probiotic dairy products in Germany - microbiological and physico-chemical parameters 125
G. Klein, W.-R. Stenzel, G. Reuter

Improvement of sensory and toxicological characteristics by N-Acetylcysteine using a canned sausage model system 127
M. Gibis, A. Schoch, A. Fischer

Carry over of additional vitamin E amounts from feeds into food of animal origin 135
G. Flachowsky, A. Berk

Determination of oligosaccharides in soy bean products using an evaporative light-scattering detector 143
A. Jaworski, H. Brückner

Analysis of vitelline membrane proteins of fresh and stored eggs via HPLC 147
A. Schäfer, W. Drewes F. Schwägele

Correlations of anticarcinogenic conjugated linoleic acid with other C18 fatty acids in German bovine milk fat 150
D. Precht, J. Molkentin

Modulation of lipids of brain, heart and liver by dietary rapeseed oil as compared to olive oil and high-oleic sunflower oil 159
N. Weber, K.D. Mukherjee

Cholesterol reduction in culture fluids by lactobacilli 165
H. Wagner

Lipase-catalyzed synthesis of designer lipids with improved nutritional properties 167
K.D. Mukherjee

Non digestible oligosaccharides are promising functional foods 170
J. van Loo

Effect of Copper in the Food Chain on Human Health (FOODCUE) 179
J. O'Connor

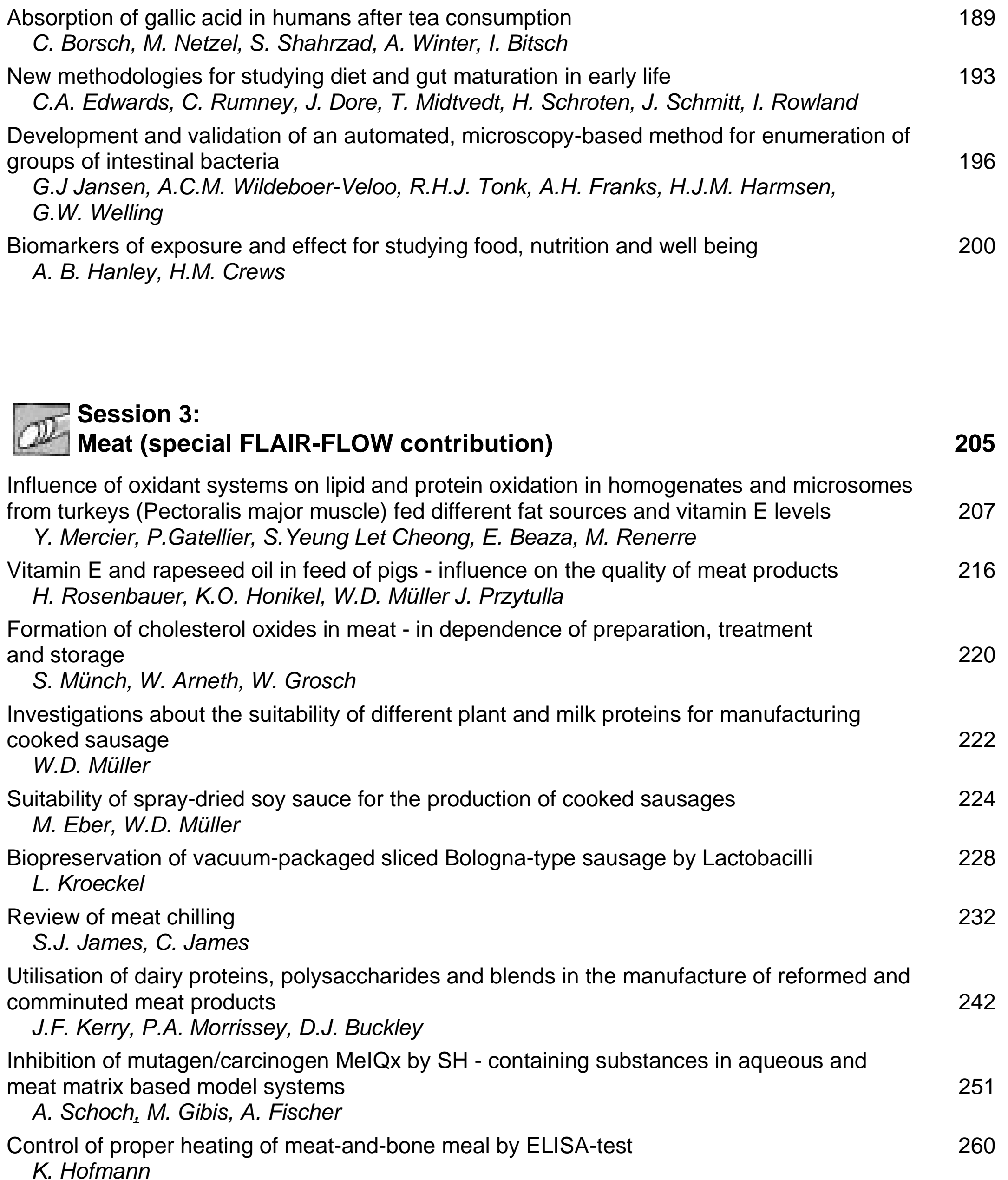
Urinary excretion of anthocyanins by one male volunteer after ingesting blackcurrant juice 183
M. Netzel, M. Janßen, I. Bitsch, R. Bitsch

Absorption of gallic acid in humans after tea consumption 189
C. Borsch, M. Netzel, S. Shahrzad, A. Winter, I. Bitsch

New methodologies for studying diet and gut maturation in early life 193
C.A. Edwards, C. Rumney, J. Dore, T. Midtvedt, H. Schroten, J. Schmitt, I. Rowland

Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria 196
G.J Jansen, A.C.M. Wildeboer-Veloo, R.H.J. Tonk, A.H. Franks, H.J.M. Harmsen, G.W. Welling

Biomarkers of exposure and effect for studying food, nutrition and well being 200
A. B. Hanley, H.M. Crews



Session 3: Meat (special FLAIR-FLOW contribution) 205

Influence of oxidant systems on lipid and protein oxidation in homogenates and microsomes from turkeys (Pectoralis major muscle) fed different fat sources and vitamin E levels 207
Y. Mercier, P.Gatellier, S.Yeung Let Cheong, E. Beaza, M. Renerre

Vitamin E and rapeseed oil in feed of pigs - influence on the quality of meat products 216
H. Rosenbauer, K.O. Honikel, W.D. Müller J. Przytulla

Formation of cholesterol oxides in meat - in dependence of preparation, treatment and storage 220
S. Münch, W. Arneth, W. Grosch

Investigations about the suitability of different plant and milk proteins for manufacturing cooked sausage 222
W.D. Müller

Suitability of spray-dried soy sauce for the production of cooked sausages 224
M. Eber, W.D. Müller

Biopreservation of vacuum-packaged sliced Bologna-type sausage by Lactobacilli 228
L. Kroeckel

Review of meat chilling 232
S.J. James, C. James

Utilisation of dairy proteins, polysaccharides and blends in the manufacture of reformed and comminuted meat products 242
J.F. Kerry, P.A. Morrissey, D.J. Buckley

Inhibition of mutagen/carcinogen MeIQx by SH - containing substances in aqueous and meat matrix based model systems 251
A. Schoch, M. Gibis, A. Fischer

Control of proper heating of meat-and-bone meal by ELISA-test 260
K. Hofmann

Session 4: Technological Methods to Improve Food Quality 265

Protein hydrolysis during the ripening of salted anchovy (*Engraulis encrasicholus* L.). An objective method to assess the ripening process. 267
M. Hernández-Herrero, A.X. Roig-Sagués, E.I. López-Sabater, J.J. Rodríguez-Jerez, M.T. Mora-Ventura

Hazard Analysis and Critical Control Point system in apple juice processing 274
G. Díaz, A. Saavedra, P. Cuevas

Controlling temperature during distribution and retail 284
A.J. Gigiel, S.J. James, J.A. Evans

Modelling of food refrigeration systems 293
S.J. James, I. Schofield

Food freezing and thawing time prediction with new simple calculation formulas application 302
F. Kluza, W.E.L. Spiess, D. Góral

Ice crystal size analysis in frozen model food gels 310
B. Woinet, J. Andrieu, M. Laurent

Modelling of quality attribute variations in food under heat treatment conditions. Important aspects. 312
I. Körmendy, L. Mészáros

Validation of food pasteurisation treatments using an amylase time-temperature integrator 322
G. Tucker P. Richardson

Optimal control of microwave combination ovens for food heating 328
B. Nicolaï

Determination of the total water content of foodstuffs by variations of the Karl Fischer titration 332
H.D. Isengard

Use of cellular systems to characterise plant tissue behaviour 337
M. Ferrando, W.E.L. Spiess

Improvement of overall food quality by application of osmotic treatments in conventional and new processes 345
D. Behsnilian, W.E.L. Spiess

Osmodehydrofreezing to improve frozen kiwifruit quality: The influence of raw fruit texture 353
D. Torreggiani, E. Forni, A. Maestrelli, G. Bertolo

Case hardening in osmotic dehydration of fruits. Effect of vacuum impregnation 363
J.M. Barat, A. Chiralt, P. Fito

Vacuum impregnation: A useful tool in candied fruit/vegetables processing 371
J.M. Barat, R. Lloría, A. Chiralt, P. Fito

BARO-Thermal processing to improve feeding value of faba bean – rapeseed concentrate 377
L. Moscicki, S. Matyka, J. Jaskiewicz, J. Pokorny

Combined microwave and air drying of apple (var. Granny Smith) 383
M.E. Martín, M.L. Vázquez, N. Martínez-Navarrete, A. Chiralt, P. Fito, J.M. Catalá, E. de los Reyes,

Modification of technological and nutritional properties of pea by germination and microwave heating 388
P. Kadlec, A. Rubecova

Pasteurisation and sterilisation with pulsed electric fields 391
P. de Jong, J. Meeuwisse, E.J.M. van Heesch, H.C. van der Horst

Use of computational models to study inhomogenities in electrical field processing of foods 394
L.J. Davies, M.R. Kemp, P.J. Fryer

Effect of low-dose gamma irradiation on *Listeria monocytogenes* inoculated onto pre-cut packaged white cabbage and radish 402
L. Mészáros, J. Farkas, Cs. Mohácsi-Farkas, É. Andrássy, T. Sáray, K. Horti

Review of decontamination techniques 410
C. James, S. J. James

Phase transitions in rectified grape must. Influence of pectin. 420
J. Martínez-Monzó, N. Martínez-Navarrete, P. Talens, A. Chiralt, P. Fito

Scuffing - Resistance of glass bottles to internal pressure and shock impact 426
S. Jirikovsky, H. Weisser

Development of ripening in manchego cheese as affected by salt content 435
Ch. Gonzalez, N. Martínez-Navarrete, A.Chiralt

Thermal diffusivity of tomato paste 443
S. Tavman, M. Sakin, I.H. Tavman

Thermal conductivity measurements of granular and powdered foods 451
S. Tavman, P. Yolci

Determination of heat inactivation kinetic parameters of a *bacillus cereus* pathogenic strain isolated from foods 461
A. Fernandez, M.J. Ocio, P.S. Fernandez, A. Martinez

Application of computational fluid dynamics (CFD) to hygienic design of food processing equipment 467
J.E. Hall, G.S. Tucker, P.S. Richardson

Improvement of the microbial safety during the processing of frozen fish from extra-European exporting countries 472
M.E. Guerzoni, A. Gianotti

Effects of novel mild preservation technology on the growth of *Listeria monocytogenes* on ready-to-use lettuce 475
G.A. Francis, D. O'Beirne

Session 5: Consumer Perception and Transfer Strategies **481**

The optimization of sweet taste quality (TOSTQ) 483
G.G. Birch

Water sorption and plasticization effect in breakfast cereals. Changes in texture 485
N. Martínez-Navarrete, R. Pedro, J. Martínez-Monzó, A. Chiralt

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Session 1:

Food Safety and Monitoring of Safety Aspects

Influence of antimicrobials on starter cultures in milk – model trials

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Abstract

In addition to the toxicological significance of residues of antimicrobials in food from animals in the case of milk the technological aspect is of great economic importance. In model trials with yoghurt and mesophilic starter cultures for numerous antiinfectives those concentrations were derived from dose/response curves which caused defined inhibition effects with respect to the values of the corresponding negative controls (20% and 50% deviation in L(+)-, D(-)-lactate-, amino N-content, differences in pH value and bacterial counts). Most often, yoghurt cultures reacted more sensitive than the mesophilic starter culture. Significant differences to the corresponding negative control were stated with yoghurt culture for the following parameter at EU MRL concentrations: L(+)-lactate: penicillin, tylosin; D(-)-lactate: oxytetracycline, spiramycin, tylosin; amino N: penicillin, spiramycin; pH value: penicillin, cloxacillin, oxytetracyclin, spiramycin and tylosin.

From these experiments it can be concluded that starter cultures applied in dairy technology are influenced in the biochemical activity by residues of antibiotics on MRL level. Therefore, the testing of milk with methods capable of detecting residues of antimicrobials at least at or below the MRL level is necessary also from a technological aspect.

1 Introduction

The toxicological evaluation of residues of antimicrobials in food include pharmacological-toxicological, immunpathological and microbiological risks. The assessment of microbiological risks include mainly pathogenic microorganisms.

In case of the foodstuff milk besides the toxicological evaluation the aspect of technological safety is of great importance. The purpose of model trials with commercially available yoghurt culture was to evaluate whether the Maximum Residue Limits (MRLs) of antimicrobials which are fixed according to EU-Regulation 2377/90 ff ensure also technological safety.

Worldwide production of cheese and fermented milk products is at 14 mio metric tons and 100 mio metric tons per year, respectively. For this, approximately 2.5 mio tons of starters are needed (1). These figures indicate which large economic losses can occur if the activity of starter cultures is impaired by residues of anti-microbially active substances.

In this paper, model studies are described with two yoghurt cultures and one mesophilic starter culture with selected antimicrobially active substances in which, in a first trial, defined inhibitory activity is determined and in a second trial, the influences of residues at EU-MRL level are tested.

2 Experimental design

Antimicrobials: Penicillin G, ampicillin, cloxacillin, dicloxacillin, oxacillin, ceftiofur[1], dapson, sulfadimidine (= methazine), oxytetracycline , erythromycin, spiramycin, tylosin, DHstreptomycin, gentamicin, neomycin, enrofloxacin, trimethoprim.

Starter cultures: Yoghurt culture V 709 and V2 (Wiesby, Niebüll, Germany); mesophilic homofermentative culture, O-culture (R-607, Chr. Hansen's Laboratory, Lübeck, Germany).

Milk: Inhibitor free from the experimental herd of the Federal Dairy Research Centre; skimmed by centrifugation, heat-treated 15 min at 80°C, addition of 3 % mother culture, mixing and distribution into Erlenmeyer flasks, addition of antimicrobial/concentration desired, and bottling à 10 ml.

Incubation: In a waterbath at 42°C until the negative control reaches pH 4.5.

Sampling: Several times during incubation period, different for the measuring parameters

Parameter: pH value, L(+)-lactate (2), D(-)-lactate (2), amino N content (3), colony counts (M17, MRS) (4) and microscopic count (Breed) (5).

Experiment 1: Concentrations tested: 0, MRL, 10•MRL and 100•MRL; 1 trial.

Experiment 2: Concentrations tested: 0 and MRL; 3 independent trials.

3 Results

3.1 Experiment 1: Determination of defined inhibitory activity

From **figure 1** it becomes evident that the chemotherapeutics sulfadimidine, dapsone and trimethoprim even in concentrations of 100•MRL do not have marked influence on the development of pH value. These substances do not seem to be of importance for technological reasons. In contrast to this oxytetracycline at MRL concentration is of pronounced influence on the development of the pH value; in milk with concentrations 10•- and 100•MRL the pH value just changed slightly during the incubation period.

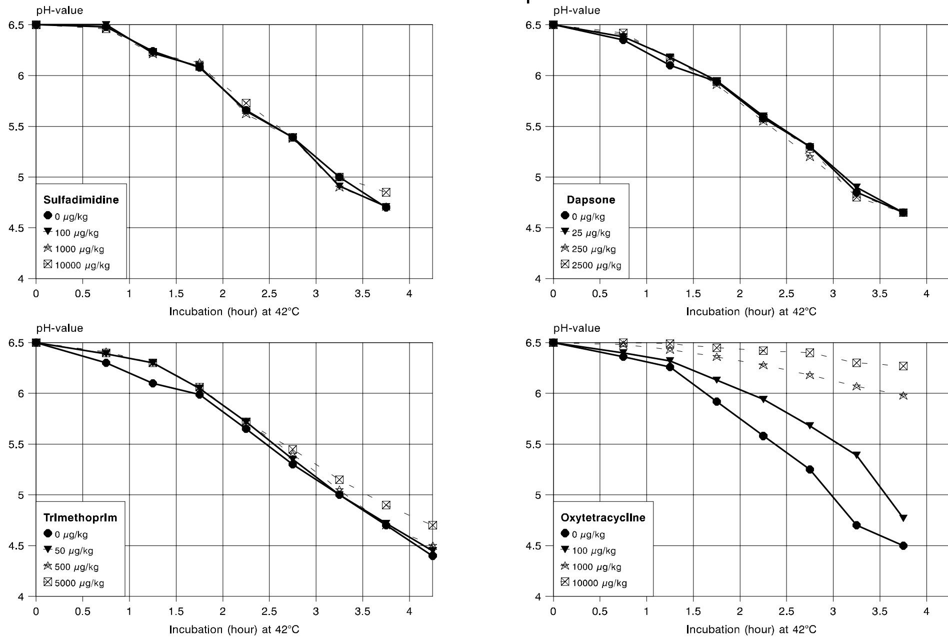
Figure 2 demonstrates that as well β-lactam antibiotics (penicillin G, cloxacillin) as also the macrolides (spiramycin, tylosin) inhibited – under technological aspects – nearly totally the decrease of pH value at concentrations of 10•MRL. The influence of MRL levels differed for the tested substances.

Figure 3 shows the influence of penicillin G on bacterial counts. After an incubation period of 2.75 hours for all tested concentrations as well the number of streptococci as of lactobacilli was less than in the corresponding negative controls; at concentrations of 10•- and 100•MRL microscopic counts were much higher than colony counts.

From graphs the lowest inhibitory concentrations – independent of the incubation period – are derived at which a marked inhibition (20 or 50 % respectively) compared to the negative control was observed. **Table 1** summarizes those antiinfectives and parameters at which inhibitory activities at < MRL level were observed. In particular with ceftiofur (only mother compound) and oxytetracycline, inhibition was observed at concentrations significantly below the MRLs.

[1] only mother compound

Influence of antimicrobials on yoghurt culture - model trials *)
Parameter: pH-value

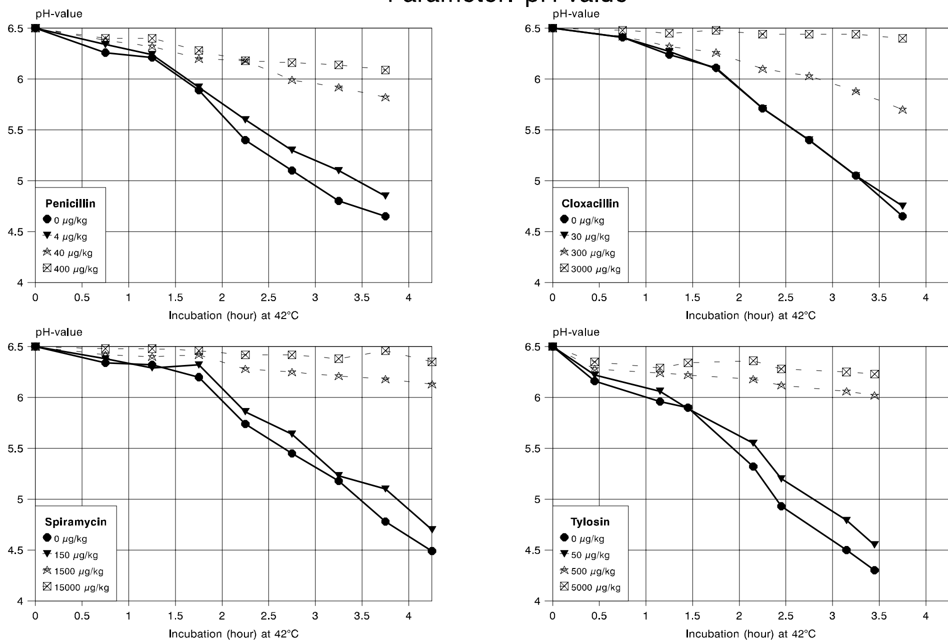


*) Culture Visbyvac 709

6_34393J

Figure 1

Influence of antimicrobials on yoghurt culture - model trials *)
Parameter: pH-value

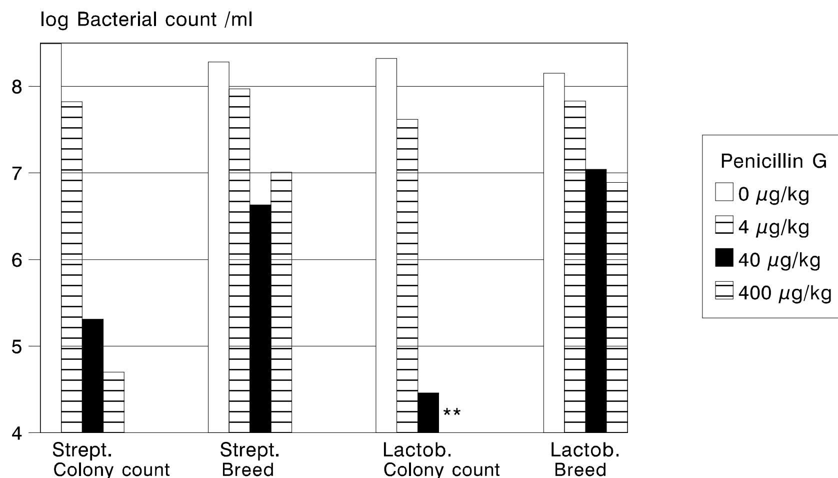


*) Culture Visbyvac 709

6_3449

Figure 2

Influence of antimicrobials on yoghurt culture - model trials
Parameter: Bacterial count (2.75 hours) Antimicrobial: Penicillin G



(*) Culture Visbyvac 709 **) < log 4,0 /ml

Figure 3

Table 1: Defined inhibition at ≤MRL level

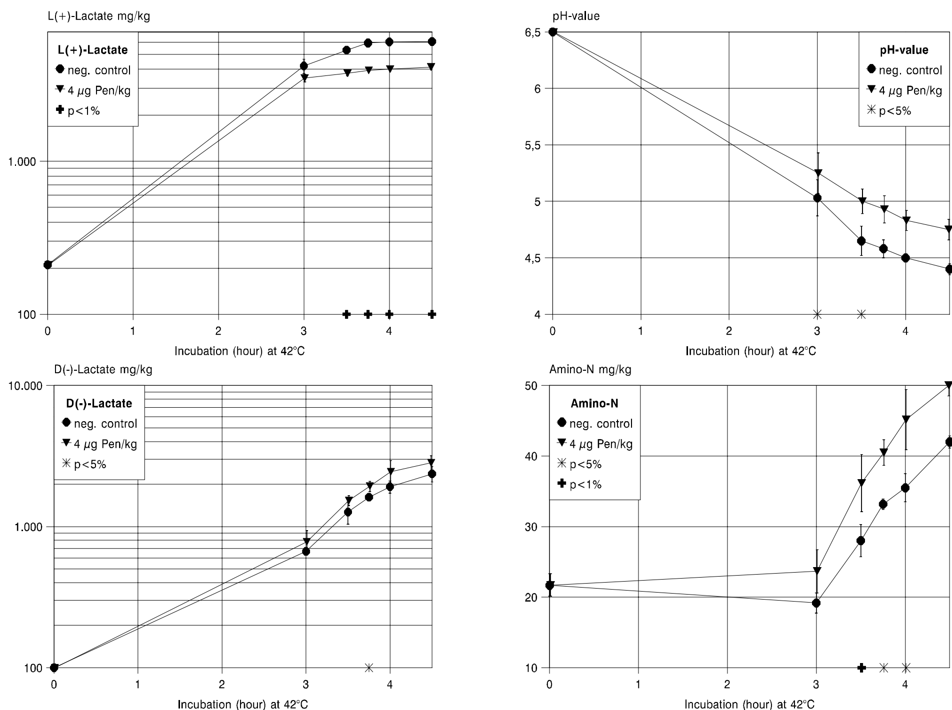
Parameter	Yoghurt culture V2	Yoghurt culture V709	Mesophilic starter
L(+)-lactate	Ampicillin, oxacillin, ceftiofur[1)], oxytetracyline, erythromycin, spiramycin, tylosin	Penicillin, cloxacillin, oxytetracycline, spiramycin	Oxytetracycline
D(-)-lactate	Oxacillin, ceftiofur[1)], oxytetracyline, erythromycin, spiramycin, tylosin, gentamicin	Oxytetracyline, spiramycin, tylosin	
Amino N	Penicillin, ceftiofur[1)], oxytetracyline, erythromycin, spiramycin	penicillin, cloxacillin, oxytetracyline, spiramycin	Oxytetracyline, Tylosin
pH Value	Oxacillin, ceftiofur[1)], oxytetracyline, erythromycin, spiramycin	penicillin, cloxacillin, oxytetracyline, spiramycin, tylosin	Oxytetracyline
Streptococci	Ceftiofur[1)]		
Lactobacilli	Ceftiofur[1)]		

[1)] only mother compound

3.2 Experiment 2: Influence of antimicrobials at MRL level

At MRL concentration level, the tested chemotherapeutics – sulfadimidine, dapsone and trimethoprim – do not have a significant effect on starter cultures whereas for all tested antibiotics – penicillin, cloxacillin, oxytetracycline, spiramycin and tylosin – significant differences to the negative control were found depending on the antimicrobial, starter culture and measurement parameters. During the trials with yoghurt cultures, penicillin, for example, impaired especially L(+)-lactate formation (**fig. 4**) whereas spiramycin slowed down the D(-)-lactate formation (**fig. 5**). With the exception of oxytetracycline, yoghurt culture V709 proved to be more frequently significantly sensitive than yoghurt culture V2 and the mesophilic starter culture.

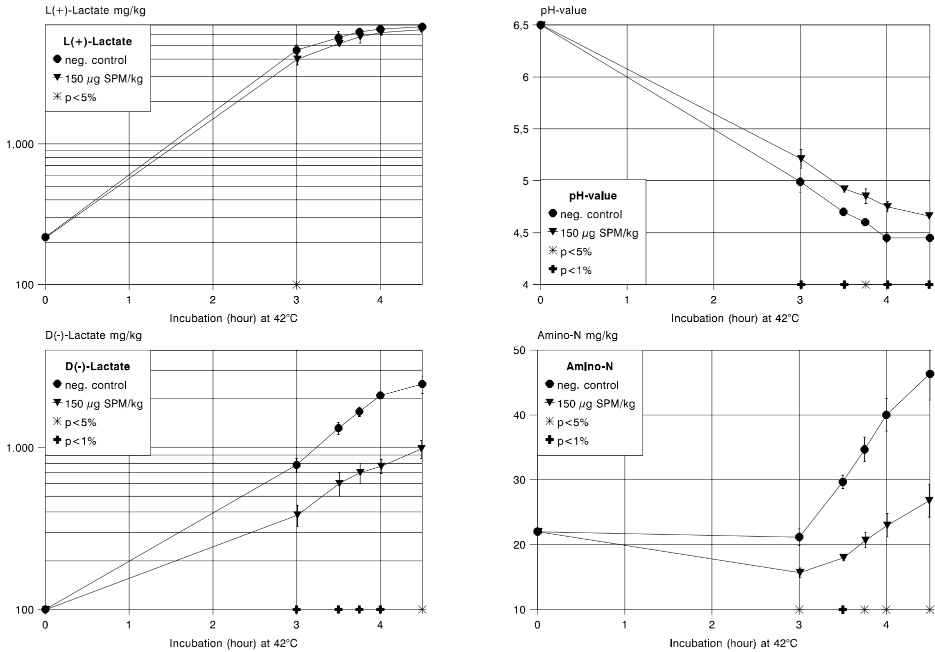
Influence of penicillin G (MRL: 4µg/kg) on yoghurt culture - model trials *)



*) Culture Visbyvac 709

Figure 4

Influence of spiramycin (MRL: 200 μg/kg) on yoghurt culture - model trials *)



*) Culture Visbyvac 709

Figure 5

Conclusions

The model trials were carried out with two yoghurt and one mesophilic starter culture under standardized conditions. The inhibitory activities were defined, and therefore comparable, for these trials for different measurement parameters. They show highly specific results for the individually tested antiinfectives, and results that can be evaluated individually for all possible combinations.

In most cases, yoghurt cultures reacted more sensitive to residues of antiinfectives than the mesophilic starter cultures as also reported by Mäyrä-Mäkinen(1). An exception to this, for example, is the inhibition of the L(+)-lactate formation by residues of oxytetracycline: yoghurt culture V709 and the mesophilic starter show a comparable sensitivity regarding this parameter whereas the second tested yoghurt culture reacts significantly less sensitive to oxytetracycline.

The tested chemotherapeutics – sulfadimidine, trimethoprim, dapson and enrofloxacin – show an inhibition activity only at very high concentrations far above the MRL level. For mesophilic starter cultures, the L(+)-lactate and amino N formation as well as the reduction of the pH value are caused by a lower concentration of these chemotherapeutics than for yoghurt cultures.

Significant differences in comparison to the negative control were observed in milk containing antiinfectives at MRL concentrations for the parameter/antibiotic combinations listed in **Table 2**.

Table 2: Antiinfective/measurement parameters/culture combinations at which significant differences at MRL level were observed in reference to the negative control

	Yoghurt culture V709	Yoghurt culture V2	Mesophilic starters
L(+) lactate	Penicillin, tylosin	Penicillin, oxytetracyclin	Oxytetracyclin
D(-) lactate	Oxytetracyclin, spiramycin, tylosin	Oxytetracyclin, spiramycin, tylosin	not tested
Amino N	Penicillin, spiramycin	Spiramycin, tylosin	Oxytetracyclin
pH Value	Penicillin, cloxacillin, oxytetracyclin, spiramycin, tylosin	Oxytetracyclin, spiramycin	Cloxacillin, oxytetracyclin

Significant differences to antibiotic residues at MRL level were observed most often with yoghurt culture V709 whereas with the mesophilic starter culture, significant differences were observed almost only with oxytetracycline residues. Whereas cloxacillin at MRL level led to significant differences in only two cases (pH value with yoghurt culture V709 and mesophilic starter culture), penicillin, oxytetracycline, spiramycin and tylosin showed significant differences in comparison to the negative controls, which were tested in parallel, for numerous parameter/culture combinations.

From these experiments it can be concluded that starter cultures applied in dairy technology are influenced in their biochemical activity by residues of antibiotics at MRL level. Therefore, the testing of milk with methods capable of detecting residues of antimicrobials at least at or below the MRL level is necessary also from a technological aspect.

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Impact of bacterial spores on food safety

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Abstract

Dormant bacterial spores display a high heat resistance and have therefore the potential to survive food preservation measures. Temperatures around 70 °C which are applied for pasteurisation of foods and inactivate vegetative cells cause dormant spores to become activated. These spores are more susceptible to germinants which trigger their resuscitation. Germinated spores can outgrow and have the potential to spoil the food. Differential scanning calorimetry of spore samples reveals an endotherm at around 50 °C which has been attributed in the literature to 'heat activation'. This transition was studied and its relevance to heat activation is discussed. Heat activation of spores at 70 °C for 1 min or 30 min resulted in no measurable effects on ^{13}C and ^{31}P nuclear magnetic resonance spectra and the phase-bright appearance of dormant spores as seen by phase-contrast light microscopy was maintained.

Introduction

Spores of bacilli differ in their characteristic to resuscitate after exposure to favourable environmental conditions. Germination can occur instantly, after a certain lag time or might not be observed at all. The process responsible to enhance gemination and shorten the initial lag period has been called activation and is usually performed by exposing spores to heat at temperatures of 65 - 80 °C for 10 - 30 min. Heat activation does not equal the trigger mechanism because it solely does not lead to germination. To ensure the detection of the majority of dormant spores, heat activation treatments have been incorporated into national standard methods for bacterial spore enumeration in milk and milk products (Moran et al., 1990). Two methods are generally applied to measure the degree of activation. Both follow the increase in germination of a spore suspension in the presence of an appropriate nutrient, the first by spectrophotometry and the second by recording the percentage of spores which form colonies when plated on a suitable medium (Keynan et al., 1965).

In cases where heat activated spores are not exposed to a suitable nutrient they will revert with time to their dormant form. Therefore heat activation has been generally described as a reversible process.

However changes on spore structure in the literature have been described which as a whole might not be 100 % reversible. The effect of the sublethal heat treatment (70 °C for 20 min) on the spore structure has been examined by Fitz-James and Srivastava (1981) and was associated with an alteration of structural proteins and enzymes found in coats of *Bacillus cereus*. They describe three changes: an increased glycosylation of coat proteins, alteration in polypeptide pattern on sodium dodecyl sulfate - polyacrylamide gels, and an increase in free SH groups of proteins. About three polypeptides leaked out in small quantities from the spore coats during heat activation. Beaman et al. (1988) also reported an effect of heating (65 °C for 20 min) on spore permeability, heat resistance and density in buoyant gradients of *Bacillus stearothermophilus* spores. The spores were permeabilized as observed by release of dipicolinic acid and minerals, they were susceptible to

lysozyme action, permeation of the gradient medium and displayed subsequently changes in their structural appearance as detected on electron micrographs. Ultrastructural changes in the subcoat region associated with activation (65 °C for 30 min) of *B. cereus* spores were described by Hashimoto and Conti (1971). Yasuda and Tochikubo (1985) detected an alteration of the binding site of glucose after heat activation of spores of *B. subtilis* which had a sufficient and reproducible germination rate without preheating. They observed that the heat treatment (65 °C for 30 min) did not alter the binding sites for L-alanine and D-alanine. Their studies revealed a small release of dipicolinic acid during heating.

Thermal analysis by differential scanning calorimetry (DSC) has been used in investigating the effects of heating on spores. An endothermic transition of spore of *B. cereus* and *B. subtilis* in water around 40 - 60 °C was described by Belliveau et al. (1992) and Maeda et al. (1975). Consistently both authors report that the endotherm was not reversible at an immediate rescan, but recovered after storage of heated spores for six weeks at 4 °C and 5 days at room temperature, respectively. In a later paper Maeda et al. (1978), the endotherm was reported to be reversible at an immediate rescan. They explained the discrepancy between this and their earlier work to the use of a less sensitive and versatile instrument in their first study.

The endotherm occurs slightly below temperatures applied for heat activation. Therefore, its relevance for an increased germination rate of spores after sublethal heating was investigated by Maeda et al. (1975). They observed almost no germination without heat activation. Heated spores in a DSC pan after passing through the endotherm displayed an increased germination rate. But heating of spores in a water bath at 65 °C for 1 h showed a twice as high germinability. In their later paper they related the reversibility of the endotherm observed by DSC to the reversibility of heat activation of spores which was measured spectrophotometrically as a decrease of germination. In this paper they claim that the endotherm recovers at an immediate rescan (0.6 °C/min to 70 °C) after cooling with a rate of 0.6 °C/min. The authors heated parallel spores for 1 h at 60 °C and showed that in comparison with a rapid cooling thereafter a cooling rate of 0.1 °C/min decreased the germination rate and that this trend was enhanced by a storage of 7 and 14 days. Their work substantiate the already established knowledge that the DSC endotherm and spore activation are reversible processes as seen by differential calorimetry and germinability measurements.

The molecular and structural origins of the endotherm are still not clear. Belliveau et al. (1992) observed a low temperature endotherm in chemically decoated spores and concluded that the coat did not contribute. Maeda et al. (1978) report an endotherm in isolated coat material, which was reversible at an immediate rescan as seen for whole spores. The transition at heat activation temperatures seems not to be attributable to spore membranes because Vary and Skomurski (1984) observed a broad endothermic phase transition between 6 - 26 °C of inner membranes isolated from *B. megaterium*.

In summary, an endothermic transition, observable by DSC, appears to have two characteristics comparable to heat activation of bacterial spores. These are the occurrence of both events due to heating and their reversibility over time. The implication is, therefore, that the molecular events associated with heat activation might be partially indicated by a DSC cooperative endotherm, emplying some kind of a thermally triggered event. In this study, we have reinvestigated the behaviour of spores in the scanning calorimeter, and simultaneously measured changes in the molecular mobility of spore components by nuclear magnetic resonance.

Materials and methods

Spores were prepared from *Bacillus subtilis* PSBlux+ (provided by Dr. P. Hill, Nottingham University, UK) and *B. cereus* T CMCC 629 at 37 °C on Heart Infusion agar by sporulating cells and harvesting spores (spore slurry). The spore slurry was washed four times with distilled water. Freeze-dried spores were prepared from the slurry using a SB Multidrier. The spores were rehydrated over water in a sealed container and the water uptake (15 %) was measured gravimetrically. All spore preparations were kept at -21 °C. The physical state of the spores was examined by a Leitz Ortholux II phase-contrast light microscope. Differential scanning calorimetry was carried out using a Perkin Elmer DSC7 and a Setaram micro-DSC. The scanning rate was 10 °C/min (sample weight 5 - 8 mg) and 0.6 °C/min (sample weight 70 mg), respectively. Nuclear magnetic resonance spectra were obtained under magic angle spinning (MAS) conditions on a Bruker MSL 300 spectrometer. The spinning speed was usually 3.5 KHz. The excitation frequencies were 75.48 MHz and 121.497 MHz for ^{13}C and ^{31}P respectively. Both single pulse excitation (SPMAS) and cross polarisation (CPMAS) techniques were used. The former is more sensitive to molecules undergoing relatively rapid reorientation whereas the latter technique is more appropriate for the study of molecules with severely restricted mobility.

Results

Differential scanning calorimetry (DSC) with spore preparations of *Bacillus subtilis* and *B. cereus* was carried out. An endotherm in the temperature range around 50 °C was observed in both preparations. The peak had a start value of 32.5 °C (s.d. 4.9) and a maximum of 50 °C (s.d. 2.9) (n = 15).

The recovery behaviour of the 'heat activation' peak was investigated for *B. subtilis* spores which derived from the same spore crop. The peak was not observed at an immediate rescan but recovered over time (Table 1). After two hours storage at room temperature it displayed a maximum at 23 °C. After 5h the peak maximum was at 28 °C. After 5 days the transition was still at lower temperatures. 10 days after heat activation the peak maxima was very close to the initial scan features.

Table 1: Recovery characteristic of the low temperature endotherm of spores of *B. subtilis* (15 % moisture content) after heating to 70 °C in a Perkin Elmer DSC with a rate of 10 °C/min. After the first scan samples were store in the DSC pan at -21 °C and rescanned after 2 and 5 h and 5, 10 and 21 days.

Sample	Treatment	Low temperature endotherm (°C)	
		Peak start	Maximum
1	first scan	26	50
1	rescan after 2 h	13	23
2	first scan	34	50
2	rescan after 5 h	13	28
3	first scan	30	45
3	rescan after 5 d	13	39
4	first scan	25	45
4	rescan after 10 d	21	39
5	first scan	31	50
5	rescan after 21 d	29	43

Heating of similar spore samples to 120 and particularly to 160 °C prolonged the peak recovery time. After heat treatment of *B. subtilis* and *B. cereus* to 160 °C and storage for 1 month at -21 °C the low temperature endotherm displayed a peak start of 14 and 20 and a maximum of 24 °C and 31 °C, respectively.

The application of a scanning rate of 0.6 °C/min in the DSC 7 led to a detection of a very broad flat peak with an onset of 0.4 °C and a maximum of 25 °C. After cooling at 0.6 °C/min and reheating with the same rate, a similar peak was observed with an onset at 1.2 °C and a maximum of 25 °C. An analysis an aliquot of the same spore preparation of *B. subtilis* (70 mg) in a Setaram micro-DSC with a scanning rate of 0.6 °C failed to show a peak.

We reported earlier (Leuschner et al., 1998) that the low temperature endotherm which was observed in the DSC7 was not affected by an initial sample temperature of 0 or 20 °C. Recent results confirmed this finding. Scans were commenced at 25 °C instead of 0 °C without observing any effects on the low temperature endotherm. A pre-scan of a sample of *B. subtilis* to the peak maximum followed by an immediate rescan resulted in a shift of the peak start and maximum to a 5 °C higher temperature.

Nuclear magnetic resonance (NMR) analysis of *B. subtilis* spore preparations were additionally performed. The samples contained 10 % moisture or excess water. NMR ^{13}C and ^{31}P analysis revealed a similar content of 'mobile' (SPMAS spectrum) and 'immobile' (CPMAS spectrum) components in samples of both water contents.

Heat activation of the 10 % moisture preparation was carried out according to the heating protocol of the DSC7 with 10 °C/min. The sample was heated in the NMR rotor in the spectrometer to 70 °C and cooled. There were no measurable effects on the ^{13}C or ^{31}P NMR spectra when compared with an unactivated sample.

In a second experiment a thick spore slurry containing excess water was heat activated and held for 30 min at 70 °C. This process corresponded to the recommended and generally applied activation procedure of bacterial spores. The NMR spectra were acquired every hour at a temperature of 4 °C after heat activation and displayed again no measurable difference from unheated samples.

Our results indicate that NMR does not measure any effects on molecular mobility within spores due to heating. DSC analysis showed a melting transition with a small entropy which was close to the detection limit of the Perkin Elmer DSC7. No enhancement of the detection of this peak could be achieved when the sample amount was increased to 70 mg and was subjected to analysis with a Setaram micro-DSC. The combination of NMR and DSC gave a general insight on effects of heat on the spore but specific changes in molecular mobility within the spore structure were not identified.

Discussion

Our results concerning the low temperature endotherm are consistent with the cited literature in that the variability of the peak location and the area are comparable with those reported earlier. The traces of Beaman et al. (1992) showed a 'heat activation' peak between 40 - 60 °C. The peak area seems large, whereas in the publication by Miles et al. (1986) only a very small 'heat activation' peak of a spore slurry of *B. subtilis* is shown. In both studies spore slurries were used but the amount used is not fully comparable. In none of the published work was the peak area measured or

taken as a measure for interpretation of results. This implies that consistently researcher did not attribute significance to the size of the area.

In none of the research papers quoted has evidence been presented that the DSC peak measures a crucial structural event in the spore which is responsible for an enhanced germinability due to heat treatment. The DSC does show a small, not well reproducible peak which occurs even below the temperatures generally used for heat activation. Maeda et al. (1975) examined the dependence of heating a spore sample in the DSC on an increase of the germination rate. However, they detect the highest germination rate after heating for 1 h at 65 °C in a water bath. This finding questions the relevance of the DSC transition for heat activation since the activation effect is increased by longer heating times, suggesting that further modifications of spore structure occur after completion of the DSC transition. Such changes of structure on heating might be detected by a change in heat capacity for spore suspensions, but this was not obvious in our experiments or reported by other workers.

In the paper Maeda et al. (1978) the reversibility of the 'heat activation' peak is related to a decrease in germinability over storage time. The observed relationship indicates the occurrence of two events at the same time which are both reversible but no specific links were identified.

Observation of ^{13}C and ^{31}P NMR spectra of moist (10-15 % water) spores or excess water containing slurries show reasonable spectra by SPMAS and CPMAS. However heating spores to temperatures and times comparable with completion of the DSC endotherm show no special changes. This is in agreement with observations by light-microscopy. There is no sudden transition from phase-bright to phase-dark images coincident with completion of the endotherm. More prolonged heating (70 °C for 30 min) normally associated with full heat activation also produced no major changes in SPMAS or CPMAS spectra, neither were spores observed to become phase-dark or grey. These results are not coincident with a major change in the spore structure e.g. complete loss of dipicolinic acid and permeability of spores to water associated with electron micrograph of 'hydrated' sections of spores as reported by Beaman et al. (1988). Such a structural change would be expected to have major effects on NMR spectra, by increasing molecular mobility; and would also imply a change in refractility caused by hydration.

Despite the role the DSC transition plays in the events responsible for achieving the activated spore state by heat, it must originate from the bulk of solid material comprising the dormant spore. A similar low temperature endotherm has been described for low moisture biopolymers (Gidley et al., 1993) and synthetic polymers (Appelqvist et al., 1993). Unfortunately, its origin in these systems is equally unclear and needs further investigation. The fact that a similar low temperature endotherm as reported for spores has been seen ubiquitously in low moisture biopolymers indicates that there is not only one polymer in spores responsible for the transition. It can originate from any of the biopolymers present in the spore and can not be attributed to a distinct compartment.

The measurement of structural events on spores during heat activation by application of NMR and DSC resulted in no satisfactory explanation of an increase in germinability. The investigation of the heat activation phenomenon has to be approached with additional measurement techniques.

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Halotolerant histamine-forming bacteria isolated during the ripening of salted anchovies

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Abstract

This study was carried out to investigate halotolerant histamine-producing bacteria isolated during the ripening of salted anchovy. Of the isolates obtained during the ripening of anchovy 1.37% showed histamine-forming activity, most of them (70%) belonging to the *Staphylococcus* genus. *Staphylococcus epidermidis* showed a powerful histamine-forming activity, producing more than 1000 μg/ml in the presence of 3% and 10% NaCl. Another powerful histamine-producing bacterium isolated during the ripening of salted anchovy was *Staphylococcus capitis*. It was able to produce about 400 μg/ml of histamine in 10% NaCl under experimental conditions. Most of these species might be expected to be found as a result of contamination of fish during capture and subsequent unhygienic handling. However, no increase in histamine content was found in any batches through the ripening process. Histamine content always was acceptable in accordance with the maximum allowable levels of histamine fixed by the Spanish and European Union regulations.

Introduction

Ripened semi-preserved anchovies are prepared from fish of the *Engraulis encrasicholus* (L) species, belonging to C*lupeidae* family by a process of salting and ripening. This process goes back to ancient times and it is a common tradition in some Mediterranean countries and in Argentina, where the specie used is *Engraulis anchoita.* In the ripening stage the general character of the anchovy is greatly changed. The finished product acquires a soft consistency along with the development of a pink colour and a strong characteristic flavour (Triqui and Reineccius, 1995). The product obtained has a high value and it is very appreciated, being one of the most expensive seafood products of the Spanish market (Perez-Villarreal and Pozo, 1992).

Histamine poisoning (scombroid fish poisoning or scombrotoxicosis) is a foodborne chemical intoxication resulting from the ingestion of foods that contain high levels of histamine (Sumner and Taylor, 1989). Histamine toxicity is increased by the presence of other amines, such as putrescine and cadaverine that inhibit histamine-metabolizing enzymes in small intestine (Taylor and Sumner, 1986). Although the exact mechanism of histamine poisoning still remains uncertain, a high histamine content in fish flesh has long been considered the most probable cause of this type of food poisoning (Lopez-Sabater et al., 1996).

During the ripening of salted anchovy an important proteolysis is observed, with liberation of peptides and free amino acids. When free histidine is found in a sufficient quantity, it can be degraded by the microorganisms or their enzymes. Consequently, histamine may be formed at this time, and eventually reach toxic levels (Veciana-Nogues et al., 1989). As a consequence, the European Union has established regulations for fish and fixed a 3-class plan for maximum allowable

levels of histamine in enzymatically ripened fish products (n=9; c=2; m=200 ppm; M=400 ppm) from *Scombridae* and *Clupeidae* families (Anonymous, 1991). Spanish semi-preserved anchovies containing 680 mg/kg of histamine were also incriminated as responsible for an incident reported in the United Kingdom (Murray et al., 1982).

The accumulation of high histamine concentrations in salted fish could be due to poor quality of the raw material, to inadequate handling or to other causes during its shelf life (Rodriguez-Jerez et al., 1993). A large assortment of bacteria has been considered as powerful histamine formers in fish, and most of them are reported to be enteric bacteria (Middlebrooks et al., 1988). However, the relationship with the histamine activity in salted fish is due more probably to the presence of halophilic/halotolerant microorganisms (Yatsunami and Echigo, 1993).

The principal aim of this study has been to identify the halophilic/halotolerant bacteria with histidine decarboxylase activity present during the ripening of salted anchovy, evaluating their histidine decarboxylase activity under experimental conditions at four different NaCl concentrations (0.5, 3, 10 and 20%).

Material and methods

Fifteen batches of ripened semi-preserved salted anchovies (*Engraulis encrasicholus*) were analyzed. Fish were caught in the Northeastern Spanish Mediterranean coast. Samples were removed every week from the same processing batch until the ripening phase was finished. This ripening period was of 9 weeks. Regular counts of slightly and moderately halophilic/halotolerant extremely halophilic bacteria in halophilic agar were realized (Baross and Lenovich, 1992). After counts, five colonies were picked at random into plates of TSA plus 3%, TSA plus 10% NaCl and halophilic agar (25% NaCl), supplemented with 0.1% L-histidine and incubated at 30ºC for 48, 72 and 96 h, respectively.

Pure cultures were tested for histamine formation in Niven medium (Niven et al., 1981) containing 2% L-histidine, and 3, 10 or 25% NaCl and incubated at 30°C for 4 days. Positive strains were confirmed by inoculating a loopful from a pure culture into 10-ml tubes of trypticase soy broth (TSB) plus 3, 10 or 25% NaCl enriched with 1% L-histidine (TSBH) and pH was adjusted to 6.3. The tubes were incubated at 30°C for 48 h. Histamine formation was demonstrated by an enzymatic method (Rodriguez-Jerez et al., 1994a).

The histamine-forming activity of confirmed strains was evaluated using the Histamine Evaluation Broth (HEB) at four different NaCl concentrations (0.5, 3, 10 and 20%). HEB containing the following composition in distilled water was developed: Tryptone (1%), potassium phosphate (0.25%), histidine (1%), pyridoxal phosphate (0.01%), and NaCl (0.5, 3, 10 or 20%). The pH was adjusted to 6.3 and sterilized at 121°C for 15 min. Finally the broth was enriched with a dextrose solution (2%) sterilized throughout a 0.22 μm membrane, to avoid the formation of interfering Amadori compound between histidine and reducing sugars during sterilization (Pavelka, 1982). Histamine concentration was assessed by the aforementioned enzymatic method.

Identification to the species level was accomplished by a variety of biochemical tests using the PASCO identification system (Difco) for Gram negative isolates and API-Staph, API-50-CHB (BIO-Merieux, Marcy-l=Etoile, France) for Gram positive isolates.

The histamine contents of raw fish and ripened salted anchovy were determined by the aforementioned enzymatic method.

Statistical methods

Regression analysis was performed to obtain the correlation between variables. Analysis of variance and Kruskal-Wallis were performed, previously verifying normality and variance homogeneity, using the Statistical Package for Social Sciences (SPSS for Windows 6.1.2).

Results and discussion

A total of 2192 isolates were obtained from the different culture media during ripening of salted anchovy. Of the total of isolates 38.73% were presumptively classified as histamine formers from the results obtained in Niven medium. However, only 14.49% and 3.53% of theses isolates were confirmed in TSBH and HEB by the enzymatic method, respectively. As consequence, a high proportion (96.47 %) of suspected histamine formers isolated from Niven medium were finally considered as false positives. Our results confirm previous observations (Roig-Sagues et al. 1997) that Niven=s medium may give a high number of false positive isolations because other alkaline bacterial products and not only histamine can be produced in this medium. Moreover, in accordance with the observations of López-Sabater *et al.* (1994) in some instances bacteria isolates unable to produce histamine can be erroneously identified as weak histamine-formers (false histamine-formers) in TSHB broth (66.1% false histamine-formers). In contrast, however, the HEB broth allowed clear separation, even between weak histamine-formers and non-histamine producing bacteria.

Most of the bacteria identified as histamine formers were Gram-positive (83% of isolates) and nearly all belonged to the S*taphylococcus* genus (76% of isolates). Only five Gram-negative strains were isolated, three of them were identified as *Pseudomonas cepaciae*, one as *Enterobacter cloacae*, and one as *Klebsiella oxytoca*. These results suggest that histamine-forming activity in ripened salted anchovy was due to Gram-positive microorganisms, represented mainly by the genus *Staphylococcus*, which involves 70% of total of identified isolates.

The proportion of histamine-forming bacteria accounting less than 1.37% of isolates. The incidence of histidine decarboxylating bacteria in our work has been lower than reported by Karnop (1988), who reported that 53% of total bacteria in semi-preserved anchovies had histidine decarboxylase activity, being *Pediococcus halophilus* the main halotolerant histamine forming bacteria.

Staphylococcus epidermis was the most frequent (63% of isolates) and active histamine former specie, with a production of 204, 571, 394 and 20 μg of histamine/ml of HEB broth at 0.5, 3, 10 and 20% NaCl, respectively (Figure 1). Statistical differences were observed among the amounts of histamine formed at four NaCl concentrations, which showed a highest histamine formation at 0.5, 3 and 10% NaCl than at 20% NaCl ($p<0.01$). One isolate of *Staphylococcus* genus was identified as *S. capitis* with a maximum histamine formation of 398 μg/ml at 10% NaCl, but only 63 and 5 μg/ml at 3% and 20% NaCl, respectively (Figure 1).

K. oxytoca and *E. cloacae* isolated in our study have also previously been reported as powerful histamine formers in fish (Lopez-Sabater et al., 1996). However, *Pseudomonas cepaciae* and *Bacillus pumilus* have never been identified before as histamine formers. Nevertheless, the histamine formers showed a weak or not histidine decarboxylase activity in the culture broth assay at four different NaCl concentrations (Figure 1).

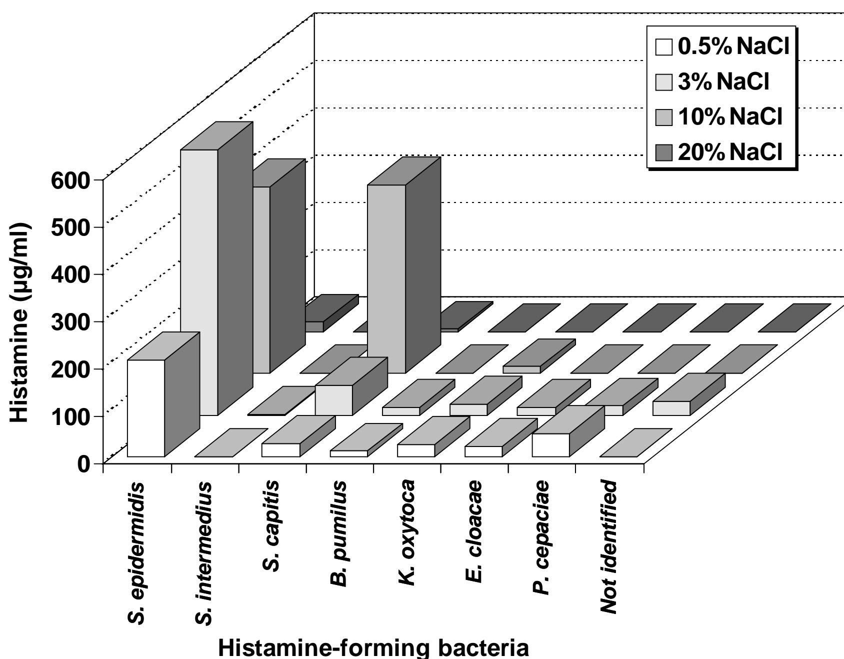

Figure 1: Histamine production in HEB at 30ºC and at four different NaCl concentrations of the histamine forming bacteria isolated during the ripening of salted anchovies.

Because of these results and the fact that the histamine-forming bacteria isolated do not appear as part of the natural microflora of newly caught marine fish, it is reasonable to assume that most of the bacterial species isolated might be expected to be found as a result of contamination of fish during capture and subsequent unhygienic handling. Since staphylococci are one of the major microbial groups inhabiting human skin, it is reasonable to expect that they would be found as part of the microflora of food products, such as ripened anchovy, which requires considerable human contact during preparation and processing. Moreover, the pH value (5.8-6.0), water activity (0.78-0.82) and NaCl concentration (18-20%) of ripened salted anchovy determine the microbial flora that can be found in salted anchovy, dominated by halophilic and halotolerant bacteria, like *Staphylococcus* spp. (Perez-Villarreal and Pozo, 1992).

The histamine levels in the raw fish were always low (< 32 mg/kg). The decrease in the histamine content were statistically significant ($p < 0.05$) during the ripening of salted anchovy. It could be due because a part of the histamine content diffuses into the brine with other nitrogen fractions during the ripening process. As reported by Karnop (1988) histamine contents were not related to the degree of ripeness. Histamine content in fish samples was below the 50 mg/kg suggested by Arnold and Brown (1978) to consider fish as edible and safe, and met the standard established by the European Union (200-400 ppm) as maximum allowable level (Anonymous, 1991).

The number of halotolerant/halophilic bacteria in our work reached 10^2-10^3 CFU/g (data not shown) and the proportion of halotolerant histamine-forming bacteria accounting for about 1.94 %. Yatsunami and Echigo (1993) have reported that when the total number of histamine-forming bacteria reached more than 10^7 CFU/g, a large amount of histamine was produced in red meat fish.

However, these authors suggested that larger amounts of histamine might be produced in fermented salted fishes if the samples were stored longer time. Normally, the sell-by-date of semi-preserved salting anchovy is 1 year. This long shelf-life might provide the histidine decarboxylation and a histamine accumulation at the end of shelf-life.

These findings conclude that the presence of sodium chloride reduces both growth and histidine decarboxylase activity in halotolerant/halophilic bacteria. In experimental conditions, NaCl concentrations in the range of 0.5-10% had stimulatory effect on the amine formation, while levels in excess of 20% inhibited the growth and the amine formation. Histamine activity of *Staphylococcus* spp. may be responsible for the high histamine levels found in salted fish. Moreover, according to observations of Rodriguez-Jerez *et al.* (Rodriguez-Jerez et al., 1994b), refrigeration temperatures for commercially produced semipreserved anchovies are recommended, especially those preserved in oil (NaCl concentration is reduced at 10-12%). At elevated temperatures, a relatively low NaCl concentration may increase histamine production and accumulation.

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Results of a feeding study concerning the carry over of toxaphene into laying hens and eggs

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Summary

There is a lot of data about the levels of toxaphene residues in fish and other marine organisms but almost nothing is known about a toxaphene burden of farmed animals, including their food products. Within a feeding study, in which laying hens got feeding stuff which was contaminated with technical toxaphene in some defined amounts, the carry over behaviour of toxaphene from the feeding stuff into the animal and from there into the egg was determined. Moreover half life times for the decline of toxaphene in some tissues could be calculated.

Introduction

Toxaphene belongs to the group of chlorinated pesticides. Beginning from the year 1947 up to its ban in the year 1982 in the USA about 500.000 tons were produced. In the former GDR further 50.000 tons were manufactured in the years from 1955 to 1990 (HEINISCH et al., 1994). Because of the ban of DDT in several countries toxaphene was used sometimes as a substitute. From the chemical point of view toxaphene is a mixture of a large number of chlorinated hydrocarbons, mainly from the substance class of the chlorinated bornanes. The number of single compounds (congeners) in this mixture is mentioned in the literature with more than 200 (SALEH et al.,1983). But recently 22 congeners were isolated from the technical mixture and their chemical structure was determined (HAINZL et al.;1995 and BURHENNE et al.,1993). With a commercially available standard mixture of these compounds a quantification of toxaphene residues based on the 22 congeners is now possible.

Material and methods

The feeding study was carried out with 89 laying hens. The toxaphene doses in the animals feed varied between 0.1 and 5 mg / kg feeding stuff. The first part of the study covered a range of 38 weeks. Samples of feces, eggs, liver, kidney, meat, fatty tissue and blood were investigated. For determining the half life times of the most important congeners in the hens the group with the highest toxaphene dose was fed in the second part of the study with uncontaminated feeding stuff for further 15 weeks.

Clean-up and analysis of samples

The lipids of the samples were extracted with hexane in a soxhlet apparatus. After removing the lipids by using a Florisil-column, the toxaphene congeners were determined with a gas chromatograph coupled to a high resolution mass spectrometer working in the EI-mode. For identification and quantification a standard mixture of 22 toxaphen congeners (Fig.1:1A) was used.

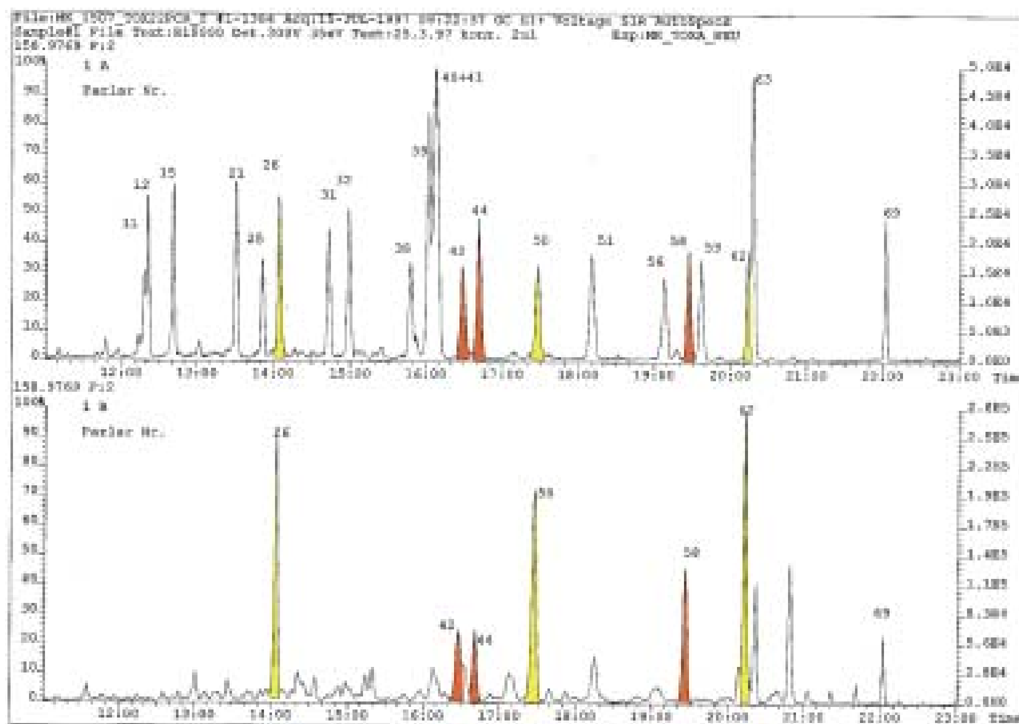

Fig 1: 1A: Chromatogram of the 22 congener toxaphen standard mixture
1B: Fatty tissue sample at the end of the withdrawal study

Results and discussion

Besides the congeners no. 26, 50 and 62 (numbers according to Parlar) which were regulated for fish in the German ‚Rückstandshöchstmengen-Verordnung' other congeners were investigated looking at their accumulation potential. As a result we found, that the congeners no. 42, 44 and 58 were also enriched in the hens tissues (Fig.1:1B Fatty tissue sample after the end of the withdrawal study). In liver and kidney the congeners no. 58 and 62 were less accumulated in relation to the congeners no. 26, 42, 44 and 50 than in fatty tissue and in yolk. Using the congener concentrations in the tissues and in the feed stuff carry over factors can be calculated. They vary for the above named congeners in fatty tissue in the range between 10 to 15, in liver and yolk between 0.9 and 1.7 and in other tissues they are clearly below 1. That means, that the investigated congeners were strongest accumulated in the fatty tissue of the hens. In the muscle meat they do not accumulate, to our present knowledge.

From the withdrawal study (KALTENECKER, SCHWIND et al.,1998) biological half life times (the time in which the concentration of a substance is reduced to half in the tissue because of the ongoing metabolism effects) can be calculated. To get a decline curve the tissue concentrations have to be drawn against a time axis. In Fig.2 this curve is plotted for the decline behaviour of the congeners no. 26, 50 and 62 in fatty tissue.

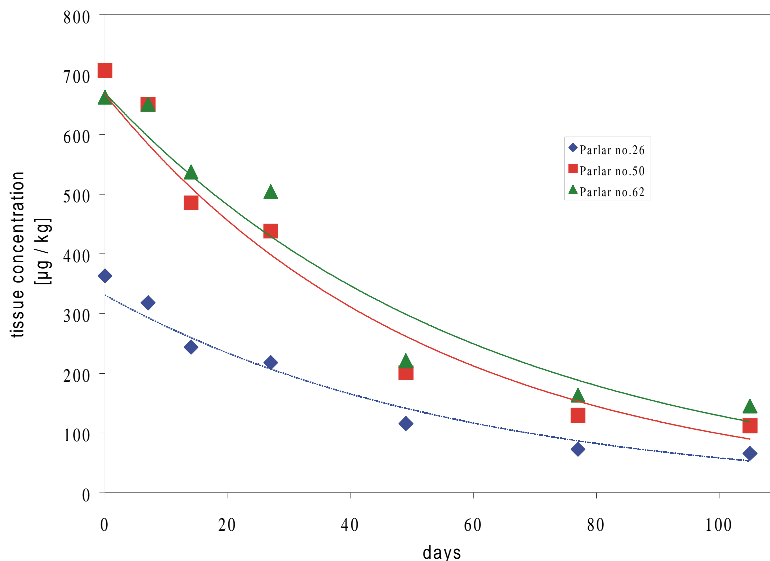

Fig. 2: Curve of the decline of the congeners 26, 50 and 62 in the fatty tissue of the hens

In table 1 half life times for fatty tissue and kidneys are shown.

Tab.1: Biological half life times in days

tissue	Parlar no.26	Parlar no.42	Parlar no.44	Parlar no.50	Parlar no.58	Parlar no.62
fatty tissue	40	25	35	36	36	42
meat	21	17	22	20	15	21
kidney	44	29	36	40	51	49

Moreover laying hens are able to reduce a considerable amount of the incorporated toxaphene by their eggs. Dependent on the congeners 22 % to 37 % of the totally uptaken congener quantity was eliminated with the yolk of the eggs.

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Carry-over of diethylhexylphthalate and aromatic nitro compounds into the milk of lactating cows

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1 Introduction and scope

The role of milk fat as an accumulating substrate for an abundance of lipophilic pollutants and residues is well documented for a considerable number of anthropogenic and non-anthropogenic chemicals. There was, however, a lack of information about the secretory carry-over of phthalic acid esters, probably the most widespread environmental chemicals world-wide, into milk fat as compared to the multiple post-secretory routes of milk contamination, beginning with the rubber parts of the milking machine. The role of the nitro musk compounds, widely used surrogates and/or fixatives for natural musk oils, is apparently of marginal importance in dairying. Nitro musk compounds show a marked bio- and geoaccumulation, are fat-soluble and can be found in freshwater fish in concentrations resembling those of the higher chlorinated biphenyl congeners. The polynitro aromatics could play a role in milk contamination, when pastures and other forage cultures are either occasionally flooded by annual floodings or regularly irrigated with water from rivers or from filter beds.

The prevalence of the phthalic acid ester diethylhexylphthalate – DEHP – in milk is regularly and originates from an abundance of possible sources, though not to be clearly identifiable due to a high background contamination. Nothing has been reported in literature about the prevalence of nitro musk compounds – musk ambrette, musk tibetene, musk xylene, musk ketone, musk moskene – in cows milk. Their findings in human substrates result to a large extent from the dermal absorption after the use of perfumes and/or (washed) clothing (RIMKUS & WOLF 1993, KOKOT-HELBLING *et al.* 1995.

The intention of the studies reported here was to quantify the secretory carry-over

- of DEHP in the milk of lactating cows after oral supplementation as compared to the difference in the DEHP content in hand-milked and machine-milked milk and
- of the five nitro musk compounds to get information over a possible contaminatory pathway to the consumer from the terrestric food chain and to derive possible similarities in the food chain behaviour of other polynitro aromatics from military explosives in polluted areas which are under recultivation.

2 Materials and methods

2.1 Diethylhexylphthalate

To overcome the considerable difficulties of a widespread and hardly to be excluded background contamination with DEHP in practically all substrates, chemicals and equipment involved, we used the tetradeuterated specimen as given in the first figure:

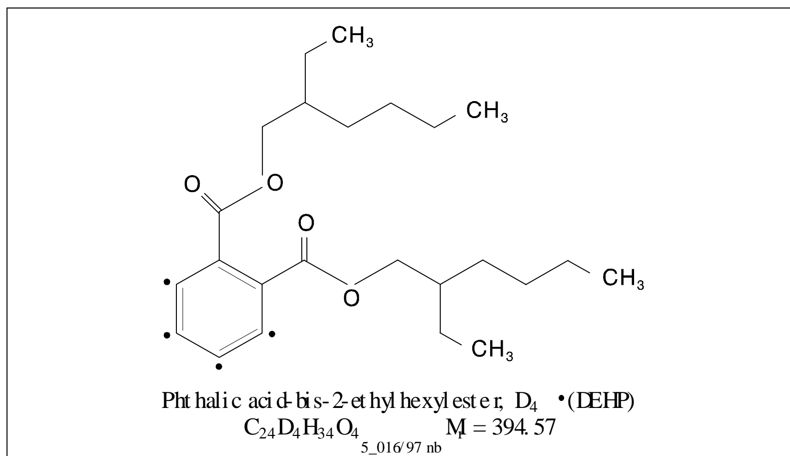


Figure 1

The use of deuterated DEHP in combination with the final determination by GC/MS excludes every unspecific background contamination completely and gives true figures for the oral carry-over.

The substance was dissolved in acetone and dosed to two lactating cows in doses of 100 and 300 mg/d, respectively, via gelatine capsules filled with starch as a carrier for the dose. The supplementation period was 10 days, the analytics in milk as outlined below were carried out in real time.

Analysis of phthalic acid-bis-2-ethylhexylester (DEHP D_4) in milk	
– **Sampling:**	Well mixed milking
– **Fat extraction:**	Cream separation and solvent partitioning
– **Gel permeation chromatography:**	Bio Beads SX3; elution volumes 95.0–160.0 ml cyclohexane/ethylacetate 1:1
– **Column chromatography:**	Florisil with 3% H_2O; elution with 3% ethylacetate in light petroleum/dichloromethane 4:1
– **Gas chromatography:**	Fused silica capillary DB5, 30 m x 0.25 mm; film thickness 0.1 µm; splitless injection; temperature programme
– **Mass spectrometry:**	Electron impact ionization at 70 eV; basis ion at m/z = 153.2(phthalic acid anhydride HD_4^+)
– **Limit of detection:**	0.2 µg/kg milk
– **Limit of determination:**	0.6 µg/kg milk
– **Recovery:**	88 %

5_018/97 nb

Figure 2

2.2 Nitro musk compounds

A possible non-specific background contamination in milk fat can arise from detergents in dairying and from the perfumes of the laboratory staff when performing the analytics. The concentrations

observed were in the low ng/kg-fat-range for the 5 compounds under study and were expected not to disturb the follow-up of the excreted amounts after oral supplementation. The molecular formulas of the 5 nitro musk compounds are given in figure 3:

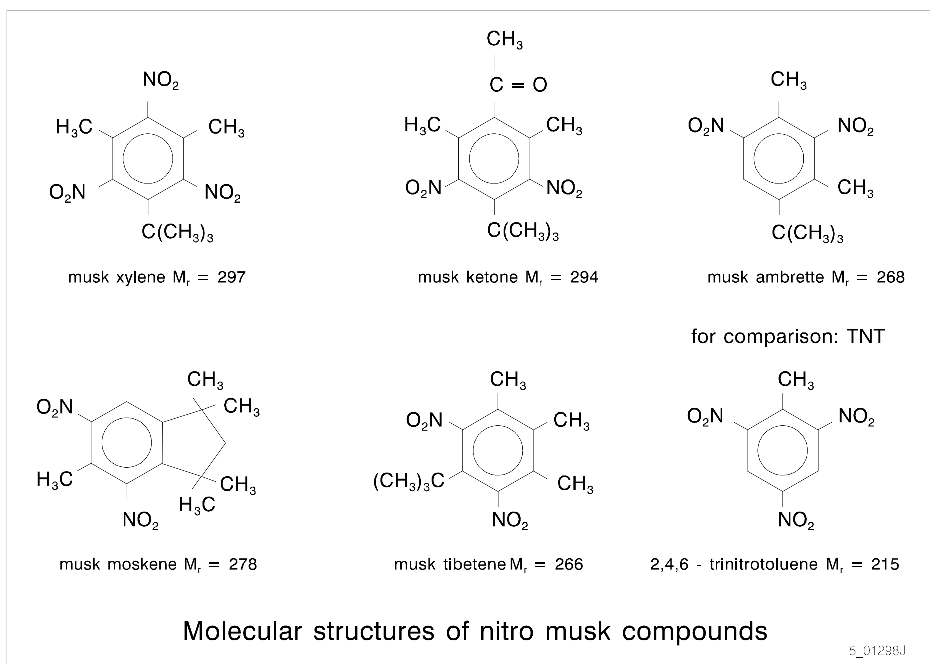


Figure 3

The substances were individually dosed in a daily dose of 150 µg/cow over 15 days. The dose was dissolved in toluene and orally administered via a starch-filled gelatine capsule after the morning milking. The analysis with GC/MS-NCI determination in the final step was carried out according to the following scheme:

Analysis of nitro musk compounds in milk fat	
– **Sampling:**	Cream separation from well mixed milking
– **Fat extraction:**	Solvent partitioning between water, acetone, n-hexane
– **Gel permeation chromatography:**	Bio Beads SX3; elution volumes 115–145 ml cyclohexane/ethylacetate 1:1
– **Column chromatography:**	Silica gel with 1,5% (w/w) water. Elution with toluene/n-hexane 35:65 (v/v) or toluene (musk ketone, -ambrette only)
– **Gas chromatography:**	Fused silica capillary DB5, 30 m x 0.25 mm, splitless injection; temperature programme
– **Mass spectrometry:**	Chemical ionization with negative ion recording, methane as reactant gas
– **Limit of detection:**	0.02 µg/kg milk fat
– **Recovery:**	82–95 %

5_......./98 nb

Figure 4

3 Results and discussion

3.1 Diethylhexylphthalate

Despite the fact that phthalic acid esters show a significant bioaccumulation in the lower links of the aquatic food chain and additionally feature an obvious geoaccumulation the dose was chosen relatively high as the ester-bond C-O-R was expected to be unstable in the rumen of the cows dosed. That this assumption was correct can be seen from the typical excretion curves as given in figure 5.

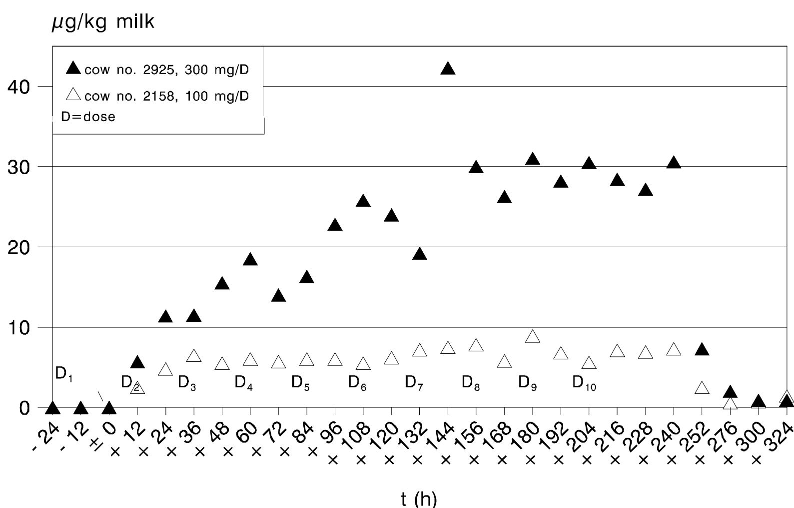
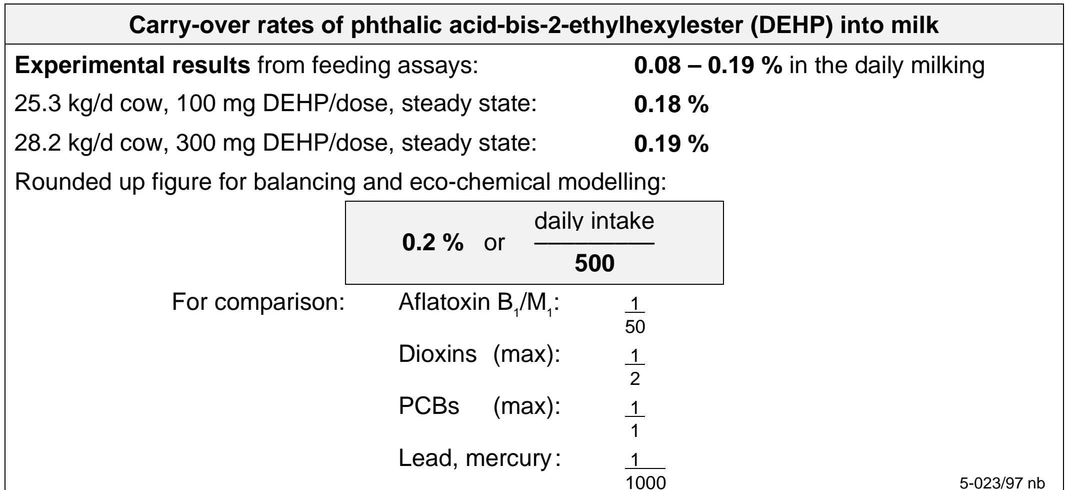


Figure 5

The plot exhibits a dose-related excretion with an equilibrium in the dosing period. The observed peak concentration for the cow receiving 300 mg DEHP-D_4/day results from an erraneously overloaded gelatine capsule and reflects nearly double the dose. As compared to the daily dose of 100,000 to 300,000 µg the excretion in the order of less than 10 to about 30 µg/kg of milk is only low. Blood, taken 22 h after the preceeding dose, exhibits only traces of DEHP-D_4, so that a rapid metabolism beginning in the rumen can be concluded. The carry-over rates as can be derived from dosage and daily milk yield are summarized in figure 6.

As compared to other lipophilic substances the carry-over rate is untypically low.



Carry-over rates of phthalic acid-bis-2-ethylhexylester (DEHP) into milk

Experimental results from feeding assays: **0.08 – 0.19 %** in the daily milking

25.3 kg/d cow, 100 mg DEHP/dose, steady state: **0.18 %**

28.2 kg/d cow, 300 mg DEHP/dose, steady state: **0.19 %**

Rounded up figure for balancing and eco-chemical modelling:

0.2 % or $\frac{\text{daily intake}}{\mathbf{500}}$

For comparison:		
	Aflatoxin B_1/M_1:	$\frac{1}{50}$
	Dioxins (max):	$\frac{1}{2}$
	PCBs (max):	$\frac{1}{1}$
	Lead, mercury:	$\frac{1}{1000}$

5-023/97 nb

Figure 6

3.2 Nitro musk compounds

The daily dose was chosen to be low due to the only occasional environmental impact on dairying. The resulting dose per kg bodyweight (b.w.) of the cows was in the order of 240 ng. Nevertheless, a lipophilic substance should build up a stable excretion on milk fat base. The observation, however, was contradictory as can be seen for 4 compounds from figure 7.

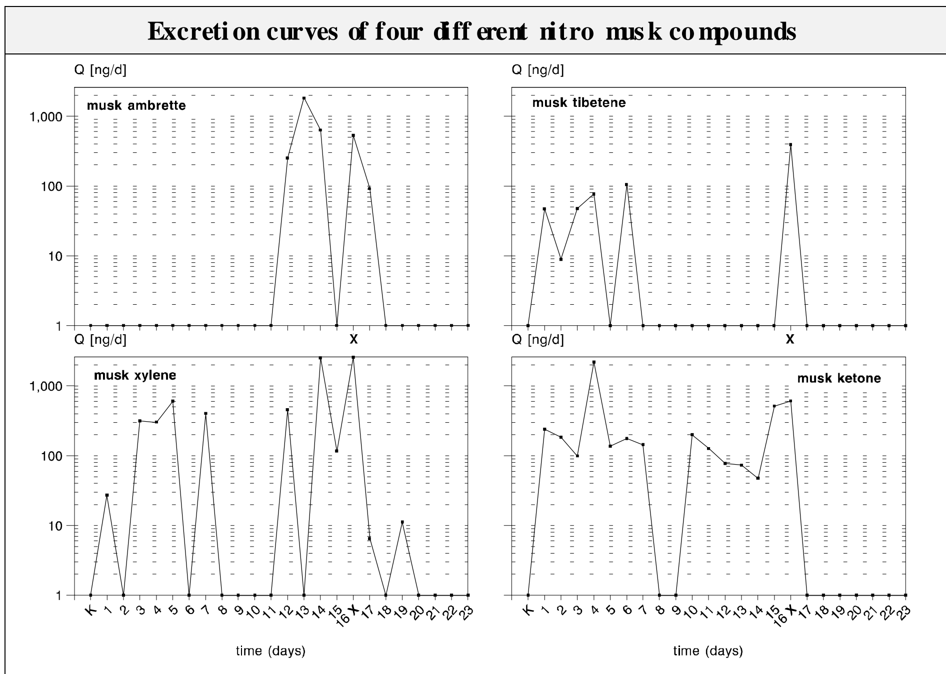


Figure 7

The y-axis shows the excretion with milk fat over the time. The plots exhibit practically only random findings, far away from the formation of a stable plateau. Only musk moskene exhibited a curve like excretion pattern. As the milk fat was analysed in real time, the excretion was continuously observed and the decision made to provocate the excretion with a single provocative dose of 15 mg. This event is marked in the plots with an X. Depending on the type of the nitro musk compound this dose was answered with more or less clear echoes. The integrated carry-over rates over the dosing period are compiled in figure 8.

Carry-over rates for nitro musk compounds into milk (lactating cows)			
Compound	Carry-over rates (%)		
	after 1st dose (150 µg)	for 15 x 150 µg/d	after provocative dose 1 x 15 mg (+ 15x150 µg)
Musk ambrette	<0.01	0.019	3.55
Musk tibetene	0.031	0.008	2.61
Musk xylene	0.018	0.042	17.1
Musk ketone	0.160	0.028	4.04
Musk moskene	0.089	0.033	2.33

Figure 8

These absolutely low percentages indicate no transfer risk for occasionally low doses taken up by the dairy cow. The considerably higher rates after the provocative dose are of no environmental significance.

4 Conclusions

4.1 Diethylhexylphthalate

The conclusions that can be derived from the feeding assay support the thesis that the secretory contamination of milk with DEHP is low and stands clearly in the background of other post-secretory contaminatory possibilities. A balancing modelling on the basis of a uniform DEHP deposition after the environmental release of the production aliquot typical for Germany features the results given in the following figure 9.

As the "normal" DEHP content is in the order between 5 and 50 µg/kg of milk, the other sources must be abundant. Another modelling with the assumption of a 10-µg/kg-gain from the milking equipment seems to be realistic as compared with the observed brittleness of the rubber parts after their service period (fig. 10).

The summarized conclusion either from the secretory or post-secretory contamination of milk with DEHP is given in figure 11.

Ecological-chemical balancing of DEHP* transfer into milk	
Annually atmogenic deposited quantity:	~ 500(+) μg/m^2
	or ~ 1.4 μg/m^2 • d (e.g. for Germany >180mt/year)
Grazing area / cow / day:	18.75 m^2
Theoretically deposited DEHP qty:	18.75 x 1.4 = 25.70 μg/day
Carry-over rate:	0.2 %
DEHP in the daily milking:	0.05 μg
or	
DEHP per litre of milk:	3.2 ng (in a 16 kg milking/day)
Conclusion: The **secretory contamination** of milk is **negligibly low** from the ubiquitous background contamination	
* Phthalic acid-bis-2-ethylhexylester	5_024/97 nb

Figure 9

Approximative balancing of the postsecretory milk contamination with DEHP* during milking		
Assumption: **5 % DEHP** in the rubber parts of cluster and milk tubes (**industry secret**)		
Weight of milk-contacted rubber parts:	1900	g
Quantity of DEHP (5 %):	95	g
Milk flow through cluster and tubes/day (10 cows, 20 kg milk each):	200	kg
Additional DEHP contamination through machine milking (estimate):	10	μg/kg
Quantity of DEHP in the daily milking:	2000	μg
Percentage of DEHP from rubber parts/day:	**0.0021**	**%** (daily wasting)
Wasting of DEHP during one lactation period per milking machine (300 days):	0.63	%
The wastings of DEHP during milking **seem plausible by their order of magnitude**. The figure is supported by the observed hardening and brittleness.		
* Phthalic acid-bis-2-ethylhexylester		5_027/97 nb

Figure 10

Conclusions to the carry-over of DEHP* into the milk

- The carry-over rate is rather low at 0.2 %
- An abundant secretory contamination of milk is unlikely to occur from the low carry-over rate
- Despite fat solubility of the substance no storage in the body of the cow is observed
- The concentrations in plasma reach at maximum 0.07 % of the dosage
- The half-life time in the milkings is less than 24 hrs
- The observed DEHP concentrations in liquid milk between 10 and 50 μg/kg result from postsecretoric contamination
- Before the background of an ADI figure between 0.6 and 1.0 mg/kg body weight a transmissible risk is unlikely for man

* Phthalic acid-bis-2-ethylhexylester 5_025/97 nb

Figure 11

4.2 Nitro musk compounds

The statement seems to be clear that nitro musk compounds show an untypical behaviour in the ruminant, even after a relatively high dosage. Still there are open questions regarding the fate of the amounts applied. The conclusion that the nitro musk compounds are degraded to a significant extent in the rumen can be supported with the only parenteral application of a phenolic nitro fasciolicide to become therapeutically effective. The last figure summarizes the results so far (figure 12).

Conclusions to the carry-over of nitro musk compounds into the milk
– The carry-over rate is substance-dependent and after low dosage (~ 240 ng/kg b.w.) between 0.008 and 0.04 %
– A provocative dose of 24 µg/kg b.w. is answered with carry-over rates between 2 and 17 %.
– Nitro musk compounds show no persistence in milk fat
– Before main exposition of humans occurs through direct dermal absorption from cosmetics and to a far lesser content from fresh water organisms

Figure 12

5 Summary

Phthalic acid esters, especially diethylhexylphthalate (DEHP) and nitro musk compounds are synthetic environmental chemicals which can become into contact with dairying and are in the case of DEHP regularly found in milk and milk products. Both groups are found in human adipose tissue as a result of foodborne and cutaneous exposition. To quantify the secretory carry-over into milk (fat), both DEHP-D_4 and five nitro musk compounds (musk ambrette, – tibetene, – xylene, – ketone, – moskene) were orally supplemented to lactating cows and the excretion with milk quantified. The carry-over rate for DEHP is little less than 0.2 %, the observed ratios for nitro musk compounds are between 0.08 and 0.04 % of the dose administered. In the case of DEHP the findings indicate the predominant role of post-secretory milk contamination. Both types of compounds build up no depots in the dairy animal.

Acknowledgement

The authors wish to thank Mr. K. Fries and Mr. G. Wulff and the staff of the experimental station for their technical assistance in GC/MS operation and accomplishment of the supplementation assay.

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Carry-over of polychlorinated dibenzo-para-dioxins into milk and prevalence in other food of animal origin in Germany

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1 Introduction and scope

"Dioxins" (polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/PCDFs)) are polycyclic aromatics altogether comprising 210 different isomers. They have not been produced as commercial products, but are formed as unwanted accompanyings during the synthesis of industrial chemicals. They are also formed during combustion processes (e.g. municipal waste incinerators). As the result of the resistance towards chemical oxidations and hydrolysis, these compounds have become widely spread into the global environment. Moreover, the 2,3,7,8-substituted dioxins/furans withstand biodegradation. In combination with high lipophilicity, this leads to effective transport in the food chain with accumulation at higher trophic levels. Nearly 90 % of the routes of exposure of human background contamination occur through food, mainly foodstuffs of animal origin.

The measures for limiting dioxin emissions pushed forward since 1991 have led to a continuous reduction of PCDD/F levels in food and thereby to a reduced consumer exposure of less than 1 pg iTE/kg b.w./d.

The above mentioned is the background of the research work on dioxins/furans started in 1991. It covers the following aspects:

– Investigations on the carry-over of selected, toxicologically important isomers into the milk of lactating cows after oral supplementation.

– A consumer-oriented monitoring of foodstuffs throughout Germany (in cooperation with other Federal Research Centres (sampling))

2 Materials and methods

2.1 Material

2.1.1 Feeding assays on the carry-over into milk after oral application

The assays were carried out with lactating cows (Holstein Friesians) according to the pattern "one cow - one isomer". The daily supplementation of 50 respectively 100 ng/isomer was given after adsorption on starch in a gelatine capsule until the equilibrium of excretion in milk fat.

2.1.2 Consumer-oriented monitoring of foodstuffs throughout Germany

2.1.2.1 Butter

In March 1995 204 samples of packed butter (creamery-, branded-, imported butter) were taken all over Germany.

2.1.2.2 Meat and meat products

In the years 1995/96 these products were sampled and analysed in cooperation with the Federal Centre for Meat Research, Institute for Chemistry and Physics, Kulmbach (sampling only). On the

basis of about 200 samples a sampling plan throughout Germany was executed. In dependence on the total population and the habits of consumption the samples were shared among the different federal counties. For the meat products common wares were selected. By proxy for poultry samples of turkey were taken. More of ecological interest 6 samples of wild boar were collected.

2.1.2.3 Fish, fish products, crustacea and molluscs

Since the beginning of 1997 this project runs in cooperation with the Federal Research Centre for Fisheries in Hamburg. The Institute for Biochemistry and Technology (Federal Research Centre for Fisheries) scheduled a plan of sampling (~200 samples) in dependence of the market shares. Until now 93 samples were taken at several passages of the research ship "Walter Herwig III" or bought on the market. The determination of fat- and water contents was realized by the Federal Research Centre for Fisheries.

2.2 Method

The analyses of the samples was according to FÜRST *et al.* (1989) in a modified version. For the determination of the isomers a quadrupole mass spectrometer (feeding assays) and a high resolution mass spectrometer (butter, meat, fish) were used.

3 Results

3.1 Transfer into milk fat after oral application

The transfer of the most important PCDD/F isomers into milk fat is very heterogeneous but there is an indication for resemblances between different isomers. If the carry-over rates are partitioned in sectors resemblances in groups are obvious (Table 1).

Table 1: Grouping of experimentally determined carry-over rates for the applied PCDD/F isomers

Sector of the carry-over rate (cor) %	Isomer	Mean of the cor in equilibrium of excretion* %
< 1	2,3,7,8– TCDF	<< 1
	1,2,3,4,6,7,8–HpCDF	<< 1
~ 1–5	1,2,3,7,8–PeCDF	0.9
5–10	1,2,3,4,7,8–HxCDF	7.7
	1,2,3,4,7,8–HxCDD	8.6
	1,2,3,7,8,9–HxCDD	8.1
10–15	1,2,3,7,8–PeCDD	14.2
	1,2,3,6,7,8–HxCDD	13.8
15–25		
25–35	2,3,4,7,8–PeCDF	25.9 (22.3–29.3)
> 35	2,3,7,8–TCDD	35.4

* Last 6 determinations

In spite of resemblances in groups this table also shows the extreme isomer-specific behaviour of the various PCDD/Fs.

3.2 Foodstuff monitoring throughout Germany

In the following tables the results of the investigations are given in international toxic equivalents (iTE) on fat basis respectively on fresh weight basis (fish, fish products, curstacea, molluscs).

3.2.1 Butter

Table 2 gives a survey of the contamination of all butter samples. 16 % of the samples are imported products.

Table 2: Polychlorinated dibenzo-para-dioxins and -furans in butter samples from Germany (March 1995) – pg iTE/g fat –

Collective	n	x_{min}	$\bar{x}_A$	x_{max}
German butter	172	0.308	0.655	1.185
Imported butter	32	0.276	0.618	1.011
Total	204	0.276	0.643	1.185

Compared to the mean value at the beginning of the nineties (1–1.2 pg iTE/g fat) the mean value of 1995 shows the distinct downward trend.

3.2.2 Meat and meat products

Table 3 summarizes the results of these investigations.

Table 3: Polychlorinated dibenzo-para-dioxins and -furans in meat and meat products from Germany (1995/96) – pg iTE/g fat –

Collective	n	x_{min}	x_A	$\bar{x}_G$	$\bar{x}_{max}$
Meat*	123	0.021	0.177	0.534	16.057**
Meat products***	84	0.058	0.153	0.236	3.931
Total	204	0.021	0.167	0.413	16.057

* Beef, pork, poultry, game
** Wild boar
*** Smoked liver sausages, Lyoner type sausages, Salami, bacon

All minima and means show a low or very low level, whereas the maxima are very different. As indicated in the table the high maximum was found in a sample of wild boar. Also the other five wild boar samples of southern Germany contained higher concentrations (1.39 pg iTE/g fat – 4.14 pg iTE/g fat). In Table 4 the meat is differentiated.

Table 4: Polychlorinated dibenzo-para-dioxins and -furans in meat from Germany (1996) – pg iTE/g fat –

Species	n	x_{min}	$\bar{x}_A$	x_{max}
Beef*	32	0.143	0.531	1.571
Pork**	58	0.021	0.174	3.026
Poultry***	27	0.045	0.280	1.788
Total	117	0.021	0.296	3.026

* Rib; ** Chuck; *** Leg of turkey

Also in this table the low minima and means are confirmed. The maximum of about 3.03 pg iTE/g fat (pork) is an exceptional case because in the majority of pork samples concentrations below 1 pg iTE/g fat were found. Table 5 presents the PCDD/F concentrations in different kinds of meat products.

Table 5: Polychlorinated dibenzo-para-dioxins and -furans in meat products from Germany (1995/96) – pg iTE/g fat –

Product	n	x_{min}	$\bar{x}_A$	x_{max}
Lyoner type sausages	40	0.072	0.168	0.903
Salami	20	0.099	0.164	0.337
Smoked liver sausages	12	0.085	0.403	2.324
Bacon	12	0.058	0.419	3.931
Total	84	0.058	0.236	3.931

Except the maxima of smoked liver sausages and bacon all quantities are low. These maxima can be made an exception of again. Otherwise the levels for smoked liver sausages ranged between 0.085 and 0.502 pg iTE/g fat, for bacon between 0.058 and 0.202 pg iTE/g fat. One of the main results of this study is that the dioxin contamination of meat and meat products is much lower than reported in literature.

Additionally a clearly expressed difference between the south and the north of Germany was detected. Meat and meat products from the south were always less contaminated.

3.2.3 Fish, fish products, crustacea and molluscs

There is an extreme variation in the fat content of fish depending on the species. What is more, it is a subject to seasonal fluctuation. Therefore the question whether the concentrations relate to the proportion of fat or to the fresh weight is absolutely crucial in making a comparative assessment of PCDD/F contamination.

Because this project is still running the data in Table 6 are provisional and include no results about fish products. The greater part of the laboratory samples are pooled samples to exclude extreme random differences in the dioxin content of individual fishes.

Table 6: Polychlorinated dibenzo-para-dioxins and -furans in fish, crustacea and molluscs (1997/98) – pg iTE/g fresh weight –

Species	Market share (%)	n	x_{min}	$\bar{x}_A$	x_{max}
Herring	22.1	20	0.286	1.105	2.885
Cod	7.7	13	0.030	0.046	0.074
Ocean pearch	7.4	15	0.066	0.233	0.706
Salmon	7.3	6	0.240	0.470	0.700
Saithe	6.8	6	0.022	0.082	0.166
Hake	5.9	1		0.076	
Mackerel	2.7	7	0.219	0.344	0.606
Greenland halibut }	2.6	9	0.094	0.692	1.386
White halibut }		1		0.180	
Plaice	1.3	3	0.186	0.284	0.336
Sardine	*	4	0.326	0.521	0.687
Cat fish	*	3	0.357	0.516	0.755
Prawns	*	2	0.109	0.164	0.219
Haddock	*	2	0.011	0.042	0.073
Squid	*	1		0.107	

* Including other species: 36.2 %

Except herring all samples show a low mean. Additionally to the maximum of herring and greenland halibut shown in Table 6 6 other herring samples and 3 other samples of greenland halibut showed concentrations higher than 1 pg/g fresh weight. Compared with prior data also here a clear decrease of PCDD/F contamination is evident.

4 Discussion and conclusions

4.1 Transfer after oral application into milk fat

The uniformly from the last 6 determinations derived and averaged carry-over rates of the 10 most important PCDD/F isomers into milk fat produce a mean carry-over rate of 11.56 %. While 84 % of the into the milk fat excreted toxic equivalents originate from 5 isomers (2,3,7,8-TCDD 13 %; 2,3,4,7,8-PeCDF 48 %; 1,2,3,6,7,8-HxCDD 11 %; 1,2,3,7,8-PeCDD 6 %; 1,2,3,4,7,8-HxCDF 6 %) the carry-over rate for these isomers can be calculated at 19.4 %. That means that at a good bioavailability about a fifth of the toxic equivalents taken up will appear in milk fat.

4.2 Foodstuff monitoring throughout Germany

4.2.1 Butter

A levelled milk fat like packed butter from retail business reflects the situation of deposition on forage plants in dependence on the situation of emission. On this reason it was attempted in Table 7 by regional partition to disclose the differences between agrarian and industrialized regions.

Table 7: Regional partition of the PCDD/F contamination in butter samples from Germany: comparison by place of production (March 1995) – pg iTE/g fat –

Region	n	x_{min}	$\bar{x}_A$	x_{max}
North + north-west	39	0.375	0.649	1.143
Centre-west	20	0.393	0.811	1.185
South	53	0.308	0.487	1.885

The means clearly show the differences between the regions. For this reason the emission/ deposition in the region concerned is the cause for the PCDD/F contamination in the milk fat.

4.2.2 Meat and meat products

The results of the dioxin analyses of meat and meat products presented in part 3 must be discussed including their certainly low content of toxic equivalents but to some extent considerable daily consumption.

Since 1991 it is endeavoured in Germany to reduce the daily exposure to dioxins/furans to below 1 pg per kg body weight. This value of 1 pg/kg b.w./d includes the PCDD/F intake with foodstuffs. Consequently proportionate highest PCDD/F concentrations of the diverse food exist.

Following these assumptions a balance modelling was made up for meat fat. If 70 pg iTE per day is the maximum tolerable value for man and 22.6 % from it originate from meat fat at most 15,8 pg iTE per day may be taken up. At a daily intake of 35.8 g fat from meat and meat products a maximum tolerable PCDD/F contamination of 0.4 pg iTE/g fat results for these products.

The studies showed that meat fat at the average is now according to this value.

4.2.3 Fish, fish products, crustacea and molluscs

Because just about 50% of the samples were analysed it is too early at the moment to draw final conclusions, but the results of the investigations justify the conclusion that estimates of PCDD/F intake through the consumption of fish have been overestimated in the past for Germany.

5 Summary

The classification of the PCDD/Fs originating from the environment, intended for preventive reasons, considered also their carcinogenic properties for man and the resulting high priority of research required in the food sector in order to minimize consumer's risks has led to a main research programme of the Institute for Hygiene of the Federal Dairy Research Centre.

Research work on dioxins/furans started in 1991 and covers the following aspects:

- Feeding assays on the carry-over into milk after oral application.
- A consumer-oriented monitoring of foodstuffs throughout Germany (in cooperation with other Federal Research Centres (sampling)).

The carry-over rates of the 10 most important PCDD/F isomers in milk fat disclosed a very heterogeneous result between <1 and 35 %, the mean value being approx. 20 %.

The need of the Federal Ministry of Food, Agriculture and Forestry for comparable data of the dioxin and furan contamination in food for legislative purposes has led to cooperative activities in this area. The Institute for Hygiene is the partner responsible for the complete analytics and the other research institutes are responsible for sample logistics. The project has been started in spring 1995 with the analyses of butter. The studies of 204 samples showed a spectrum ranging between 0.276 and 1.185 pg iTE/g fat, the arithmetic mean being 0.64 pg iTE/g fat. The following studies of 207 samples of meat and meat products led to a mean value of 0.41 pg iTE/g fat for both. The values ranged between 0.021 and 16.057 pg iTE/g fat. The still running analyses of fish, fish products, crustacea and molluscs show until now (93 samples) a mean of 0.47 pg iTE/g fresh weight.

These findings have shown that the measures for limiting emissions pushed forward since 1991 have produced their minimizing effects. The actual daily intake of PCDD/F equivalents for the consumer in Germany is meanwhile significantly less than 1 pg iTE/kg b.w./d.

Acknowledgement

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Toxin production of *Bacillus cereus* isolates from a milk powder plant

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1 Introduction

Bacillus (B.) cereus known to cause spoilage in a variety of foods is of high significance in food hygiene by causing food-poisoning mostly in connection with rice, but also with milk and milk products.

Milk powder as a basic material for the production of sensitive food like babyfood and desserts is of main interest in this context.

Types of food-borne illness caused by *B. cereus* toxins are diarrhoea or emesis or a combination of both (6). Toxins identified are two enterotoxin complexes and two single toxins.

Two enterotoxin complexes

They show highest toxicity if all three components of each complex are present.

- One is hemolysin BL with three single components (B, L_1, L_2) and molecular weights (MW) of about 37.5, 38.2 and 43.5 kDa respectively (1). The L_2 component is recognised in the Oxoid RPLA® kit (2). The three components together possess hemolytic, cytotoxic, dermonecrotic, and vascular permeability activities. They induce rapid fluid accumulation in ligated rabbit ileal loops (1).
- The other is a non-hemolytic complex with MW's of 39, 45 and 105 kDa. Neither of the fractions is as cytotoxic alone as when the fractions are combined. The 45 kDa protein is the main antigen detected in the Tecra® ELISA ("diarrhoeal toxin"). The 39 kDa protein showed some similarity to the L_1 protein of hemolysin BL (2).

Two single toxins

No routinely applicable tests for the detection are available.

- The ring structure of the emetic toxin is known, the MW is 1.2 kDa. The so called cereulide is a dodecadepeptide, it structurally resembles the antibiotic valinomycin and also causes swelling of the mitochondria of HEp-2 cells. AGATA et al. suggest that cereulide causes emesis through the 5-HT_3 receptor and stimulation of the vagus afferent. By heating for 30 min. at 121°C the activities of both vacuole-formation and emesis effect were never lost (3).
- The enterotoxin T was discovered in 1995. It causes VERO-cell cytotoxicity, fluid accumulation in ligated mouse ileal loop, was positive in vascular permeability assay, and lethal to mice upon injection. The protein has a calculated MW of 41039 Da (4).

Within the scope of this paper the toxin production of *B. cereus* strains isolated from milk powder and the environment of the production unit is examined.

2 Materials and methods

2.1 B. cereus strains

106 *B. cereus* strains isolated from 1057 samples of milk powder
139 *B. cereus* strains isolated from 171 samples from the environment } same dairy

4 reference strains (Dr. A. Christiansson, Swedish Dairies' Association), used as controls

2.2 Tests for the detection of toxins

- Detection of cytotoxicity (VERO-cell test):

– A VERO-cell (African green monkey kidney cells) culture was overlaid with sterile filtered culture supernatant of each *B. cereus* strain. After 48h the degeneration of the VERO-cells was microscopically evaluated.

– The release of lactate dehydrogenase (LDH) of the VERO-cells was measured photometrically (LDH-L testkit, Sigma). Damage of cellmembranes leads to significant increase of LDH (5).

- Detection of the diarrhoeal enterotoxin:

– The Tecra® ELISA (Bioenterprises, Australia) was used according to the manufacturers' instructions. It detects the 45 kDa component of the non-hemolytic enterotoxin complex (2).

3 Results

3.1 Cytotoxicity (n = 245 B. cereus strains)

3.1.1 Microscopic evaluation

- Judgement:

– Different criteria of cytotoxicity are roundness of the polygonal cells, retraction of the runners, vacuolisation of the cytoplasm, and death of the VERO-cells.

⇒ Cell damage was caused by 117 of the tested *B. cereus* strains as demonstrable by microscopy.

3.1.2 LDH-release

- Judgement:

– Measurement of the positive-control is taken as 100%, the single reading of each strain is set in relationship to the positive-control.

– A statistically significant increase of LDH-release >20% was defined as positive (T-test) (Figure 1).

⇒ 142 *B. cereus* strains were LDH-positive.

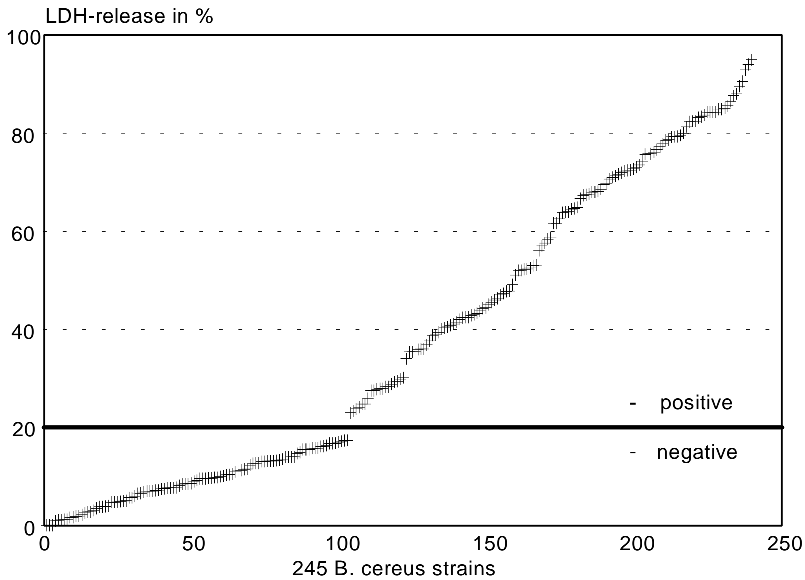

Figure 1: Release of lactate dehydrogenase (LDH) from VERO-cells, ascending order of the results of the *B. cereus* strains (n = 245)

3.2 Diarrhoeal toxin (Tecra® ELISA)

- Visual evaluation was done according to the manufacturers' instructions (panel 1-2 negative, 3-5 positive).
- For quantitative evaluation of the ELISA a standard was used (value of record: 1ng toxin/ml) (Table 1, Figure 2)

Table 1: Qualitative and quantitative evaluation of the Tecra® ELISA

qualitative panel	quantitative ng/ml	n
1	0-0.5	7
2	1.6	1
3	2.5-5.7	6
4	4.3-63.9	127
5	31.0-303.8	111

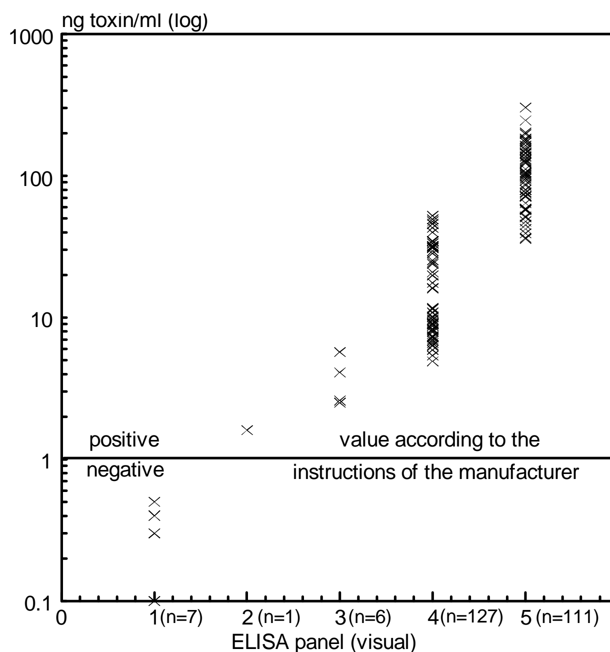

Figure 2: Value of the Tecra® ELISA

4 Conclusions

In the VERO-cell test supernatants of 142 *B. cereus* strains (n=245) showed cytotoxicity as evaluated by LDH-release and 117 strains of these also by morphological evaluation. 237 *B. cereus* strains were positive in the Tecra® ELISA including all strains positive in the VERO-cell test (Table 2).

Table 2: Summarized results of toxin detection produced by *B. cereus* from a milk powder plant

Test		positive (%)	negative (%)
VERO-cell test	morphological evaluation	117 (48)	128 (52)
	LDH-release	142 (58)	103 (42)
Tecra® ELISA		237 (97)	8 (3)

Maximal cytotoxicity of the non-hemolytic enterotoxin complex depends on the presence of all three components, although binary combinations cause some activity at higher levels (2). The 45 kDa protein of this complex is the main protein reacting with the Tecra® ELISA (2), however, it is only one component and not mainly responsible for cytotoxicity by itself. Therefore the VERO-cell test gives a more reliable estimation of cytotoxicity.

Hemolysin BL, enterotoxin T and the emetic toxin which were not determined may also be of influence on the VERO-cell test. Hemolysin BL and enterotoxin T both have cytotoxic activity (1, 4) and the emetic toxin has a HEp-2 cell-vacuolation factor (3).

Nevertheless, to perform a proper risk assessment on the hazard of food poisoning due to a certain *B. cereus* strain an estimation of cytotoxicity by the VERO-cell test is superior to the Tecra® ELISA.

5 Summary

Bacillus (B.) cereus is known to cause spoilage and food-poisoning in a variety of foods including milk and milk products.

Typical forms of intoxication with *B. cereus* are diarrhoea and/or vomiting.

An especially concerned product in this context is milk powder because it is used as a basic material in sensitive foods like babyfood and desserts.

Two enterotoxin complexes (hemolysin BL, non-hemolytic toxin) each consisting of three components are described and two single toxins: the emetic toxin and enterotoxin T.

In 96.7% (237 out of 245) of the *B. cereus* strains examined the diarrhoeal enterotoxin was detected qualitatively by the Tecra® ELISA. The quantitative record of toxin gave a range from 0 to 303.8ng/ml. 58.0% (142) of the collected strains showed cytotoxicity in the VERO-cell test. All those strains were positive in the Tecra® ELISA either.

The Tecra® ELISA detects only a single component of the non-hemolytic complex, but the toxicity depends on the presence of all three components. A result of more concern for the health of the consumer can be expected from cytotoxicity testing applying the VERO-cell test. Hemolysin BL, enterotoxin T and probably the emetic toxin are also of influence on this test.

Acknowledgements

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Chiral amino acid analysis related to food quality and food authenticity

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Introduction

Food quality and food authenticity will certainly continue to be of paramount importatnce in the next millennium. The aim of this paper is to show new perspectives and methods for quality and authenticity control of food and beverages based on the pattern and enantiomeric distribution of amino acids (AAs).

It has been shown that during fermentation of foodstuffs D-AAs are generated by the action of bacterial racemases on L-AAs and by autolysis of bacteria. Most bacteria contain free D-AAs in their cytoplasm as well as peptide-bonded D-AAs in their cell wall peptidoglycan [1]. Therefore, it has been suggested that D-AAs in food can be used as chemical markers indicating the presence and activity of bacteria [2-8].

In recent investigations of fermented foods it has been shown that in balsamic vinegar, soy sauces and wine the amounts of D-AAs increased with age [9-12].

In extension of our previous work on chiral AA analysis of fermented foods [2-5,9,11] we have now systematically investigated different groups of vinegar and beer for the presence of AA enantiomers. We used GC on a chiral stationary phase coupled with selected ion monitoring mass spectrometry (SIM-MS) for detection and quantification.

Experimental

Chemicals and solvents

All chemicals and solvents were of analytical grade. Dichloromethane (DCM), 2-propanol (2-PrpOH), 1-propanol (1-PrpOH), acetyl chloride (AcCl), HCl, 32 % (all from Merck, Darmstadt, Germany), trifluoroacetic acid anhydride (TFAA), pentafluoropropionic acid anhydride (PFPA), 2,6-di-*tert.* butyl-*p*-cresol (BHT).

Standards: AA Standard H (Pierce, Rockford, IL, USA) consisting of Gly and the L-enantiomers of Ala, Val, Thr, Ile, Pro, Ser, Leu, Asp, Met, Glu, Phe, Tyr, Lys, Trp, Arg, His (2.5 mM each) and L-Cystine (1.25 mM). AAs are abbreviated according to three-letter-nomenclature; special AAs are abbreviated as follows: pipecolic acid (Pip), γ-amino butyric acid (GABA), ornithine (Orn) (all from Fluka); L-norleucine (L-Nle) (Merck) was used as internal standard (IS).

Sources of samples

Balsamic vinegars (n = 6), wine vinegars (n = 8), spirit vinegars and blends of spirit and wine vinegars (n = 5) were purchased in local retail outlets. Bottled and canned beers (bottom fermented beers (n = 5), Altbier (n = 5) and Berliner Weisse (n = 4)) were also purchased in local retail outlets. Lambic beers (n = 5) were purchased from a specialized shop.

Isolation of AAs and preparation/derivatization procedures

After addition of L-Nle (100 µl of a 10 mM solution in 0.01 M HCl) as internal standard, vinegars or beers (1 - 5 ml) were subjected to Dowex® 50W X8 cation exchange treatment as described [9].

For GC analysis the AAs of vinegars and beers were converted into their *N(O)*-pentafluoropropionyl 2-propyl esters or *N(O)*-trifluoroacetyl 1-propyl esters, respectively.

Aliquots of 0.6-1.0 µl were subjected to GC/MS (vinegar) or GC-FID (beer) at a split ratio of 1:30 to 1:50. MS was run in selected ion monitoring (SIM) mode.

Quantification was carried out with the internal standard (IS) method according to ref. [9]. Relative amounts of D-AAs were calculated according to the equation $\%D = 100 \times A_D/(A_D+A_L)$, where % D is the relative amount of the D-enantiomer, and A_D and A_L are the peak areas of the D- or L-enantiomer, respectively.

Due to derivatization conditions Asn and Gln, if present, are converted into Asp and Glu. Hence, the calculated amounts of Asp and Glu represent the sum of acid and acid amide. His, Trp, Cys and Arg were not determinable satisfactorily under the routine derivatization conditions described.

Data are the average of two parallel preparation/derivatization procedures.

Capillary gas chromatography

A Shimadzu GC-17A gas chromatograph and a Shimadzu QP-5000 mass spectrometer or a Shimadzu GC-14A with flame ionization detector (FID) and integrator C-R3A (Shimadzu, Kyoto, Japan) were used. The column was a fused silica Chirasil-L-Val capillary column (25 m x 0.25 mm ID, Chrompack, Middelburg, The Netherlands). For chromatographic conditions see ref. [9].

Results and discussion

Evaluation of vinegars

The absolute and relative amounts of D-AAs of selected vinegars are presented in Table 1. Among all vinegars highest amounts of D-AAs were determined in balsamic vinegars. This is due to the very special production process and prolonged maturation times [9,10].

Remarkably, high amounts of D-Pro (up to approx. 200 mg/l in a sample matured for 25 years) were found. Besides D-Ala, D-Asp and D-Glu, which are characteristic bacterial markers, traces of other D-AAs were found. In balsamic vinegars of defined age in particular amounts of D-Ala and D-Pro increased during maturation for 5, 10, 12, and 25 years, respectively. Total amounts of AAs ranged from 1000 - 2000 mg/l. A typical chromatogram of a balsamic vinegar is shown in Fig. 1.

In wine vinegars D-Ala, D-Asp and D-Glu were the dominating D-AAs. Red wine vinegars also contained significant amounts of D-Pro (1.8 - 3.8 mg/l), whereas in white wine vinegars no D-Pro was found. Traces of some other D-AAs occur in several wine vinegars. The total AA amounts, however, vary from 135 to 387 mg/l, due to the diversity of vines, raw materials and treatments used for the production of wines and wine vinegars.

Remarkably, some of the vinegars made of spirits, or mixtures of spirits and wine, did contain only trace amounts of D- and L-AAs, but high amounts of L-Glu. This indicates addition of monosodium

glutamate as flavour enhancer. In one case high amounts of L-Glu were found (Fig. 2), but addition of glutamate was not declared by the manufacturer.

Vinegars made of spirits exclusively did contain only trace amounts of Pro. In contrast, vinegars made from blends of 25-33 % wine and spirits must contain at least approx. 50 mg/l of L-Pro. This is due to the fact that Pro is the major AA in grape juice and, therefore, in wine (see Table 1). However, one blended vinegar labeled as consisting of 33 % wine did contain no more than 4 mg/l Pro. This indicates that a much lower percentage of wine must have been added for production of this vinegar.

Table 1: Absolute and relative amounts of AAs in selected vinegars

AA*	Balsamic vinegar			White wine vinegar			Spirit vinegar			Blend (spirit/wine)		
	amount			amount			amount			amount		
	(L) [mg/l]	(D) [mg/l]	D [%]	(L) [mg/l]	(D) [mg/l]	D [%]	(L) [mg/l]	(D) [mg/l]	D [%]	(L) [mg/l]	(D) [mg/l]	D [%]
Ala	182.4	15.3	7.7	9.2	0.6	6.4	0.9	0.1	10.0	6.6	1.9	22.4
Val	68.7	0.5	0.7	5.0	n.d.	-	0.4	n.d.	-	4.3	n.d.	-
Thr	54.1	n.d.	-	6.6	n.d.	-	0.2	n.d.	-	4.4	n.d.	-
Gly[a)]	41.0	-	-	8.0	-	-	0.4	-	-	5.3	-	-
Ile	40.6	n.d.	-	3.9	n.d.	-	0.0	n.d.	-	2.9	n.d.	-
Pro	653.6	30.8	4.5	256.0	n.d.	-	1.2	0.1	7.7	59.8	n.d.	-
Pip	24.6	n.d.	-	4.9	n.d.	-	0.0	n.d.	-	1.2	n.d.	-
Leu	72.7	2.2	2.9	9.8	n.d.	-	0.5	n.d.	-	5.8	n.d.	-
Ser	72.7	1.5	3.8	8.9	n.d.	-	0.3	n.d.	-	4.6	n.d.	-
GABA[b)]	115.5	-	-	7.9	-	-	0.2	-	-	4.2	-	-
Asp[c)]	79.4	8.7	9.9	11.4	0.4	3.4	1.0	0.1	9.1	5.7	0.3	5.0
Met	20.3	n.d.	-	0.8	n.d.	-	0.5	n.d.	-	0.4	n.d.	-
Phe	49.2	1.9	3.7	8.7	0.1	1.1	0.3	n.d.	-	3.5	n.d.	-
Glu[d)]	79.1	6.7	7.8	13.4	0.7	5.0	847.4	1.4	0.2	13.2	0.9	6.4
Tyr	30.1	0.9	3.0	7.7	n.d.	-	0.1	n.d.	-	2.4	n.d.	-
Orn	81.8	7.1	8.0	8.5	n.d.	-	0.4	n.d.	-	8.3	n.d.	-
Lys	51.9	1.1	2.1	14.4	0.1	0.7	0.3	n.d.	-	5.0	n.d.	-
Σ	1717.7	76.7	4.3	385.1	1.9	0.5	854.1	1.7	0.2	137.6	3.1	2.2

* Amino acids (AAs) are listed according to their elution order on GC; [a), b)] non-chiral AAs; n.d.= not determinable, (limit of determination approx. 0.1 mg/l), %D = 100 x D/(D+L), [c),d)] representing Asp+Asn and Glu+Gln respectively; due to derivatization conditions; His, Trp, Cys and Arg were not determinable satisfactorily.

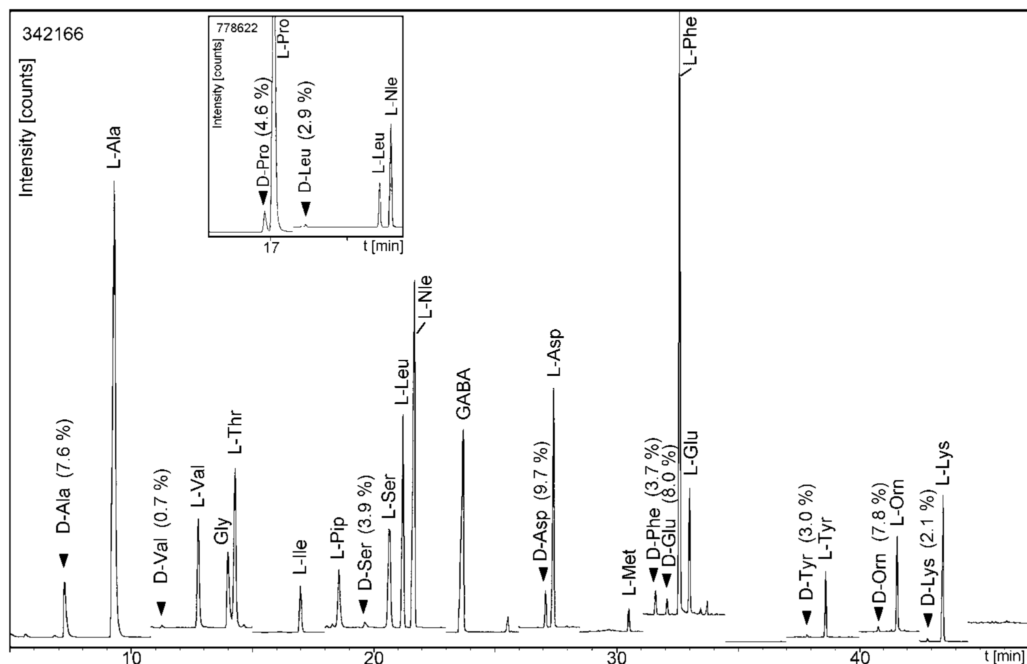

Fig. 1: GC/SIM-MS of a balsamic vinegar; insert shows a separate GC run with a lower injection volume in order to prevent column overloading due to high amounts of L-Pro; AAs separated on Chirasil-L-Val as PFP-AA-2-propyl esters (for abbreviations see Experimental).

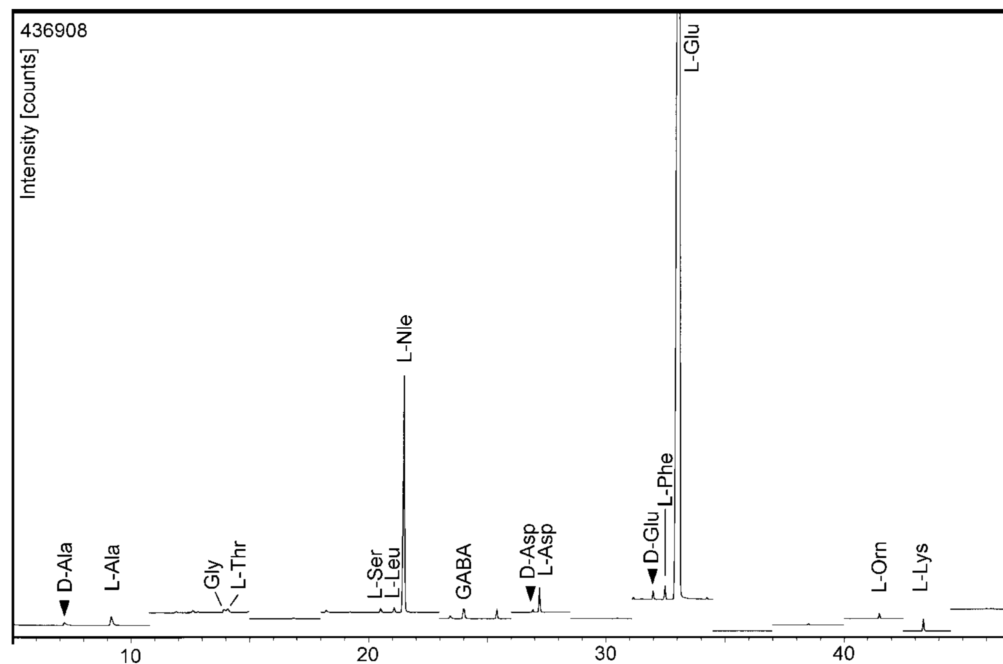

Fig. 2: GC/SIM-MS of a spirit vinegar; AAs separated on Chirasil-L-Val as PFP-AA-2-propyl esters (for abbreviations see Experimental)

Evaluation of beers

The Total AA content of the beers ranged from 530 to 2020 mg/l. L-Pro was the main component with the exception of two Lambic beers, where L-Asp was the major AA (see Fig. 3 and Table 2). Lowest absolute and relative amounts of D-AAs were detected in bottom fermented beers like Pilsener beer (8-13 mg/l and 0.7-2.1 %, respectively). In all beers the marker D-AAs (D-Ala, D-Asp, D-Glu) were present. Besides that, Berliner Weisse beers also contained high absolute and relative amounts of D-Pro (12 - 56 mg/l and 4.9 - 21.7 %, respectively), in particular those which had been flavoured with woodruf or raspberry syrup. The high amounts of D-AAs might be explained by the lactic acid fermentation with *Lactobacillus* spp. taking place in addition to the alcoholic fermentation by *Saccharomyces cerevisiae*. The different AA pattern of Lambic beers might be due to the addition of fruits like cherries or raspberries to the mash. The use of wild yeasts for spontaneous fermentation of Lambic bees does probably not effect the D-AA distribution in comparison to beers fermented with pure cultures of *Saccharomyces cerevisiae.*

Table 2: Absolute and relative amounts of AAs in selected beers

AA*	Pilsener			Altbier			Lambic			Berliner Weisse		
	amount (L) [mg/l]	amount (D) [mg/l]	D [%]	amount (L) [mg/l]	amount (D) [mg/l]	D [%]	amount (L) [mg/l]	amount (D) [mg/l]	D [%]	amount (L) [mg/l]	amount (D) [mg/l]	D [%]
Ala	273.5	4.2	1.5	87.1	5.2	5.7	61.2	10.9	15.1	54.9	23.7	30.2
Val	128.3	n.d.	-	58.1	n.d.	-	44.5	n.d.	-	35.2	n.d.	-
Thr	9.3	n.d.	-	7.4	-	-	20.2	n.d.	-	14.7	n.d.	-
Gly[a)]	99.6	-	-	69.5	-	-	29.6	-	-	48.9	-	-
Ile	35.6	n.d.	-	11.7	n.d.	-	24.7	n.d.	-	25.3	n.d.	-
Pro	623.7	1.7	0.3	534.0	n.d.	-	91.7	0.6	0.5	208.7	56.0	21.2
Leu	60.7	n.d.	-	31.6	n.d.	-	41.6	n.d.	-	50.2	n.d.	-
Ser	30.2	n.d.	-	37.5	n.d.	-	22.1	n.d.	-	20.8	n.d.	-
GABA[b)]	123.5	-	-	65.6	-	-	28.1	-	-	82.1	-	-
Asp[c)]	43.3	3.9	8.2	29.8	5.1	14.9	622.2	10.3	1.7	33.3	5.5	14.3
Met	14.4	n.d.	-	4.8	n.d.	-	13.6	n.d.	-	14.5	n.d.	-
Phe	75.5	n.d.	-	60.8	1.3	2.1	27.2	n.d.	-	42.4	1.2	2.7
Glu[d)]	96.1	3.1	3.2	42.8	3.9	8.6	36.4	2.2	4.0	58.9	8.4	12.3
Tyr	70.0	n.d.	-	60.8	2.0	3.3	14.6	1.0	4.5	6.9	n.d.	-
Orn	6.1	n.d.	-	5.4	n.d.	-	3.1	n.d.	-	10.4	n.d.	-
Lys	85.4	n.d.	-	19.0	1.6	6.5	8.6	n.d.	-	43.9	1.5	3.9
Σ	1775.4	12.9	0.7	1125.9	19.1	1.7	1089.3	25.0	2.3	751.0	96.1	11.4

* Amino acids (AAs) are listed according to their elution order on GC; [a), b)] non-chiral AAs; n.d.= not determinable, (limit of determination approx. 0.1 mg/l), %D = 100 x D/(D+L), [c),d)] representing Asp+Asn and Glu+Gln respectively; due to derivatization conditions; His, Trp, Cys and Arg were not determinable satisfactorily.

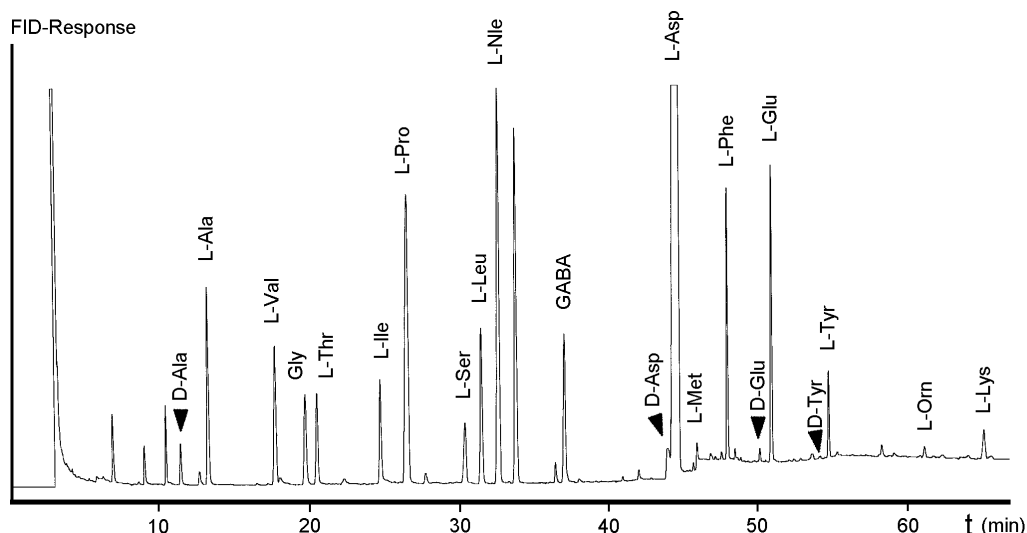

Fig. 3: GC-FID chromatogram of a "Lambic" beer; AAs separated on Chirasil-L-Val as TFA-AA-1-propyl esters (for abbreviations see Experimental).

Conclusions and perspectives

The data show that chiral AA analysis of food and beverages is a promising tool for the assessment of food quality and authenticity. AA analysis reveals the addition of flavour enhancers like monosodium glutamate. The pattern of AAs can be used as fingerprint for the origin of raw materials. Therefore, chiral AA analysis could serve as control for food declaration according to food laws. Presence of D-AAs (D-Ala, D-Asp and D-Glu) indicates microbial fermentation. Thus, D-AAs are considered as indicators of naturally fermented foods and beverages. D-Pro, in particular, might serve as chemical marker for ageing and ripening of foodstuffs. This is of interest since the value of many foodstuffs is depending on their maturation. On the other hand D-AAs might also be used as markers for microbial food spoilage.

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Application of two novel PCR assays for the rapid detection and discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group

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Summary

Psychrotolerant strains of the *Bacillus cereus* group (*Bacillus cereus, Bacillus thuringiensis* and *Bacillus mycoides*) are frequently associated with the spoilage of pasteurised food and dairy products. Moreover, many of these strains are pathogenic, with their proliferation in food often resulting in the production of enterotoxins and leading to food poisoning. Detection of psychrotolerant *B. cereus* group strains in food products is at present extremely slow using conventional microbiology. This is due to an inability to discriminate these cold-adaptive strains from their mesophilic counterparts by means other than growth at low temperature, which at 7°C may take up to 14 days for detection. Here we report the development of two separate PCR assays that, using either major cold-shock protein or 16S rDNA specific primers, are capable of both rapidly identifying bacteria of the *B. cereus* group and of discriminating between psychrotolerant and mesophilic strains. It is intended that these assays should help towards more accurately predicting the shelf-life of refrigerated pasteurized food and dairy products, and reduce the incidence of food poisoning by such strains. The reliability of both assays has been demonstrated by testing 194 *B. cereus* group strains.

Introduction

Psychrotolerant (psychrotrophic) bacteria have been recognized as a recurring problem in the refrigerated storage and distribution of perishable food products, a problem that is particularly pertinent to the dairy industry (9). Research into the bacterial spoilage of dairy products has focused predominantly on post-pasteurization contaminants such as *Pseudomonas*. Improved processing conditions for milk and other dairy products has, however, meant less interest in these nonheat-resistant contaminants and more attention being directed towards psychrotolerant sporeformers such as *Bacillus* (9). Of particular importance are strains of the *Bacillus cereus* group, which includes *B. cereus, B. thuringiensis* and *B. mycoides*. These are very closely related bacteria that, in the case of the former two species at least, are known to cause food poisoning through the production of enterotoxins (3, 7). Incidence of food poisoning by *B. cereus* vary considerably, being reported by Granum *et* al (1997) to account for 33% of the total cases of foodborne illness in Norway (1988-1993), 47% in Iceland (1985-1992), 22% in Finland (1992), 8.5% in The Netherlands (1991), and 5% in Denmark (1990-1992).

The detection of psychrotolerant strains of the *B. cereus* group in dairy products is at present extremely slow (taking up to 14 days), due to the inability to discriminate psychrotolerant and mesophilic strains by means other than growth at low temperature. In order to develop a rapid detection method for such bacteria, at least some molecular aspects of psychrotolerance need to be known. Studies directed at understanding the molecular mechanisms adopted by *Bacillus* for dealing with growth at low temperature (i.e., cold adaptation) have focused on the roles played by ribosomes

and by a family of small polypeptides termed the major cold-shock proteins (4, 8). Both of the latter determinants are significantly induced by a temperature downshift and play a fundamental role in the bacterium's survival at low temperature (1, 5). These molecules were therefore made the focus of our attention, since it was suspected that subtle differences in their regulation and/or structure might contribute to the psychrotolerance of strains of the *B. cereus* group.

In this study, data is presented showing that PCR targeting of particular psychrotolerant and mesophilic signatures within the 16S rDNA or the major cold-shock protein gene *cspA*, allows rapid discrimination of these two thermal groups of bacteria. These assays have been developed and evaluated testing 194 *B. cereus* group strains.

Materials and methods

One hundred and ninety four strains of the *B. cereus* group were involved in the development and evaluation of both PCR assays. The majority of these strains (150) were gained from a variety of collections, with an additional 44 *B. cereus* group strains isolated from food and soil samples prepared in this study. In addition to testing nearly 200 strains of the *B. cereus* group, 32 non-*B. cereus* group strains were also tested using both PCR assays to establish specificity. *B. cereus* and *B. mycoides* were isolated on *B. cereus* selective agar (PEMBA, Oxoid), with these plates being incubated for two days at room temperature. Growth tests were performed in Plate Count Broth (PCB). An overnight culture of bacteria was grown in 5 ml PCB, shaken at 150 rpm and 30°C. 10 µl of this overnight culture served as inoculum for the growth test, which was performed in 5 ml PCB, shaken at 120 rpm and 4°C, 7°C or 10°C.

Multiple alignments of both 16S rDNA sequences and *cspA* sequences from several different mesophilic and psychrotolerant strains of the *B. cereus* group revealed the existence of temperature growth-type specific signatures. In each case, these sequence differences were used to design pairs of oligonucleotide primers for specific PCR amplification of DNA from either psychrotolerant or mesophilic strains. Sequences of the mesophilic and psychrotolerant 16S rDNA and *cspA* specific primers are given in von Stetten *et al* (manuscript submitted for publication) and Francis *et al* (in press), respectively. The principles of these assays are illustrated in Figures 1 and 2.

PCR was performed with a Techne Progene automated thermocycler with 0.2 ml thin walled PCR tubes (Advanced Biotechnologies). Reactions were carried out in 50 µl volumes containing 5 µl of 10 × PCR buffer (supplied with *Taq* DNA polymerase; Eurogentec), 2.0 mM $MgCl_2$, 50 pmol of each oligonucleotide primer, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Eurogentec), 1 U of *Taq* DNA polymerase (Eurogentec), and a pin-head sized aliquot of bacteria picked from a PEMBA plate. Bacterial cells were lysed by heating the mixture at 95 °C for 5 min. Amplification of 16S rDNA or *cspA* was then attempted with 30 cycles at 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s (Taq polymerase was added at 80°C during the initial cycle), followed by a final extension step at 72 °C for 2 min. A 5 µl volume of each PCR product was electrophoresed through a 2 % agarose gel, which was ethidium bromide stained and visualised using a Pharmacia Image Master (Pharmacia Biotech).

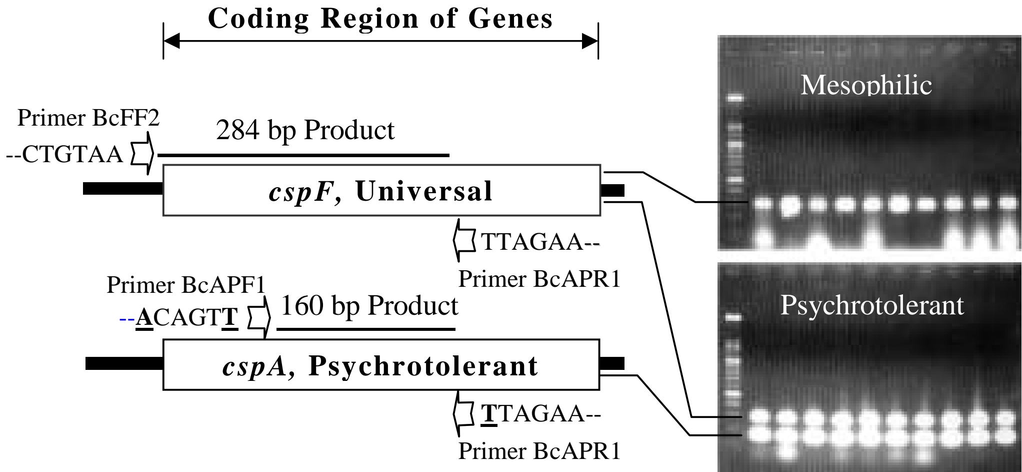

Fig.1: (taken from ref. 2)**:** The *cspA*/*cspF* assay. Left of the figure shows a schematic diagram of the *cspA* and *cspF*, the two genes used as PCR target sequences for the differentiation of psychrotolerant and mesophilic strains of the *B. cereus* group. The approximate positions at which each of the three primers hybridizes to each of the genes and the size of the resulting PCR products are indicated accordingly. Signature bases, allowing PCR amplification of psychrotolerant *cspA* only, are underlined and in grey. Right of the figure are two pictures of an ethidium bromide stained agarose gel with examples of DNA amplification from psychrotolerant and mesophilic strains of the *B. cereus* group gained using this PCR assay.

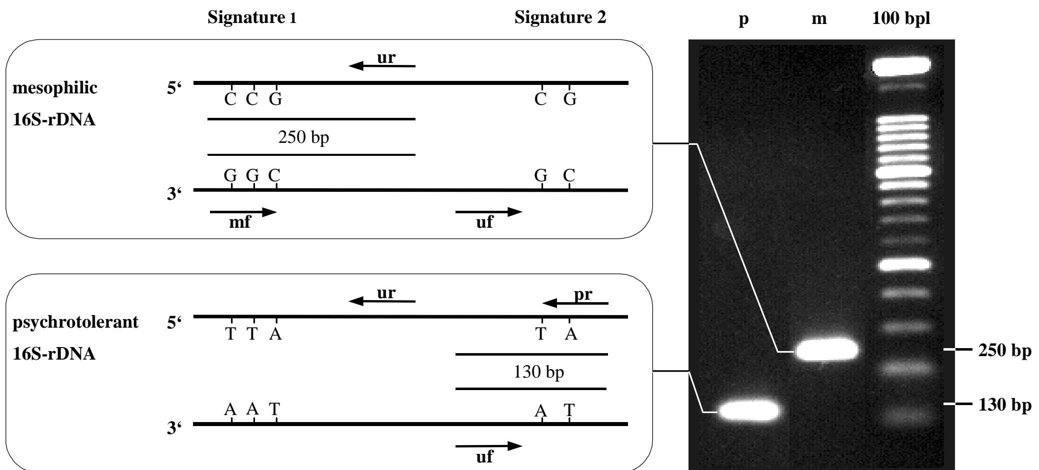

Fig. 2: (taken from ref. 11)**:** The 16S-rDNA assay. The mesophilic-specific forward primer (mf) only anneals when a mesophilic signature 1 is present, the psychrotolerant-specific reverse primer (pr) only anneals if a psychrotolerant signature 2 is present. The universal reverse and forward primers (ur, uf) anneal in either case. The distance of their binding site to the concerned signature determines the length of the amplified DNA. A 250-bp fragment detected on the agarose gel indicates a mesophilic target (lane m), a 130-bp fragment indicates a psychrotolerant target (lane p). The molecular weight marker is a 100-bp ladder (100 bpl).

Results and discussion

Examples of PCR products amplified from different strains of the *B. cereus* group using the *cspA* assay are shown in Figure 1. In all of the 194 *Bacillus cereus*-group strains tested, 100% correlation was seen between both sets of PCR data and the growth data with regard to classifying the strains as psychrotolerant or mesophilic. All strains grew at 25°C. 51 of these strains were psychrotolerant, showing growth at 7°C, with 41 of these strains also able to grow at 4°C. 45 of the remaining 143 mesophilic strains were tested for growth at 10°C, all except 7 (isolates from Thailand) of these 45 strains grew.

Due to the high degree of conservation found between 16S rDNA sequences in closely related bacteria, 5 of the 32 investigated non-*B. cereus* strains gave a PCR product: *Bacillus megatereium*, which gave a psychrotolerant specific product, and *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis* and *Lactobacillus plantarum*, which each gave a mesophilic specific product. For this reason the foregoing enrichment should be selective, e.g. for sporeformers. In contrast, none of the non-*B. cereus* strains gave a PCR product using the *cspA* assay.

Both PCR procedures developed in this study for detecting and discriminating psychrotolerant and mesophilic *B. cereus* group strains are rapid and cost effective. The most laborious step in this procedure is the initial isolation of pure cultures. Isolation of these bacteria on selective agar took two days, due to the incubation at ambient temperature to ensure the recovery of all *B. cereus* group strains. Refinement of the PCR to allow quantification of both psychrotolerant and mesophilic templates simultaneously, using a technique such as the 5' nuclease assay (12), would eliminate the time necessary for the initial isolation of bacteria. Such a refinement could allow separation of the bacteria directly from a food or soil matrix by using techniques such as immunomagnetic capture, centrifugation, or two-phase partitioning (6, 10). Such approaches are currently under investigation.

We believe that development of the above PCR assays will significantly reduce the time necessary for screening pasteurized food and dairy products for contamination by psychrotolerant strains of the *B. cereus* group. It is also possible that employment of this assay during food processing might allow the number of such contaminating bacteria to be more accurately estimated, reducing the incidence of food poisoning by these potential pathogens.

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Multiplication of Staphylococcus (S.) aureus and production of enterotoxins during the experimental manufacturing of Camembert cheese

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1 Introduction

The enterotoxins (SE) A-E produced by Staphylococcus (S.) aureus world-wide belong to the most frequent causes of food intoxications. One of the origins for raw milk and raw milk products may be cows suffering from subclinical mastitis. About 10^6 cfu/ml/g are sufficient to produce an amount of enterotoxins which may be dangerous for humans (1). Because of similar conditions for the production thermonuclease (TNase) may be used as a screening for the potential presence of enterotoxins in the product (3). It is important to know that especially in soft cheeses because of the high water content and pH value during the ripening process there are a lot favourable conditions for the multiplication of S.aureus (4). In the EU Directive 92/46 (6), therefore, limits are given for S.aureus in raw milk used for manufacturing of raw milk products (m = 500, M = 2 000) and for cheese from raw milk and thermized milk (m = 1000, M = 10 000). If M is exceeded the presence of toxin has to be examined in the product. Aim of the presented work was to show the kinetics of multiplication and of enterotoxin production of S.aureus during the experimental manufacturing of raw milk soft cheese under different conditions.

2 Material and methods

2.1 Material

- Reference strains of S.aureus producing SEA (staphylococcus enterotoxin A) and SEE (Staphylococcus enterotoxin E)
- inhibitor free raw bulk milk for cheese production from cows of the experimental station of the institute
- starter cultures (Probat 505-Wiesby);
- P.candidum (SC, DIP D1-Wiesby)
- rennet extract standard (Chr. Hansen)

2.2 Methods

The manufacturing of raw milk soft cheese was done according to Prokopek and Voss (7).

- thermization of the raw milk to 32° C and additional inoculation of S.aureus culture of reference strains with different counts
  starter culture (concentration 0.75 - 1.6 %, preincubation: 1 or 3 days at 22° C in UHT milk) and P.candidum culture (0.025 %) at manufacturing step 0.5 h
- after filling the cheese curd in forms three turnings after every hour
- ripening (2. to 11. day): 18° C, 80 % relative air humidity, wrapping of the cheeses
- storage (12.-32. day): 15° C, 20 % relative air humidity

The steps for taking samples during manufacturing are given in Table 1.

Table 1: Sampling during the manufacturing of raw milk soft cheese

manufacturing step		time of sampling
bulk milk before thermization		0 h
after addition of S.aureus, starter and mould culture		0,5 h
after addition of rennet		1,5 h
after cutting of cheese curd	whey	2,5 h
	curd	2,5 h
after filling in form	whey	3,5 h
	curd	3,5 h
after third turning		6,5 h
after brine bath		8,5 h**
2., 4., 6., 8., 11., 18., 25., 32. day		indication of manufacturing (d)

All samples were examined according to official standard methods (8) for:

Count of coagulase positive staphylococci

- preparation of the cheese samples (L 03.001-1)
- determination of the count of coagulase positive staphylococci (L 01.00-24)
- differentiation of colony types on BAIRD PARKER-medium
- determination of TNase (L 01.00-33)
- determination of coagulase (L 01.00-24) for TNase positive colonies

Isolation of TNase directly from the food (L 01.00-33)

- the enterotoxins were determined directly from the food for SEA and SEE by methods (sandwich ELISA) which were developed in the institute (9, 10)
- measuring of pH value and temperature

All these measurements were done at the steps given in Table 1.

3 Results

The different lots of cheese varied regarding the count of enterotoxigenic S.aureus strains (2 x 10^3 - 4.5 x 10^4 cfu/ml) and the mentioned starter activities and concentrations.

3.1 Course of pH values

Starting in raw milk with pH 6.8 the value decreased till 6.5 h resp. 8.5 h to pH < 5.0. These values were constant up to the 6. resp. 8. day, then increased during the following steps and reached 6.0 - 7.5 between 25. - 32. day.

3.2 Course of S.aureus counts (see Figure 1)

Regarding different conditions the geometric mean values of the different lots are demonstrated starting at 0.5h after inoculation of the test organisms. Lots with identical test conditions are summarized in Figure 1. A general tendency of multiplication during the first days of manufacturing is a clear increase up to 8.5h, followed by a plateau and then within the last weeks of storage again by a further multiplication.

The counts in the whey are not shown in the figure. After 3.5h they were generally two potencies lower than in the cheese curd, which may be caused by a physical enrichment.

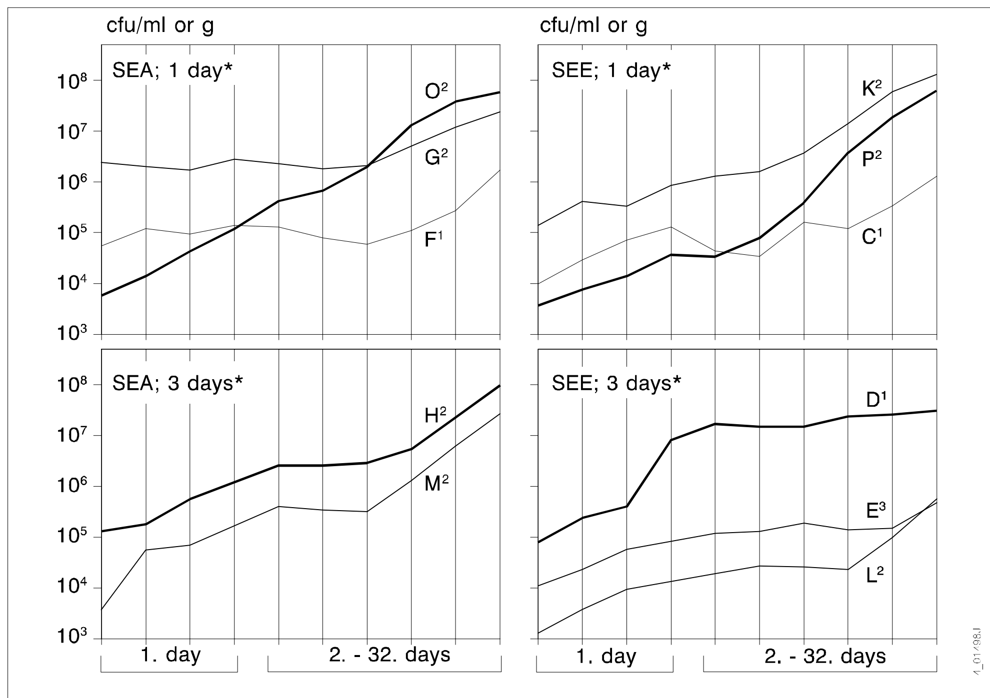

Fig. 1: Manufacturing of raw milk soft cheese. Course of S.aureus counts (cfu/ml/g) after inoculation as geometric means.
Starter concentration (%): 1) 1.0; 2) 0.75; 3) 1.6
* Starter pre-incubation: lots F, G, O and C, K, P (1 day), lots H, M and D, E, L (3 days)

The influence of the initial counts of S.aureus (0.5h), the concentration (0.75%, 1.0%, 1.6%) and activity (pre-incubation one or three days) regarding the growth course and count of S.aureus at the end of ripening may be interpreted as follows.

The initial count seems to have less importance if the starter concentration is equal (e.g. lots O^2, G^2; H^2, M^2; K^2, P^2). It shows that the lowest starter concentration allows the highest final count of staphylococci. Comparing the activities of starter (1 or 3 days) the fresh one day starters, obviously, for lot F an G prevent a multiplication of S.aureus in spite of higher initial counts. The staphylococci of lot O, however, show a continuous multiplication in spite of a low beginning, maybe caused by the lower starter concentration. Summing up the starter concentration seems to have more influence than the starter activities.

3.3 TNase and enterotoxin production (see Figures 2 and 3)

The most important question of these examinations was to see the possible production of enterotoxins during a usual manufacturing process of raw milk soft cheese and the prove of TNase as a screening for potential enterotoxin presence also under the identical combinations as described above.

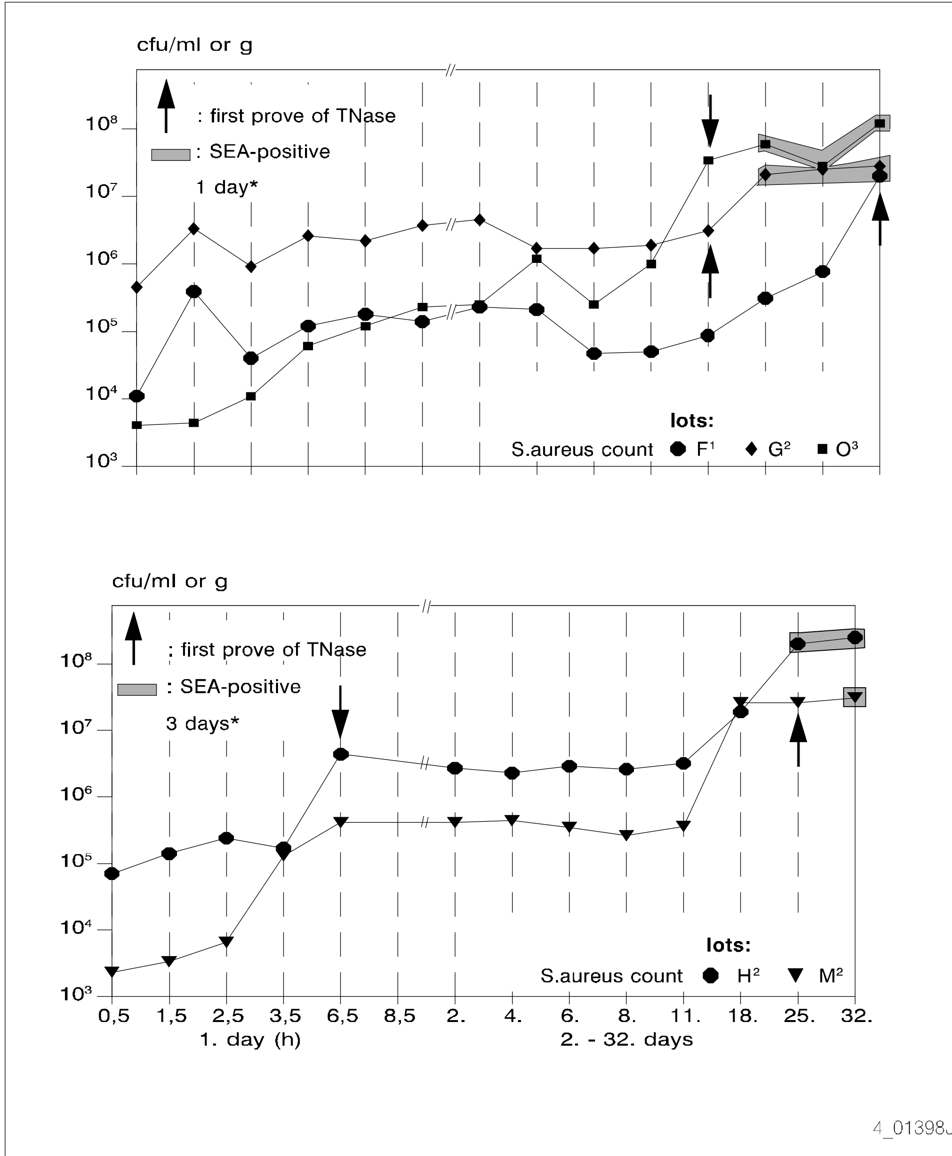

Fig. 2: Manufacturing of raw milk soft cheese with inoculation of an SEA-producing S.aureus First ocurrence of TNase-and Enterotoxin in relation to the growth of staphylococcus starter concentration (%): [1] 1.0; [2] 0.75; [3] 1.6

* starter pre-incubation: lots F,G,O (1 day), lots H,M (3 days)

In ten of eleven lots during the manufacturing and ripening enterotoxin could be found. All SEA samples showed also a positive TNase except one lot (F) earlier to the presence of enterotoxin. This, however, was not true for the SEE samples, where TNase was detected at the same time or later as enterotoxin. As it is known from the literature TNase and/or enterotoxin only can be detected if more than at least 10^6cfu/g or ml are present. However, detectable amounts only are present if the bacteria had the chance to multiply several times in the food. That means that high initial counts also need a distinct time of incubation for production of these substances. A direct correlation to initial count of staphylococci, starter concentration and activity is not to be seen. These factors only are relevant for the multiplication of the staphylococci and these consequently are responsible for TNase and toxin production.

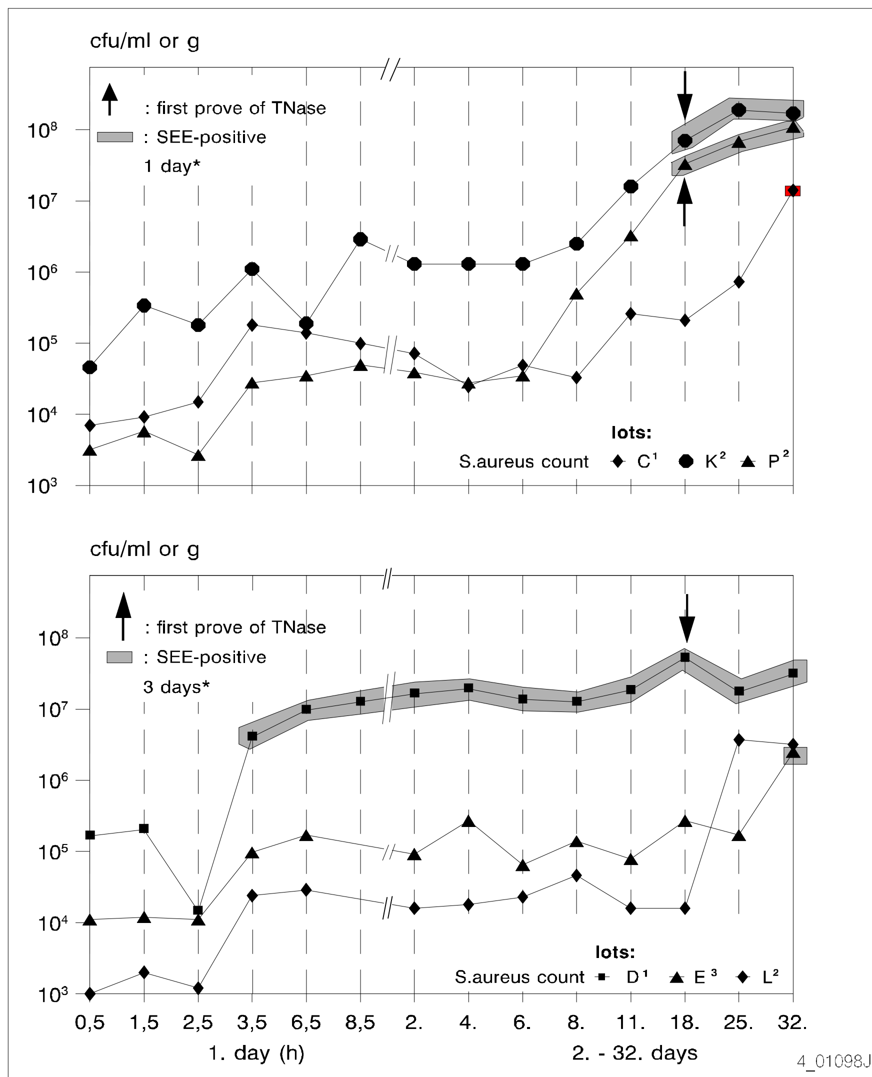

Fig. 3: Manufacturing of raw milk soft cheese with inoculation of an SEE-producing S.aureus First ocurrence of TNase-and Enterotoxin in relation to the growth of staphylococcus starter concentration (%): [1] 1.0; [2] 0.75; [3] 1.6
* starter pre-incubation: lots C, K, P (1 day), lots D, E, L (3 days)

4 Discussion

On the background of the EU Directve 92/46 resp. the German MilchVO '95 (5) it should be shown whether the demanded maximum values for S.aureus in raw milk and raw milk soft cheese are realistic under practical conditions. This concerns especially the multiplication of S.aureus and the production of enterotoxins and TNase. For this purpose enterotoxin producing reference strains in different initial counts were experimentally inoculated in raw ex-farm bulk milk for manufacturing raw milk camembert cheese. Besides the varying S.aureus counts different starter concentrations and activities were used.

The increase of S.aureus counts during the first day of manufacturing was also described by others (12, 13, 14) and may be explained besides the multiplication by a physical enrichment by the cheese curd (15, 16). Generally, this phenomenon depends on the kind of cheese and its stage during ripening and storage. In hard cheese a decrease is usual (16, 17) while at a higher water activity in soft cheeses counts of 10^6 - 10^7 cfu/g were observed (18, 19).

In the beginning of manufacturing the concentration and activity of starter cultures have a clear effect on the multiplication of pathogens (16, 20, 21, 22). In our experiments the concentration seems to have a more distinct influence than the activity. When low concentrations and/or low activities were used yet in the early stage of manufacturing S.aureus counts of $>10^6$ cfu/g can be reached. Especially, under non-professional conditions on farms this may happen. The counts of S.aureus in our experiments exceed by far the maximum (M) of 10,000 cfu/g as it is demanded in the EU Directive 92/46. These counts in any case are high enough to produce detectable amounts of enterotoxin (2).

The described relation of TNase- and enterotoxin production regarding the S.aureus counts of at least 10^6/g (2, 16) could also be proved by our own experiments as well as the fact that TNase mostly occurs earlier than the enterotoxin (13). In ten from eleven lots enterotoxin could be detected in a more or less early stage of manufacturing.

Regarding the standards for raw milk cheese on the level of manufacturing our results show that a maximum (M) of 10,000 cfu/g acc. to the EU Directive easily may be exceeded latest at the end of the ripening . Similar results were obtained from examinations for Brie and red smear cheese produced from naturally contaminated raw milk in dairy plants (23). For the postulated examination for toxins if M is exceeded, therefore, the TNase test as a rapid and reliable screening can be recommended.

Summing up, from our experiments it could be demonstrated that during the manufacturing process even at "allowed" counts of S.aureus in the milk may lead to a multiplication which enables the production of detectable amounts of enterotoxins. The methods used in the presented paper are feasible and reliable also for routine laboratories to fulfil the demands of the EU Directive.

5 Summary

The EU-directive 92/46 has laid down criteria for the presence of S.aureus in raw milk used for the production of raw milk products and in raw milk cheese. The regulations were transferred to German national law by means of the Milk Ordinance ‘95. The scope of the present investigation was to monitor the kinetics of multiplication and production of enterotoxins during the experimental manufacturing of camembert-cheese from artificially inoculated milk.

For the experimental production of camembert cheese, raw milk was used containing levels of 10^3-10^5 cfu S.aureus/ml after artificial inoculation with SEA- or SEE producing reference strains. The conditions of the starter culture varied in concentration (0.75 - 1.6 %) and time of incubation (1 or 3 days). Thermonuclease and/or enterotoxins were produced in 10 out of 11 lots. They even occurred in cheese manufactured from milk with low initial counts of 7.0 x 10^3 cfu S.aureus/ml, using starter culture (1.0 % concentration; 1 day incubation).

Thermonuclease- and/or enterotoxin-positive samples in all cases contained counts of > 10^6 cfu S.aureus/g and were observed mostly in the last stages of ripening (days 18-32). If milk with high initial counts of 10^4 - 10^5 cfu S.aureus/ml was used in combination with a long incubation period of starter cultures (3 days), S.aureus reached counts sufficient for thermonuclease and/or enterotoxin already on the first day of manufacturing. On the other hand, neither thermonuclease nor enterotoxin was detected in one lot produced from milk with 1.0 x 10^3 cfu S.aureus/ml, although the starter cultures were less active (0.75 % concentration, 3 days incubation).

The thermonuclease-test is recommended as an additional method for the screening or the confirmation of the occurrence of enterotoxins in cheese.

The present combination of methods is suggested as a basis for the development of a routine method according to the Milk Ordinance'95.

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Antibody capture ELISA for the sensitive direct detection of heat-stable toxin of Escherichia coli from soft cheese

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1 Introduction

Within the genus Escherichia coli (E.coli) during the last decades different mechanisms of pathogenicity could be detected (EPEC, EIEC, ETEC, VTEC/EHEC). On the background of the EU Milk Hygiene Directive 92/46 in certain milk products the presence of pathogenic E.coli strains has to be investigated if a maximum count (M) is exceeded. For the direct isolation and identification of heat-stable toxin (ST) of enterotoxigenic E.coli (ETEC) from soft cheese a method should be developed. Normally, in food no toxins are produced although toxigenic strains are present and multiplying. To save time and material the laborious isolation of respective E.coli colonies should be avoided to get quicker results regarding positive products. The idea was to incubate the untreated sample in special media to enhance the production of toxins and to detect these by an antibody capture ELISA.

2 Material and methods

2.1 Immunoassay

As an antigen for immunization E.coli heat-stable enterotoxin Sta (SIGMA E 5763) was used and transformed to an immunogene by a modified method according to AVRAMEAS (1) by linkage to glutaraldehyde.

Immunization of rabbits was done every ten days starting with 70 µl of the immunogen and continuing with 140 µl, 280 µl and 560 µl. The immunogene was coupled to Alugel S (Serva) and Freund adiuvans. A booster shot was set after 4 and 12 weeks after last immunization. The determination of the antibody titer and the performance of the ELISA was described previously (4).

2.2 Sample preparation for toxin detection

Sample material: Three soft cheeses with different contamination flora from the retail.

Inoculation of 10 g cheese in 90 ml prewarmed EE broth (Oxoid BM 317) with a ST-producer in different bacterial counts (10^3, 10^2, 10^1/g cheese).

Homogenization two minutes in the stomacher

Incubation in a shaking water bath, 4h at 37° C

Subculturing of 4 µl of the filtrate in 4 ml Caye 2-broth

Incubation 18h at 37° C in a shaking water bath

Spin down of the bacteria, 15 min at 3000 g

Dilution of the supernatant 1:5 in Caye 2-broth for the prevention of unspecific reactions

Detection by ELISA

3 Results

3.1 Immunoassay

Principle of evaluation

The extinction values are given as relative optical density (%OD). These are calculated by setting the zero values (control) as 100 % because it is a competitive ELISA. The meaning of %OD is that the extinction values of more or less positive samples are brought into percentage relation to 100 % (control). By this principle accidental differences from day to day are levelled out. In Figure 1 the following results are given:

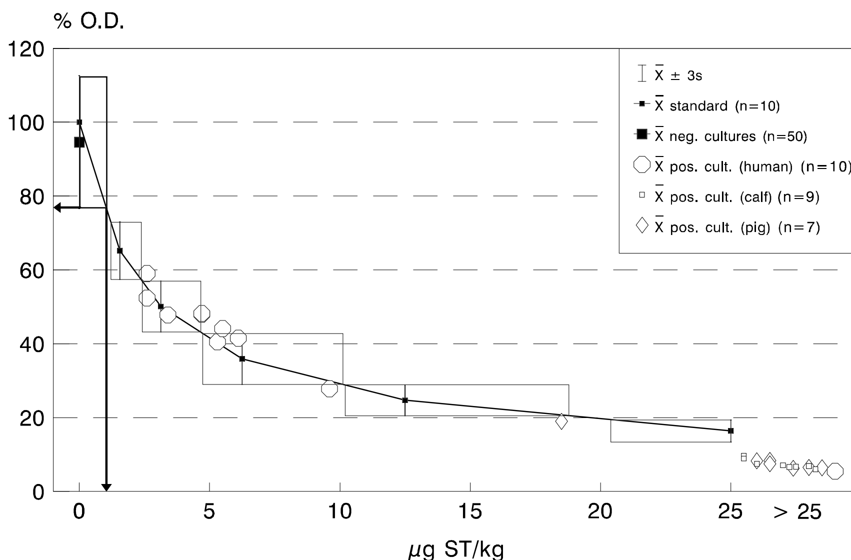

Figure 1: Standard graph of an E.coli ST-ELISA including readings of ST-negative and ST-positive reference strains

Pure toxin in dilution steps from 0 to 25 ng/ml was measured ten times independently. Considering a threefold standard deviation the results are shown in the graph. The height of the boxes indicate the threefold standard deviation at a given concentration. As one sees they do not touch one another which enables a quantitative identification. Additionally, the values allow the determination of the threshold (± 3s) between positive and negative samples expressed as %OD.

The obtained values of 15 different ST-negative strains from 3-5 repetitions showed a mean of 94.6 %OD compared with the 100 %OD from toxin free controls. By subtraction this resulted in a sharp threshold between positive and negative samples at 76.6 %OD regarding ± 3s. (see horizontal arrow).

Readings of positive reference strains (26 values from 12 strains of 1-5 tests) are indicated by different symbols regarding the origin from humans, calves and pigs. It is to be seen, that the strain from humans show a distinct lower toxin production than those of bovine and porcine strains.

The detection threshold (sensitivity) is to be estimated at about 1.0 µg ST/kg if you throw a line from the point of intersection of the threefold deviations of the zero-values and the positive readings (see vertical arrow).

The titer of antiserum is between 1:10,000 and 12,000.

The specificity of the test was checked by two independent tests using as substrates choleratoxin, all known S.aureus enterotoxins; all readings were negative. Out of 15 verotoxin (Shigatoxin) producing strains 13 were negative and two positive, obviously producing both toxins.

The repeatability is indicated by the coefficient of variation (cv) of the standard readings with pure toxin (n = 10 per concentration) of 5.36 and the ST negative cultures (n = 50) of 6.34.

Although for routine analysis it is sufficient to have a clear cut between positive and negative samples ST dilutions from 0-25 ng/ml were tested in a blind study by three independent repetitions: 57 of 60 samples could be determined within the respective $\pm$ 3s section quantitatively.

According to a routine test three isolates each of 70 French raw milk soft cheeses were examined and showed to be clearly negative.

3.2 Toxin identification in cheese

By artificially inoculated soft cheeses even 10 cfu of ST producing E.coli per gram could be identified in spite of a high contamination flora of coliforms and ST negative *E.coli* (Table 1).

The low amount (4 µl) of culture from EE broth to be subcultered in Caye 2-broth resulted from separate tests. Using e.g. 0.33 and 0.16 ml the test was not to read, obviously, because of too high amounts of sample matrix.

Table 1: Identification of heat-stable toxins (ST) free soft cheese with different levels of contamination flora after artificially inoculation with ST producing E.coli

Contamination flora/g		ETEC (ST)	ST
Coliforms	E.coli	g/cheese	ng/ml
4.3×10^4	2.4×10^3	6.5×10^2	> 25
		7.6×10^1	1.7
		7.3×10^0	0
2.4×10^5	3.8×10^1	7.6×10^2	> 25
		7.9×10^1	> 25
		7.6×10^0	3.1
4.3×10^4	1.5×10^2	1.4×10^3	> 25
		1.5×10^2	> 25
		1.1×10^1	9,0

4 Discussion

Similar immunoassays described in the literature (2, 5-9, 10, 11) are mainly based on selfmade antigens by complicated procedures. In contrary to this we used a commercially available toxin for immunization and coating of the solid phase for the ELISA. The necessity of a simple test results from the EU Directive 92/46 where the identification of toxigenic E.coli is asked. The advantage of the test described is that those strains may be identified also in heavily contaminated samples. Because, obviously, in spite of the presence of toxigenic strains no toxin is produced in the substrate, normally you have to isolate at random suspicious colonies by laborious enrichment methods. This problem was avoided by stomaching and incubation of the sample in a selective medium (EE broth) to enhance the growth of E.coli and subculturing in Caye 2-broth for optimum toxin production. The toxigenic strains present in the sample thereby can be detected directly by

their toxin. Even seven cfu/g of soft cheese could be detected in spite of a high contamination flora. These principle has proved yet for heat labile toxin of ETEC strains (3). The advantage without any doubt is a saving of time and material and offers a more reliable test than the at random isolation of e.g. 5 colonies from a sample from a certain dilution step. This principle may be transformed to other toxigenic bacteria species in food hygiene.

5 Summary

On the background of the EU Milk Hygiene Directive 92/46 a simple, sensitive and specific ELISA for the detection of heat-stable Escherichia coli toxin (ETEC-ST) was developed. The principle is a competitive antibody capture test. For immunizing rabbits a commercially available toxin (Sta, SIGMA) was used. The test allows to detect within a few hours the toxin from a culture supernatant in a sensitivity of 1 ng/ml - qualitatively and quantitatively. The at random isolation and further handling of suspicious colonies from a selective medium normally is very laborious. Therefore, an additional method was developed by incubating the sample, e.g. 10 g of cheese in the selective EE-broth which is subcultured and incubated again in Caye 2-broth for enhancing the toxin production where from the toxin can be identified. Even at a contamination flora of more than 10^5 coliforms/g and 10^1 E.coli yet 7 toxin producing E.coli/g in the original flora could be detected. This method enables a large saving of material and time and, additionally, is more reliable to detect toxin producers in a sample than the at random isolation of suspicious colonies.

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Influence of sample sizes and processing of milk powder for the isolation of Salmonellae

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1 Introduction

For the isolation of Salmonellae from milk powder normally 25 g samples should be examined. This results in an enormal need of time and especially of material in routine analysis. Therefore, it is permitted by different standard methods to pool samples. Of course, the pre-enrichment solution (double buffered peptone water-PW) always has to be used in a tenfold amount independent of the size of samples. However, it is not indicated up to how many multiple samples may be examined simultaneously. Therefore, an examination was done to find out the effect of pooling on the isolation rate of Salmonellae.

2 Materials and methods

The basic material for these examinations was a naturally contaminated skim milk powder of one day-lot of 20 tons which was made available to us from a dairy plant. The advantage is in comparison to experimentally contaminated powder that you will have the typical clusters of Salmonellae which makes the isolation and the statistics so difficult. But on the other hand you have realistic conditions as they appear in routine analysis. The material we used were 6 bags (A-F) à 25 kg from the mentioned lot.

For one run 3 kg were taken and divided in different groups of sample sizes a follows: 1 x 750 g, 3 x 250 g, 6 x 125 g and 30 x 25 g each corresponding to a batch of 750 g. Additionally, the original powder was „diluted“ 1:2 and 1:4 with Salmonella-free powder which, however, had a high contamination flora of 3.4×10^3 - 2.3×10^4 cfu/g.

The isolation procedure was a simplified standard method which has proved since years to be as effective:

- Resuscitation of the sample (1:10) in double-buffered peptone water, 8-24 h at 37°C;
- subculturing of 0,1 ml in 10 ml Rappaport-Vassiliadis medium, 24 h at 43°C;
- subculturing by streaking of XLD agar, 18-24 h at 37°C;
- subculturing of subspicious colonies on blood agar, 18-24 h at 37°C;
- confirmation by bacteriological and/or serological (polyvalent sera) and/or biochemical (API 20E) tests

3 Results

By a modified MPN method Salmonellae counts could be detected up to 22,5/500 g.

Table 1 shows the positive results in the original milk powder from the different bags A-F from three independent examinations.

Table 1: Number and percentage of Salmonella positive samples from three independent examinations of original skim milk powder

bag	750 g n = 3	250 g n = 9	125 g n = 18	25 g n = 90
A	3	7	13	12
B	3	7	10	15
C	3	8	9	23
D	3	7	13	26
E	3	6	9	11
F	3	6	4	7
pos./n	18/18	41/54	58/108	94/540
% pos.	100	75.9	53.7	17.4

It is to be seen that the positive scores increase according to the size of sample, i.e. the 750 g samples show the best results. The 17,4 % positives of the 25 g samples, however, do not mean, that the probability to detect Salmonella is only as low. The 30 small samples all are from one batch, which therefore has to be discarded, even if only one of the 30 samples would be positive. On the other hand, however, the results show that it is a professional error to examine only one 25 g sample from a lot as it is, unfortunately, done by some investigators. Normally, nowadays skim milk powder, especially high heat powder, has a very low contamination flora of only some hundreds/g. To see the influence of low Salmonella numbers and a higher contamination flora the original powder was diluted with „dirty“ powder 1:2 and 1:4.

Table 2 shows the results of a 1:2 diluted powder from 3 independent examinations per bag. The principle relation according to the size of samples was comparable to the results of the original powder, but on a lower level. Even two batches (750 g in bag F and 125 g in bag A) showed false negative results in all repetitions.

This tendency continues in the 1:4 diluted powder as it is to be seen from Table 3.

These results show as it was expected at least theoretically that the positive results decrease depending on the decreasing size of the sample and additionally on the dilution. A statistic evaluation cannot be done due to the few numbers of data.

An evaluation can be done, however, by the following aspects:

- All bags contain Salmonellae;
- the total of 3 kg as a basic material for one examination splitted in the mentioned sample sizes may to be seen as one independent „lot“ as it could be in practice;
- for every of this lots 750 g have to be investigated by one of the mentioned sample size models;
- the lot has to be refused if only one sample within the lot is Salmonella positive;
- in these investigations therefore 18 lots were available (3 independent examinations of 6 bags).

Table 2: Number and percentage of Salmonella positive samples from three independent examinations of 1:2 diluted skim milk powder

bag	750 g n = 1	250 g n = 3	125 g n = 6	25 g n = 30
A I	0	2	0	2
A II	1	1	0	2
A III	0	0	0	1
B I	1	0	1	4
B II	1	1	1	1
B III	1	1	3	1
C I	1	2	1	3
C II	1	2	0	1
CIII	1	1	2	1
D I	1	3	1	2
D II	1	2	3	2
D III	1	1	0	3
E I	1	0	0	3
E II	1	3	0	0
E III	0	2	2	3
F I	0	1	3	1
F II	0	1	0	2
F III	0	1	1	0
pos./n	12/18	25/54	18/108	32/540
% pos.	66.7	44.4	16.7	5.9

Table 3: Percentage of Salmonella positive samples depending on sample size and dilution

milk powder	750 g	250 g	125 g	25 g
original	100 %	75.9 %	53.7 %	17.4 %
1:2	66.7 %	44.4 %	16.7 %	5.9 %
1:4	44.4 %	25.9 %	16.7 %	4.4 %

Under this aspect the Salmonella positive lots depending on the dilution and sample size are summed up in Table 4.

Table 4: Percentage of Salmonella positive „lots“ depending on sample size and dilution

milk powder	750 g	250 g	125 g	25 g
original	100 %	100 %	100 %	100 %
1:2	66.7 %	83.3 %	55.6 %	88.9 %
1:4	44.4 %	55.6 %	72.2 %	72.2 %

On this basis, consequently, all lots of the original powder had to be assessed as Salmonella positive, independent of the sample size, but on condition that the total of 750 g is examined by one of the splitting models. The second best score of 88.9% was with the 25g samples.

For the transfer of these results to routine analysis the following recommendations could be made:

For the assessment of one lot of milk of high quality concerning the reliability and the enormous saving of material and processing time it may be recommended to examine a single batch of 750 g. In this case it is necessary to use about 10 l flasks for the 7,5 l volume of pre-enrichment and shake them several times during incubation.

If you have milk powder of less quality the examination of 30 x 25 g per lot seems to give better results regarding the total lot.

Accompanying to this trials another question was dealt with: according to some standard methods IDF, ISO, LMBG § 35 (1, 5, 6) for some substrates it is allowed to use distilled water or a salt solution for pre-enrichment instead of peptone water. The background of this is that the substrate milk powder itself contains enough of amino acids, oligo peptides etc.

The results of a comparison is shown in Table 5:

Table 5: Comparison of different pre-enrichment (resuscitation) solutions for the isolation of Salmonellae (positive samples)

solution	milk powder original n = 30	milk powder 1:2 n = 30
distilled water	14	2
salt solution	13	0
peptone water	13	3

Since years we, therefore, use a double buffered salt solution corresponding to the double buffered peptone water but without the peptone itself.

4 Discussion

The main question of this investigation was to see the relation of the multiple number of pooled 25 g milk powder samples and the efficacy of positive Salmonella isolations. The aim could be saving enormously material and time for the examination, when e.g. the FOSTER plan (3) has to be carried out. Simular investigations were done by others (2,7,8).

Our results show that an optimal recommendation is depending on the quality of milk powder. For a high heat powder which usually contains a very low contamination flora of only some hundreds/g it is obvious to use the biggest batch of 750 g in 7.5 l pre-enrichment solution in a 10 l vessel. For worse powder with a high contamination flora and less Salmonellae the examination of 30 x 25 g per lot seems to show better results. But these kinds of powders are very rare in food hygiene. The reason we used the 750 g batch as the biggest amount may be explained by the FOSTER plan. There you need for the highest quality level 60x25 g per lot, which means an amount of 1.5 kg. In many dairy

plants, nowadays, autosamplers are used according to Habraken et al. (1986), which deliver aliquots of about 2.5 g during the drying process for test samples. By this a much higher chance to detect Salmonellae is given. Therefore statistically the 750 g batch is sufficient.

The additional examination to save a lot of material and money is the use of a salt solution or distilled water for resuscitation of milk powder. Both modifications are real improvements to simplify the isolation procedure of Salmonellae detection.

5 Summary

The aim of these examinations was to see the effect concerning the detection of Salmonellae by different pooling of milk powder. In three independent trials for each examination 3 kg of naturally contaminated skim milk powder were taken and divided into batches of 1x750 g, 3x250 g, 6x125 g and 30x25 g. The original powder additionally was "diluted" 1:2 and 1:4 using heavily contaminated but Salmonella-free powder containing a contamination flora from 3.4×10^3 - 2.3×10^4 cfu/g. The isolation procedure was done by a modification of the usual international standards. In the original powder from all 750 g samples (n = 18) (6 bags and three independent investigations) Salmonellae could be isolated (100%). From the same batch from 540 (6x90) 25 g samples only 17.4% were positive. In the 1:2 powder the relation of results were comparable but on a lower level. This continued with the 1:4 dilution. However, it is to emphasize that not only e.g. 17.4% of Salmonellae were detected. In principle, according to the FOSTER plan 30x25 g has to be examined for one lot. If only one of these is positive the total lot has to be refused. Therefore the results have to be interpreted regarding not the single sample than the total lot. Additionally, the effect of replacing peptone water for resuscitation in milk powder by salt solution or distilled water was investigated. The results were comparable which enables to save a lot of expenses. A recommendation for a more material and time saving method is made.

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A post-modern food control labs network in the Argentine Northwest

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Abstract

The “Post-industrial Rapid Food Lab” has been conceived for Latin America’s regions, and designed for small communities (< 100,000 inhabitants) with very limited budgets. It can be handled by two food scientists, and two food examiners. As an official laboratory, it tests the food consumed in a district and fulfils the task of enforcing food legislation.

It is a post-modern view point; the food problem is seen as preventive, in real time, on going and dependent upon local circumstances.

Besides being a survey that is descentralized, cheap and rapid, the lab allows the control and guidance of new food factories set up in Catamarca Province, guaranteeing the quality of goods at the very moment of trading in the domestic as well as the international international market (Mercosur).

1 Introduction

Catamarca’s National University has sketched a food laboratory model, the “Post-industrial Rapid Food Lab”, that is especially suited to Latin America, particularly for small communities (<100,000 inhabitants). Based on H. Deelstra’s concepts and considered an official laboratory, it tests food consumed in a district and fulfils the task of enforcing food legislation. From a theoretical point of view, this type of Lab introduces a post-modern view of food control: little units with great efficiency. In Latin America, there are burocratic national or provincial labs settled in big cities and monitoring food at a distance with slow inspection procedures. Regions depend on hypothetic budgets that never come or come too late. In the proposed new scheme, every zone has its highly trained food examiners and scientists, together with their rapid Lab, that in time is connected to state Labs just in case of higher complexity. A *preventive approach* to the food problem is achieved, in real-time, on going and based on the local situation. Moreover, FAO’s equivalency concept of analytical methods justifies the settlement of these official Labs, technically up-to-date but suited to each district. This paradigm requires highly trained technicians, specially taught and guided by the University.

2 Deelstra’s model

In his primitive food control plan, H. Deelstra thinks about the Lab that is accessible, rapid and cheap; this would mean - in Latin America - *possible.* In the third world, food situation is often chaotic, and sometimes goes unknown. All kind of adulterations are practised; food and water contamination exists. Cost-benefit relation is high, sometimes enormous. A rather small investment (that the officials ignore or reject when they are politically corrupt) could be a great support not only for domestic consumption control but also for the export trade. Besides, it could help with setting up food factories, by training consumers in sanitary or nutritional problems. Deelstra states three main goals of food control:

1. To promote safe and well presented foods, so the consumer is protected from adulteration, contamination and putrefaction.
2. To control exported or imported foods, ensuring their quality and healthiness.
3. To assist local food factories, in order to generate trading in the international market.

Normally, in Deelstra's opinion, effective food control requires labs, instruments and technicians. If there are enough funds for the labs, sophisticated equipment must be avoided, for two reasons: the scarcity of food scientists highly trained in analytical chemistry; and the conditions of labs which are not the same as in developed countries (need for air conditioned, gas tubes - for instance for gas chromatography-stable current, etc.). You must, on the contrary:

- Apply modern kits based in biochemical or biotechnological methods, to solve problems.
- Search, as the funds are limited, for coherent problems that, for instance, quantify the impact of a specific contamination in safety of foods being consumed domestically: such as the micotoxins in tropical countries.
- Use semi-quantitative or quantitative TLC techniques.
- Always apply simple, cheap and rapid methods.

This conceptual approach gives rise to our "Rapid Labs".

3 Division of the lab

The "Rapid Food Lab" is divided into four areas: *Foods (in general), Microbiology, TLC and Pesticide Residues.* These modules work as follows:

1. FOODS: Food screening methods.
2. MICROBIOLOGY: The indicator micro-organisms are identified.
3. TLC: This technique facilitates the identification of prohibited compounds and semi-quantitative determination of admitted ones.
4. PESTICIDE RESIDUES: The RBPR system gives, in a few hours, the overall toxicity of insecticides and fungicides in fruits and vegetables. If a certain value is exceeded, the shipment with toxic results is prevented from trading for further chemical analysis.

This Lab can be seen as a "food cell", included in the food security network. These units cluster around a state or provincial laboratory, which is connected at the same time with other state or national labs. In Argentina, the comprehensive national network is responsible for sending the information related to food adulteration and forbidding trade.

Frequently this information is produced by small labs, in places where transgressors send the adulterated food trusting that it will not be detected. The "Rapid Food Lab" needs highly trained and ethically irreproachable food examiners. They must be aware of food legislation and its enforcement, trained in some important laboratory techniques to detect problems in shipments (that could then be prevented from trading) and be qualified in sanitation and food hygiene.

Food scientists and examiners are now being trained in Catamarca National University, which acts as a generator of knowledge. These professionals are considered imperative for the performance of "Rapid Food Labs".

4 Towards a new philosophy in food control

Nowadays there is a post-modern philosophy of uncertainty and suspicion in the consumer, which states new paradigms for food control. After seeing with astonishment the great ecological and food catastrophes of the modern age (Seveso, Bophal, Sandoz, Tchernobyl, Toxic Oil Syndrome in Spain, Mad Cow Disease, etc.), the consumer awareness of toxic risks has increased dramatically. Obsessed by chemistry, he is concerned with nutrition, security, sanitation, legal outlooks, methods of analysis, agricultural outlooks preceeding food processing, risks of bio-technology, in one phrase: The end quality of the food.

This new critical awareness means new responsibilities in food control: the consumer, whose culture allows him to select the safest and most nutritious food, collaborates with the food scientist as he fights against fraud. The Lab cannot be bound to modern utopias, as its work is now post-modern, meaning descentralized, upstream, flexible, and innovative. It becomes adapted to a complex reality, in which the rigidity of planning changes to the ductile design of analysis.

Due to this concern for the micro and the efficient, the Post-industrial Rapid Food Lab shows - at the end of millenium - worry for safe and nutritious goods and a guaranty for trading regional food products.

Detection of residues of quinolones in milk

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Abstract

For the detection of enro- and ciprofloxacin (EFX, CFX) residues in milk on the level of an expected MRL of <10 µg/kg as well screening (microbial inhibitor test with E.coli, ELISA) as well as confirmation (HPLC) methods were developed and applied to the examination of milk of 3 cows treated with Baytril®. The detection of further quinolones by these methods was tested in orientating experiments. HPLC analysis is performed after solid phase extraction with oxalic acid/triethylamine/acetonitrile as an eluent and fluorometric detection (295 nm/495 nm); limit of was ≤2.5 µg/kg for CFX, EFX, marbofloxacin and danofloxacin. In the milk of the treated cows CFX, EFX, CFX metabolite M1 and two unidentified substances were detected. No false negative results of the screening methods with respect to HPLC values ≥3.0 µg/kg were observed.

Introduction

Enrofloxacin (EFX) belonging to the group of quinolones is widely used in the treatment of bacterial infections in veterinary medicine, but it is not (yet) licensed for the treatment of lactating cows. A biologically active metabolite ciprofloxacin (CFX), which has been restricted to use in human medicine, is excreted with milk after EFX treatment. A maximum acceptable daily intake (ADI) value of 18.75 µg EFX + CFX/person was derived. According to EU Regulation 2901/93 a maximum residue limit (MRL) of 30 µg/kg is fixed for muscle, liver and kidney. Based on a daily food intake of 500 g of combined tissue and 1500 ml milk following the ADI-concept a MRL of about 2.5 µg EFX + CFX/kg for milk has to be expected. The MRL of marbofloxacin (MFX) in milk is 75 µg/kg (EU Regulation 2017/96).

Methods for an integrated detection system (1) which comprise a microbiological inhibitor test for screening, an antibody-capture immunoassay (ELISA) for preliminary confirmation and a HPLC method for confirmation were developed and put to test with milk samples of EFX-treated cows. The detection of further quinolones by these methods was tested in orientating experiments

Materials and methods

Quinolones:

EFX, CFX and metabolites M1-M4 of CFX were donated by Bayer, Wuppertal (DE), danofloxacin (DFX) by Pfizer, Karlsruhe (DE), MFX by Laboratoire Pharmaceutique Véterinaire, Lure Cedex (F) and Sarafloxacin (SFX) by Abbott Laboratories, Chicago (US) and flumequin (FQ) was purchased at Sigma (DE). For further dilutions 1 million µg/kg stock solutions in alkaline methanol were prepared.

Treatment trial:

3 healthy cows (somatic cell counts <50 000/ml, milk yield 19-23 kg/day) of the experimental herd of the Federal Dairy Research Centre were treated i.v. with 20 ml of Baytril® (Bayer, DE); samples were taken before treatment, 2, 4, 6, 8, 10 hours after treatment and afterwards during normal milking time (14/10 hours), stored at -18°C and analyzed within at 3 weeks at maximum.

Microbial inhibitor test:

Agar diffusion test at pH 0.5 with *Escherichia* coli ATCC 11303 (2) as test microorganism and bromcresol purple as indicator in microtitre plates. Milk samples are heated (10 min at 80°C) before analysis. On each test plate negative and positive controls (10 μg EFX/kg) have to be analyzed.

Incubation:

1 h at 6°C and 18 h at 37°C. Test results are evaluated visually (purple = positive, yellow = negative) or by photometric measurement by an ELISA reader (3). The following detection limits (μg/kg) were determined: CFX 4, DFX 8, EFX 10, FQ 500, MFX 10 and SFX 20 (4).

ELISA:

Antibody-capture immunoassay for EFX with 100% cross reaction with CFX and limit of detection (LOD) of 1.56 µg EFX equivalents/kg (5).

HPLC:

The vacuum manifold was an Adsorbex solid-phase extraction unit (Merck). The SPE columns contain 500 mg Chromabond C18ec (Macherey-Nagel). A model L-6200 gradient pump, F-1080 fluorescence detector, AS-2000 autosampler, D-6000 interface, LC-organizer and L-5025 column thermostat were supplied by Merck. The analytical cartridges were EcoCART 125 mm/3 mm (Merck, DE) with Superspher 60RP 8ec.

SFX (50 µg/kg milk) was added to the milk sample as an internal standard. A mixture of 5 ml milk, 3 ml acetonitrile (ACN), 2 ml trichloracetic acid and 5 ml water was centrifuged and filtered. To 12 ml filtrate 0.5 ml sodium acetate buffer and 10 ml water were added. SPE-column conditioning was carried out with 5 ml ACN and 5 ml water. Sample elution was effected with 2 ml buffer (oxalic acid, triethylamine, pH 2.9)/ACN (30:20); the eluate was concentrated by vacuum concentration; the volume was made up to 2 ml with eluent (final concentration 2:1). The mobile phase was a gradient of ACN and water or eluent respectively at a flow rate of 0.5 ml/min. The column was thermostated at 30°C, the injection volume was 10 µl. The detector was operated at excitation and emission wavelengths of 295 nm and 495 nm, respectively. If also DFX, MFX and FQ have to be detected the wavelength have to be changed to 325 nm/365 nm after 21 min and the analytical time is lengthened to 30 min. For evaluation, peak areas were included into linear regression equations derived from the analysis of aqueous standard solutions. The price of consumables per sample were calculated to be <9 DEM.

Fig. 1 shows a chromatogram of aqueous standard solution (2.5 µg/kg). Analyzing CFX 4 peaks appear; one could be identified as CFX metabolite M1, whereas the two others had different retention times than the tested CFX metabolites M1-M4. **Tab. 1** comprises the data of the examination of blind and spiked samples and the derived recovery values.

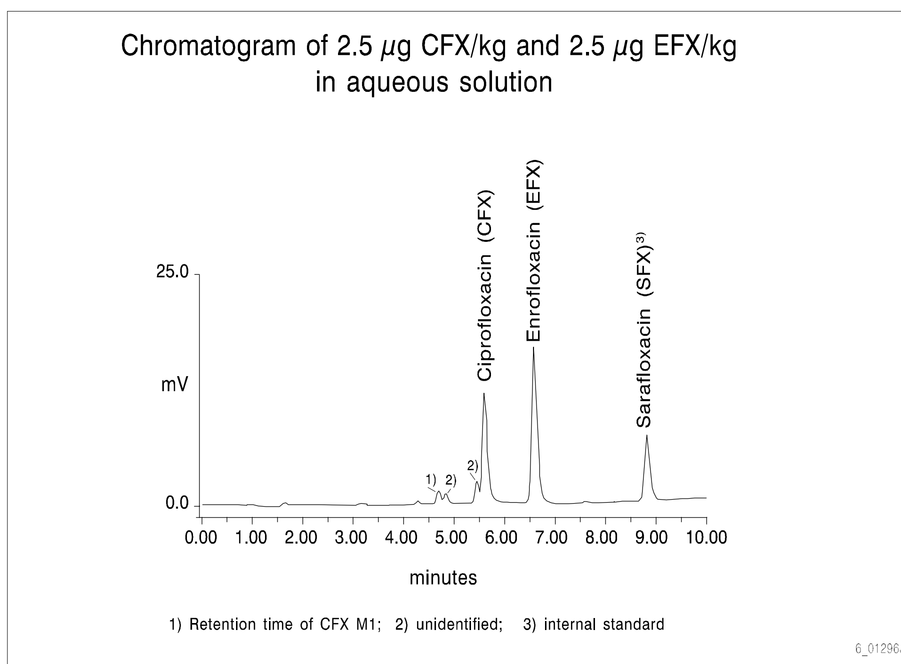

Figure 1

Table 1: Characteristics of the HPLC method for the detection of quinolones in milk

	Retention time (min)	LOD[1)] µg/kg	LOQ[1)] µg/kg	Recovery %	Recovery µg/kg	Repeatability s_r	Repeatability µg/kg
MFX	9.7	≤1	≤2.5	108[2)]	75.0	0.3	2.5
CFX	10.2	≤1	≤2.5	68	2.5	0.3	2.5
DFX	10.8	≤2	≤2.5	101	2.5	0.3	2.5
EFX	12.6	≤1.5	≤2.5	72	2.5	0.1	2.5
SFX	19.0		<50[3)]	76	50.0	–	–
FQ	26.2		<50[3)]	–	–	–	–

[1)] X_Q of negative milk + 3/6 s respectively; [2)] without SPE, [3)] preliminary

Treatment trial

Fig. 2 shows a chromatogram of a milk sample from the anamnestic phase and of a milk sample collected 24 hours *post applicationem*. For this experiment the analytical conditions were adjusted to the detection of CFX, EFX and SFX. It becomes obvious that in the milk sample of the treated cow several peaks appeared. One showed the same retention time as CFX metabolite M1 whereas the others could not be identified.

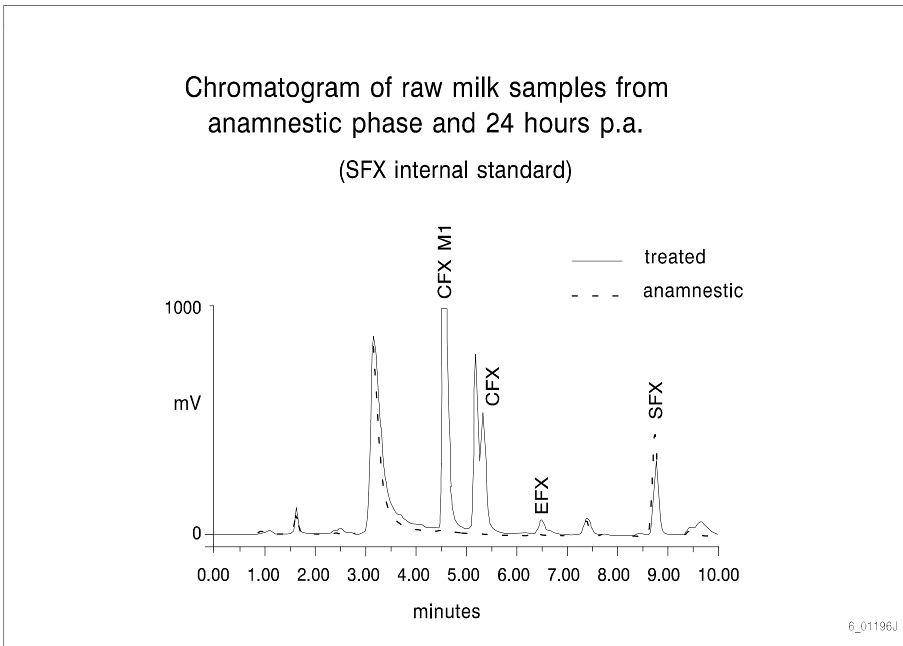

Figure 2

Tab. 2 summarizes the results of the treatment trial. It becomes evident that in the milk of cows treated with EFX its metabolite CFX can be detected in higher concentrations and for a longer period than EFX as also reported by Tyczkowska et al. (5). 82 hours p.a. CFX was <LOD and below the expected MRL of 2.5 µg/kg. The screening test with *E.coli* was positive in all samples with $\geq$3 µg CFX/kg and negative in samples $\leq$2 µg CFX/kg. The results of the ELISA were >LOD in all samples with at least 2 µg CFX/kg; 5 samples, in which no quinolones were detected by HPLC, showed values close to the LOD of the ELISA; in no case false negative results with respect to the HPLC values were observed.

Table 2: Excretion of enro- and ciprofloxacin (EFX, CFX) after i.v. treatment of 3 cows with Baytril®

Time after treatment (h)	EFX (µg/kg)[1)]	CFX (µg/kg)[1)]	ELISA (µg/kg)	*E.coli* test[2)]
-24	<LOD	<LOD	<LOD	0
-14	<LOD	<LOD	<LOD	0
0	<LOD	<LOD	<LOD	0
2	300	1863	>100	3
4	61.3	1232	>100	3
6	17.2	615	>100	3
8	9.8	273	>100	3
10	5.5	230	>100	3
24	<LOD	19.7	9.4	3
34	<LOD	11.0	6.7	3
48	<LOD	6.0	5.3	3
58	<LOD	4.6	4.5	3
72	<LOD	2.8	4.3	2
82	<LOD	<LOD	2.4[3)]	0
96	<LOD	<LOD	<LOD	0

[1)] Recovery rates considered; [2)] Number of positive results out of 3; [3)] n = 2, n = 1 <LOD

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Proficiency testing of microbial inhibitor tests[2]

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Abstract

Seven microbial inhibitor tests (BR-Blue Star, BR-AS, BR-EC, BR-6, Charm AIM-96, Delvo SP and *B.cereus* microtitre test with indicator) were examined in 8-9 participating laboratories with respect to the detection of 5 antimicrobials (oxytetracycline, sulfadimidine, sulfadimethoxine, gentamicin, spiramycin). Surprisingly high were the differences in readings of the test results between the participating laboratories. Including all tests the extreme values between laboratories were 10% and 40% positive results respectively. Causes of this different interpretation might be subjective interpretation of colour and/or adjustment of correct reading time. Defining the sensitivities of this proficiency study as those concentrations were at least 80% of the results were indicated as positive the following antimicrobials can be detected at ≤ MRL level: Oxytetracycline (*B.cereus*), gentamicin (Charm AIM-96), sulfadimethoxine (BR-Blue Star, BR-AS, BR-EC, Charm AIM-96, Delvo SP). None of the included inhibitor tests is suitable for the detection of sulfadimidine and spiramycin.

Introduction

In the context of the checks provided for in Article 14 of the EU-Milk Hygiene Directive 92/46 the EU Member States have to ensure that tests are carried out to detect residues of antimicrobial substances exceeding maximum residue limits (MRLs) which are fixed according to EC-Regulation 2377/90. Within the IDF (International Dairy Federation) integrated system for the detection of residues of antimicrobials, which comprises the application of different methods and the definition of shared responsibilities of the parties of concern, microbial inhibitor tests play an important role as screening methods which is demonstrated in **fig. 1** (1). The interpretation of the graph is that milk samples are screened by microbial inhibitor tests and in dependency on further need - quality payment, self control in the dairy, food inspection - positive samples are further analysed by more sophisticated methods which allow identification and quantification. As there are not available microbial inhibitor tests providing satisfying detection limits for all antimicrobials in question, e.g. chloramphenicol, it is necessary to start examinations with specific and sensitive tests to detect those residues.

Factors and procedures which have to be considered when evaluating microbial inhibitor tests and consistently when interpreting test results are summarized in an IDF Standard (2). One item is the evaluation of tests within collaborative studies. The purpose of the proficiency study presented here was to evaluate the sensitivities of several microbial inhibitor tests for the detection of antimicrobials other than ß-lactam-antibiotics when analysed in different laboratories.

[2] The experimental work was initiated by a group of experts at the Federal Institute for Health Protection of Consumers and Veterinary Medicine, Berlin/DE. The authors are obliged to the members of that group for their participation in this study.

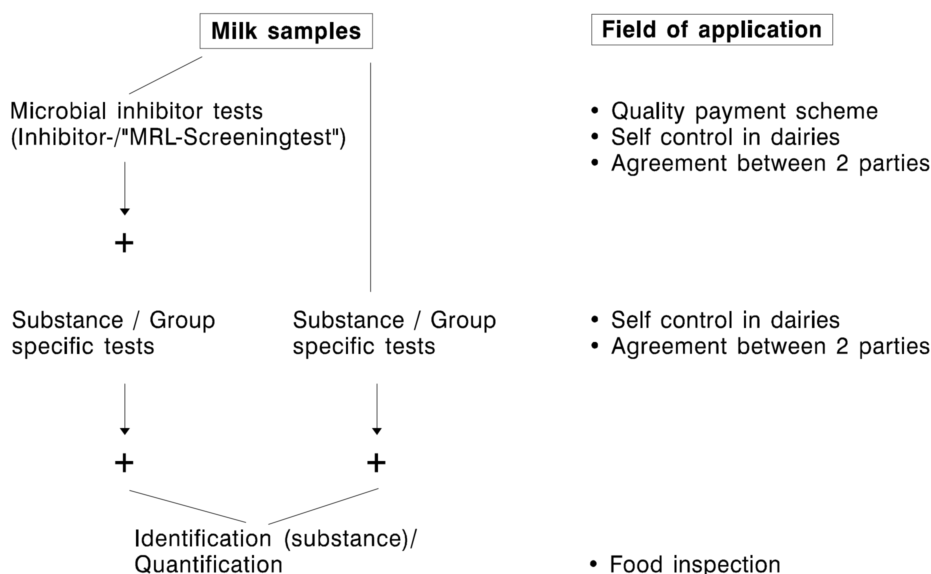

Figure 1: Position of microbial inhibitor tests within an integrated detection system

Experimental design

Three different batches each of the following microbial inhibitor **tests**[3] were included: BR-Blue Star[4], BR-AS[3], BR-EC[3], BR-6[3], Charm AIM-96[5], Delvo SP[3] and *Bacillus cereus* microtitre test (3).

For the preparation of the **test samples** raw milk from the experimental herd of the Federal Dairy Research Centre, which was not treated with antimicrobials in the preceding 4 weeks was skimmed by centrifugation. 3.3 ml portions of negative and test samples with the antimicrobials/concentrations listed in **table 1** were dispensed into tubes and lyophilized. The test samples for the 3 trials were prepared at the same time; they had to be reconstituted by 3 ml water on the day of use.

The lyophilized test samples coded at random, test kits and test protocols were mailed individually for each of the 3 kit batches to 8-9 participating laboratories. Due to the experimental design 4 results per substance/concentration and test and trial for every participating laboratory were obtained. The participating laboratories had to use their own negative/positive control samples and to indicate the test results as negative or positive.

[3] We thank the companies which provided us with the commercially available test kits free of charge.
[4] Gist Brocades, Delft/NL;
[5] Charm Sciences, Inc., Malden, USA.

Table 1: Antimicrobial/concentration combinations (µg/kg) of the test samples and MRLs and FDA safe/tolerance levels (µg/kg)

Substance	Supplier	Concentrations tested	EU-MRL[1)]	Codex MRL[2)]	FDA safe/ tolerance[3)]
Oxytetracycline	Serva 31357	30, 100, 150, 200	100	100	30/0
Sulfadimidine	Serva 35635	10, 100, 150, 200	100	25	10/0
Sulfadimethoxine	Sigma S7385	10, 100, 150, 200	100	–	10/10
Gentamicin	Serva 22185	30, 100, 200, 400	100	100	30/0
Spiramycin	Sigma S9132	75, 150, 200, 300	200	100	–
Negative milk	–	–	–	–	–

1) EU-Regulation 2377/90 ff
2) Codex Committee on Residues of Veterinary Drugs in Food
3) CFR 21 and CVM correspondence

Results

The **variation between test kit batches/trials** expressed in percentage of positive results within test and antimicrobial is summarized in **table 2**. From this table it becomes obvious that the variation between test kit batches depends on the kind of test and antimicrobial under study and has therefor to be evaluated individually for each antimicrobial/test combination. The greatest variation between batches were observed on the following combinations:

Oxytetracyclin: BR-EC
Gentamicin: Delvo SP
Sulfadimidine: BR-EC
Sulfadimethoxine: Charm AIM-96.

With regard to the total results it is striking that in the first trial/batch the lowest and in the third batch the highest number of positive results were indicated.

Table 2: Percentage of positive results within test and antimicrobial (n=144*) with respect to the test kit batch/trial

Antimicrobial Test	**Oxytetra-cycline**			**Genta-micin**			**Spira-mycin**			**Sulfa-dimidine**			**Sulfadi-methoxine**			**Total**		
Batch/Trial No.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
BR-Blue Star	10	0	31	5	14	14	0	0	5	27	47	40	72	68	68	23	26	33
BR-AS	8	2	8	6	5	8	0	1	0	28	31	38	74	64	73	23	20	25
BR-EC	**26**	**38**	**51**	17	17	24	10	9	14	**43**	**59**	**40**	72	74	78	34	39	41
BR-6	56	60	63	0	0	0	1	0	0	1	0	4	0	0	0	12	12	13
Charm AIM-96	26	34	35	70	79	74	1	1	0	29	20	28	**35**	**50**	**51**	32	37	38
Delvo SP	14	8	19	**22**	**20**	**49**	5	3	11	60	60	66	76	75	76	35	33	46
B.cereus	75	69	72	8	6	3	10	8	4	8	9	5	10	10	6	23	21	18
Total	31	30	40	17	19	24	4	3	5	28	33	32	49	49	52	26	27	31

*) Charm AIM n = 128

The **differences** in indicated positive results **between** the **participating laboratories** were evident for all substances and tests under study. The results are summarized in **table 3**.

Table 3: Extreme values of positive results (%) in participating laboratories with respect to the inhibitor test (n = 240/test and lab)

Test	Min	Max	Lab.No.
BR-Blue star	7	42	6/2
BR-AS	6	37	6/9
BR-EC	13	50	6/9
BR-6	4	14	6/7
Charm AIM-96	13	51	6/9
Delvo SP	17	52	6/3
B.cereus	14	44*	6/3

* Probably problems with test procedure as numerous negative samples were evaluated as positive by lab 3

The detection of oxytetracycline, gentamicin, sulfadimidine and sulfadimethoxine is demonstrated in **fig. 2** in form of dose (µg/kg) - response (% positive results) curves and the **detection limits** are summarized in **table 4**. Negative samples were evaluated as positive in some cases; this proportion was relatively high in the case of *B.cereus* test and lab. 3. The detection limits for this proficiency study were defined as the intersections of 80% and 90% positive results lines respectively and concentration. The spiramycin concentrations tested were not detected by any test. The demands according the EU and/or Codex MRLs (see **table 1**) were fulfilled for the following combinations of antimicrobial and test:

Oxytetracycline:	B.cereus
Gentamicin:	Charm AIM-96
Sulfadimidine:	–
Sulfadimethoxine:	BR-Blue Star, (BR-AS), BR-EC and Delvo SP
Spiramycin:	–

Table 4: Detection limits* (µg/kg) of oxytetracycline, gentamicin, sulfadimidine and sulfadimethoxine by various inhibitor tests

	Oxytetracycline		**Gentamicin**		**Sulfadimidine**		**Sulfadimethoxine**	
	80%	**90%**	**80%**	**90%**	**80%**	**90%**	**80%**	**90%**
BR-Blue Star	>200	>200	>400	>400	>200	>200	**85**	**100**
BR-AS	>200	>200	>400	>400	>200	>200	**90**	125
BR-EC	>200	>200	>400	>400	>200	>200	**85**	**100**
BR-6	135	150	>400	>400	>200	>200	>200	>200
Charm AIM-96	>200	>200	**90**	**100**	>200	>200	>200	>200
Delvo SP	>200	>200	>400	>400	135	200	**80**	**90**
B.cereus	**80**	**90**	>400	>400	>200	>200	>200	>200

* Detection limits within this proficiency study are defined as the intersections of concentration and 80 and 90% positive results lines respectively

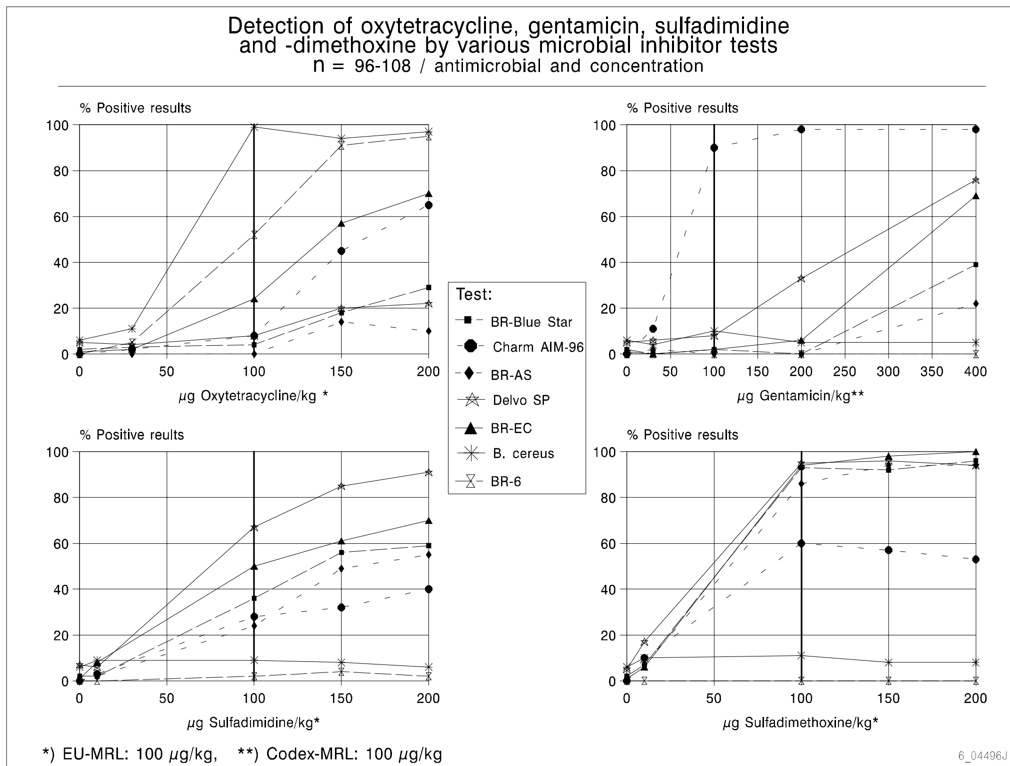

Figure 2

Conclusions

In interpreting the results it has to be kept in mind that the antimicrobials and concentrations included are more difficult to detect by microbial inhibitor tests with *B. stearothermophilus* as for example ß-lactam-antibiotics. Further it has to be considered that most often positive control samples containing penicillin are used and that due to the different mode of action of antimicrobials on the test microorganisms the colour change might differ.

Within the 3 trials the percentage of samples indicated as positive increased in the overall evaluation. Reasons might be

- different sensitivities of the test kit batches
- stability of the test samples and/or
- "learning effect“ of the participating people.

The variation between test kit batches/trials proved to be dependent on the test and antimicrobial under study. For some combinations the difference between the percentage of positive results of the 3 trials were in an order of magnitude of >20%. These results emphasize the need to check the stability between test kit batches with various antimicrobials.

The variation in test interpretation between the participating labs was surprisingly high. The differences between the maximum and minimum percentage of positive results were - with exception of BR-6 with only a low number of positive results - $\geq$30 %. The minimum figures were indicated in the same lab for all tests applied. Reasons of the different test interpretation might be

- adjustment of the correct incubation period and/or
- interpretation of the colour.

These unsatisfying reproducibilities of test results stress the need for better and feasible possibilities to standardize the test procedure including test interpretation by objective readings as for example ELISA reader (4, 5).

Defining the detection sensitivities of this proficiency study as those concentrations where at least 80 and 90% of the results were indicated as positive respectively (see **table 4**) the following tests are suitable with respect to the detection of the MRL level (EU and/or Codex):

Oxytetracycline: *B. cereus*

Gentamicin: Charm AIM-96

Sulfadimethoxine: (BR-AS), BR-Blue Star, BR-EC and Delvo SP.

The test kits under study detect different kinds of antimicrobials with the required sensitivities. Taking into account only positive test results all tests failed to detect sulfadimidine and spiramycin.

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Characterization of microbial transglutaminase

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Introduction

Transglutaminase catalyzes cross-linking of proteins by formation of isopeptide bonds (ε-(γ-Glu)Lys bonds) between protein-bound glutamine and lysine residues. Microbial transglutaminase (TG) from *Streptoverticillium sp.* is available as an additive for meat products to improve firmness. Here we present first data on the characterization of this enzyme.

Materials and methods

A "ready-to-use" preparation for the production of frankfurter type sausage („Activa WM", AJINOMOTO Europe Sales GmbH, Hamburg) was used for the studies. Protein was determined by a modified method according to Bradford [1]. Results were calculated using the absorbance quotient of A_{590nm} and A_{450nm}. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [2] on a gel with 14 % T (concentration of acrylamide and bisacrylamide) and 0,4 % C (concentration of bisacrylamide) at a constant current of 20 mA. Enzyme activity was determined by a colorimetric hydroxamate procedure described by Folk *et al.* [3].

Results and Discussion

Protein determination

The commercial available transglutaminase preparation consists of a sugar matrix in mixture with the enzyme to simplify dosage and processing. The determined protein content was only 0,25 %.

Molecular Weight Determination

On a 14 % SDS polyacrylamide gel 40 µg respectively 80 µg (0,1 µg and 0,2 µg protein) of the TG preparation were separated (Fig. 1) into two bands. The band at 10 kD was not identified, but its concentration was much lower than that of the main component showing a band at 42 kD. The latter value corresponds with the published molecular weight of transglutaminase.

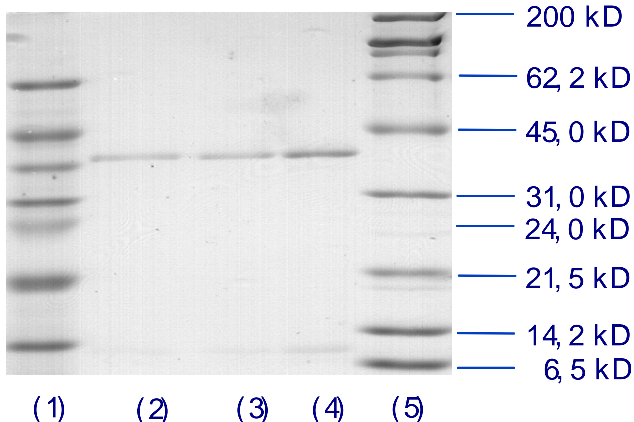

Figure 1: SDS-PAGE of the TG preparation for frankfurter type sausage on a 14% gel, stained with „Coomassie Brilliant Blue“. (1) Molecular weight marker I; (2) molecular weight marker II; (3) 40 µg TG preparation; (4) 40 µg TG preparation; (5) 80 µg TG preparation; (6) molecular weight marker I; (7) molecular weight marker II

Activity Determination

The TG activity test consists of two reactions. First the enzyme catalyzes the reaction of carbobenzoxi-L-glutaminglycine and hydroxylamine to hydroxamic acid. Together with Fe(III) the hydroxamate forms in a second step a red-coloured complex, which was determined at 502 nm. (Fig. 2).

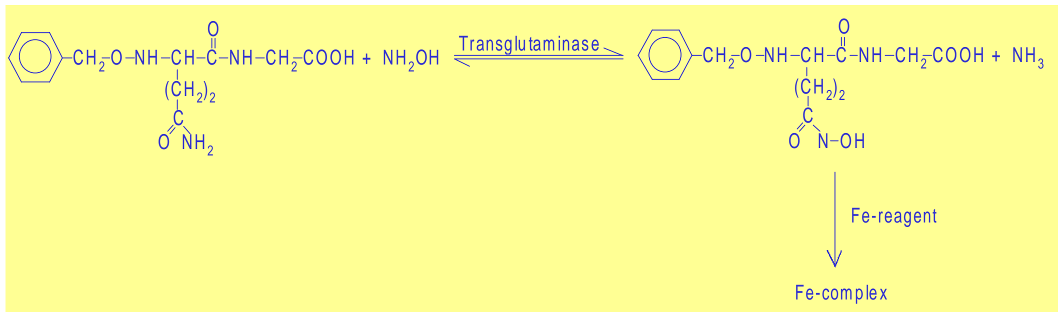

Figure 2: Reaction system for the determination of TG activity.

The Reaction rate was calculated from the quotient of the difference of the absorbance at 502 nm and a defined reaction time. The resulting reaction rates were plotted against substrate concentration (Fig 3). 1 ml test volume contained 250 µg of TG preparation (0,625 µg protein), 50 µmol hydroxylamine in 100 mM TRIS-buffer pH 8.0 at 37 °C. Incubation time for enzyme reaction was 10 minutes. Under this conditions a linear dependence between reaction rate and substrate concentration was measured between 0,5 µmol/ml and 5 µmol/ml.

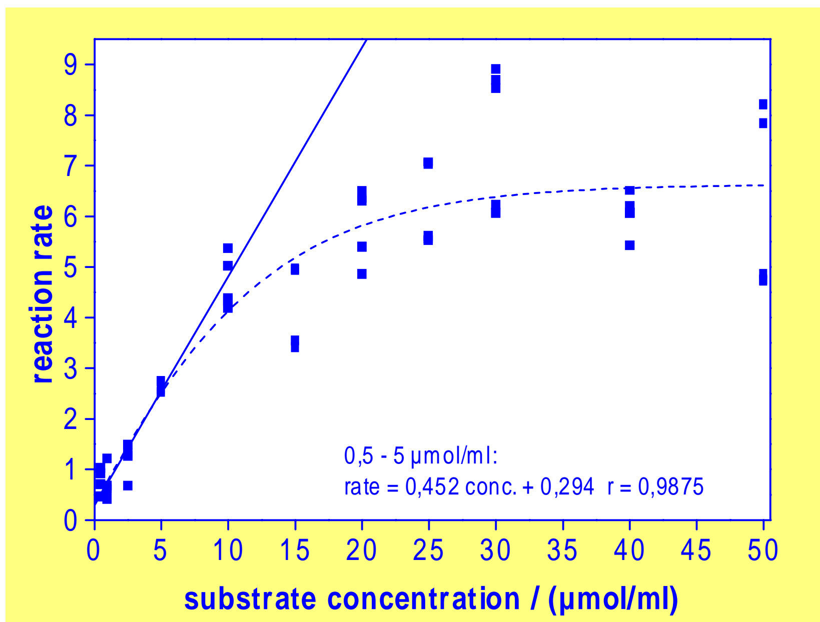

Figure 3: TG reaction rate in dependence of substrate concentration.

The influence of different parameters on TG activity e. g. temperature, pH, and salt concentration will be investigated in future experiments. The enzyme preparation will be characterized also by other kinetic data (V_{max}, Michaelis-Menten-Constant). The aim of further studies is to develop a method allowing the determination of TG activity in meat products.

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Session 2:

Food, Nutrition and Well Being

Biogenic amines and the quality of food

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Introduction

Biogenic amines (BAs) are alkaline compounds occurring in food and beverages. Amounts usually increase on fermentation of food or in the course of food spoilage [1]. Therefore, amounts and ratios of BAs serve as indicators for the quality and hygiene of foodstuffs. Histamine (Him), putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm) are the most abundant BAs in fish and fish products. Based on these characteristic BAs so-called biogenic amine indices (BAIs) are calculated [2]. In particular for assessing the quality of tuna a BAI is calculated by adding the amounts of tyramine (Tym), Put, Him, and Cad [3].

Because of the toxicological effects caused by the uptake of large amounts of Him, Tym, 2-phenylethylamine (Pea) and tryptamine (Trm), their amounts in food should also be limited [4,5]. For analysis of BAs preferably chromatographic techniques like high-performance liquid chromatography (HPLC) are used. Since most BAs occurring in food do exhibit neither satisfactory absorption in the ultraviolet or visible range nor show fluorescence, their determination is usually carried out by HPLC after chemical derivatization. Because of its high selectivity and sensitivity most analysts are using this method for the separation and quantification of BAs.

We present and discuss data on the BA content of food samples determined by HPLC and pre-column derivatization with oxycarbonyl chlorides (“chloroformates”). These reagents react with in 3 – 10 min at ambient yielding stable derivatives [6-9].

Experimental

Instruments

For HPLC an instrument comprising a Model L-7612 solvent degasser, L-7100 pump with low-pressure gradient-former, 7250 programmable autosampler, L-7300 column oven, and L-7480 fluorescence detector with 12 µl flow cell were used. This chromatograph was controlled by D-7000 HPLC System Manager including chromatography data station software (LaChrom® system from Merck-Hitachi, Darmstadt, Germany, and Tokyo, Japan). This instrument was used for the analysis of NOC- and FMOC-derivatives of BAs.

For analysis of PNZ-derivatives a HP 1100 series HPLC comprising Model G1322A degasser, G1311A pump with low-pressure gradient-former, G1313A autosampler, G1316A column thermostat, G1314A diodearray detector with 13 µl flow cell, and software HP ChemStation for LC (Rev. A.04.02) were used (Hewlett-Packard, Waldbronn, Germany).

Derivatized BAs were analyzed on a Superspher® 60 RP-18e column (250 x 4.6 mm ID, 4µm) (for NOC- and PNZ-derivatives), or a Superspher® 60 RP-8 column (250 x 4.6 mm ID, 4µm) (for FMOC-derivatives). Columns were provided by Merck.

Solvents and chemicals

All chemicals were of the highest purity available and were purchased from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), and Fluka (Neu-Ulm, Germany). Doubly distilled water was used for preparing all aqueous solutions. The reagent 2-Naphthyloxycarbonyl chloride (NOC-Cl) was synthesized from 2-naphthol and phosgene in toluene according to the literature [7].

Preparation of sodium acetate and borate buffers and reagents

Sodium acetate (NaOAc) buffer was prepared from AcOH (1.0 mol) in water (950 ml) by titration with NaOH (20%, w/v) to the pH-value needed and final dilution to 1 l by addition of water.

Potassium borate buffer was prepared from boric acid (33.43 g, 0.5 mol) in water (950 ml) by titration with KOH (20%, w/v) to the pH-value needed and made up with water to 1 l.

As derivatizing reagents 5 mM NOC-Cl (MeCN), 3 mM FMOC-Cl (acetone) or 25 mM PNZ-Cl (acetonitrile) were used and an aqueous solution of glycine served as scavenger reagent.

For standards of BAs (10 mM) the corresponding hydrochlorides were dissolved in 0.1 M HCl. Suitable concentrations for calibration were prepared by appropriate dilution. As internal standard 10 mM 1,6-diaminohexane (Dhx) in 0.1 M HCl was used.

Procedures of derivatization and chromatographic conditions have already been described in detail for NOC-Cl [7], PNZ-Cl [8], and FMOC-Cl [9].

Sources of samples and treatment for analysis

Food samples were purchased in local retail outlets in Germany.

To aliquots of liquid samples (1 ml), ethanol (0.5 ml) and Dhx solution (internal standard, 50 µl) were added and adjusted with 0.1 M HCl to a final volume of 10 ml. After centrifugation at 3500 x *g* (20 min), 20 µl of the supernatant were analyzed. For matrices containing low amounts of BAs, 2 ml samples were used. Ethanol (0.5 ml) and Dhx (50 µl) were added, and the volume adjusted to 10 ml with 0.1 M HCl.

Hydrochloric infusions of tea were made by extraction of tea leaves (1 g) with boiling 0.1 M HCl (35 ml) for 20 min and filtration. After cooling to room temperature the filtrate was adjusted to 50 ml with 0.1 M HCl and an aliquot of 40 ml was evaporated to dryness. The residue was solved in 0.1 M HCl (2.5 ml) and centrifuged. Aliquots (20 µl) of the supernatant were analyzed.

Vinegars, red wine samples and fermented cabbage juices were analyzed using *para*-nitrobenzyloxycarbonyl chloride (PNZ-Cl) as reagent. UV absorption at 265 nm was used for monitoring. Fruit juices were derivatized with NOC-Cl and hydrochloric tea infusions with FMOC-Cl. Both derivatives were detected by fluorescence.

Results and discussion

Food samples were analyzed by HPLC after derivatization with oxycarbonyl chlorides The data are compiled in Table 1.

In fruit juices high amounts of Put were determined (with highest amounts in citrus juices) but only trace amounts of other BAs were detected. A chromatogram of an orange juice after derivatization with NOC-Cl is shown in Figure 1.

In red wine samples high amounts of Put and Trm as well as Him (up to 11.2 mg/l) were measured. As a result of the synergistic effects of other BAs and alcohol, wines with amounts of Him of this order of magnitude can cause toxic effects such as headache, vomiting and diarrhoea. Therefore, the amounts of Him in wines in various countries are not allowed to exceed 2 – 10 mg/l [10]. A chromatogram of a red wine from the Rioja region (Spain) after derivatization with PNZ-Cl is shown in Figure 2.

The content of BAs in vinegars is low in most cases. As an exception, extremely high levels of Him (129.3 mg/l) and Tym (415.8 mg/l) were detected in a saltened rice vinegar from China. These high amounts indicate that the product was spoiled.

Table 1: Amounts (mg/l for liquid samples, µg/g for tea) of biogenic amines in different food samples. Tryptamine, serotonin and octopamine were determined only in samples analyzed with the PNZ-method (i.e. wines, vinegars and fermented cabbage juices).

Biogenic Amine	Fruit Juices	Wines	Vinegars	Fermented cabbage juices	Teas
Cadaverine	n.d.	n.d. - 1.4	n.d.	n.d. - 20.3	n.d. - 3.2
Histamine	n.d.	n.d. - 11.2	n.d. -129.3	19.4 - 92.3	n.d. - 6.6
Octopamine	n.a.	n.d.	n.d. - 4.6	n.d. -118.5	n.a.
2-Phenylethylamine	n.d. - 0.8	n.d. - 3.8	n.d.	n.d.	n.d. - 4.2
Putrescine	2.1 - 68.6	n.d. - 39.3	n.d. - 49.1	24.2 -693.5	n.d. - 29.9
Serotonin	n.a.	n.d. - 6.7	n.d. -504.5	18.2 - 67.8	n.a.
Spermidine	1.1 - 3.6	n.d. - 1.6	n.d. - 1.9	n.d. - 4.2	3.6 - 66.4
Spermine	n.d.	n.d.	n.d.	n.d. - 0.4	16.9 - 76.4
Tryptamine	n.a.	n.d. - 16.3	n.d.	n.d. - 11.4	n.a.
Tyramine	n.d.	n.d. - 5.1	n.d. -415.8	29.6 - 68.7	n.d. - 72.1

n.d. = not detected, n.a. = not analyzed

As a typical example for fermented foodstuffs a lactic fermented cabbage juice demonstrates the increase of the amounts of BAs during bacterial fermentation. High levels of Put and Ocp, as well as Him, Tym and Seo, were detected. In some of the juices analyzed, amounts of Him and Tym might cause toxic symptoms.

The analysis of BAs in hydrochloric infusions of green and black tea in most cases shows high amounts of the non-toxic polyamines Put, Spd, and Spm. Only in infusions of black tea higher amounts of Tym were detected. A chromatogram of an HCl extract of a black tea from Ceylon after derivatization with FMOC-Cl is shown in Figure 3.

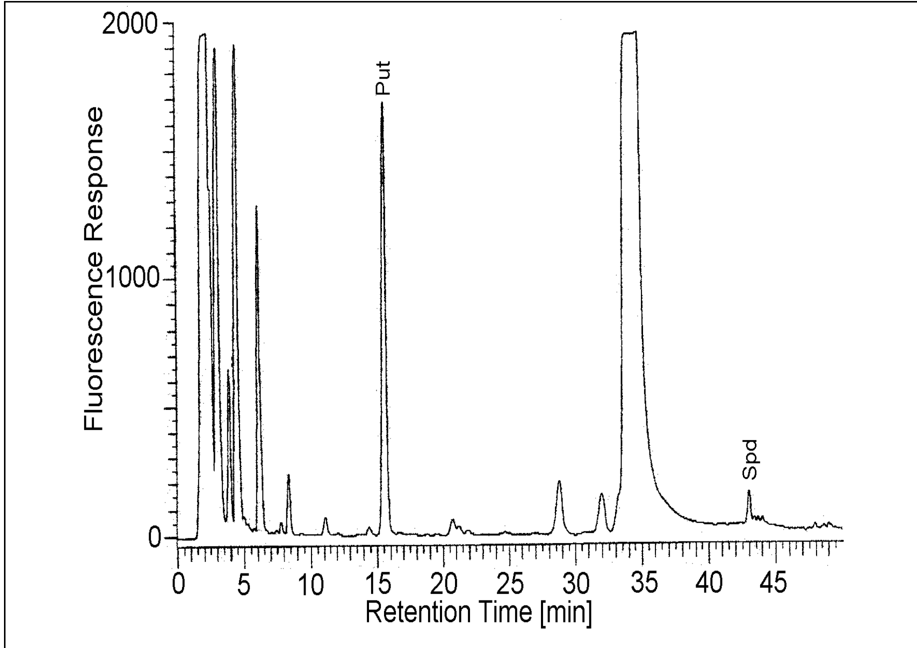

Fig. 1: HPLC of an orange juice after derivatization with NOC-Cl. For chromatographic conditions see [7].

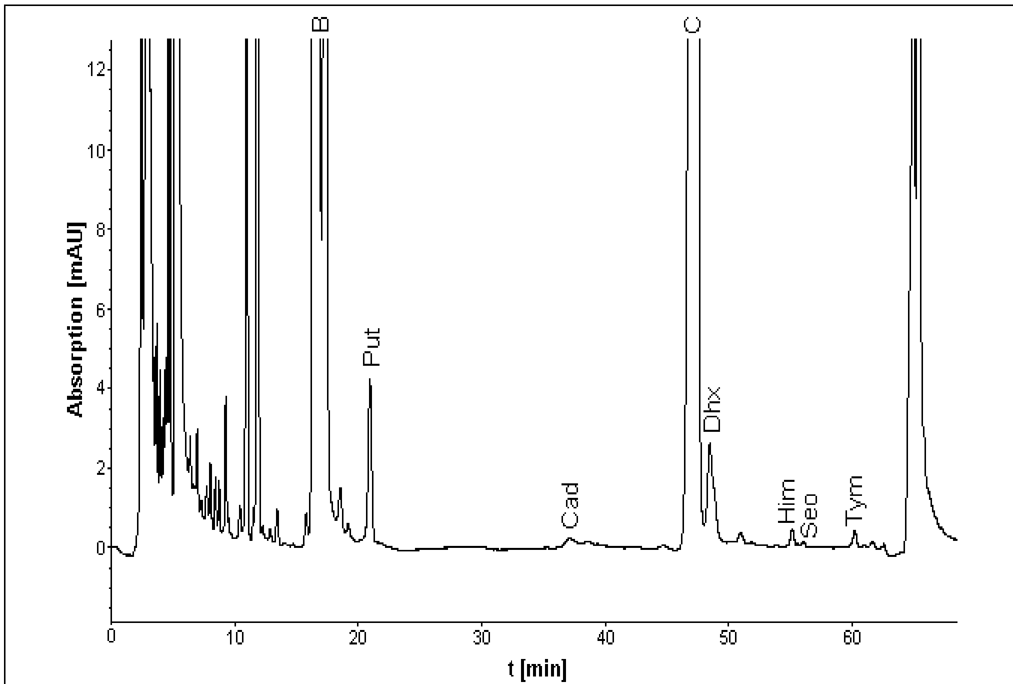

Fig. 2: HPLC of a red wine (Rioja, Spain) after derivatization with PNZ-Cl. For chromatographic conditions see [8].

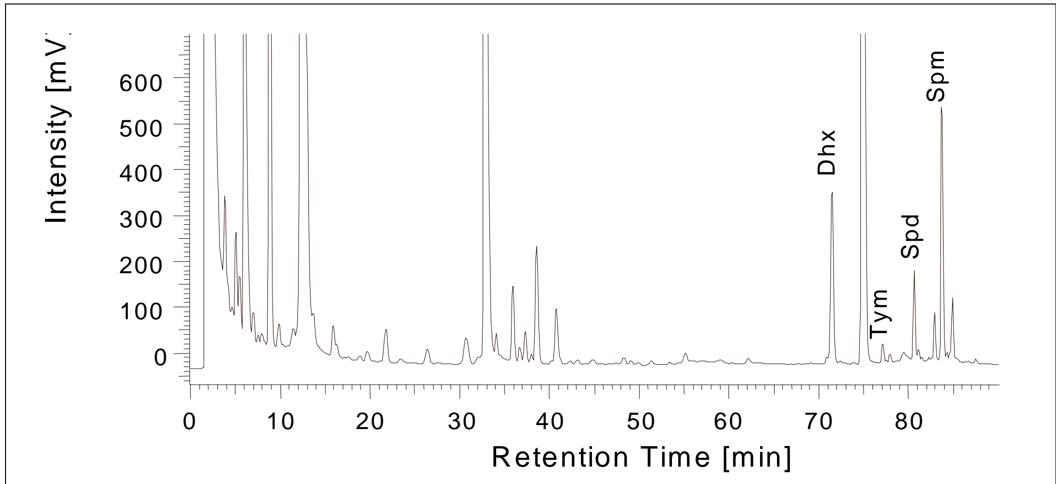

Fig. 3: HPLC of a HCl extract of a black tea (Ceylon) after derivatization with FMOC-Cl. For chromatographic conditions see [9].

Conclusions

In fruit juices Put and Spd are naturally occurring BAs. Higher amounts in particular of Him and Tym are the result of bacterial contamination and indicate spoilage. In wines usually only low amounts of BAs are detectable. The amounts of Him should be determined because of its synergistic effects with other BAs and alcohol. In vinegars, like in wines, the concentration of BAs is low. Again, increased concentrations of BAs are indicators of spoilage. In leaves of green (non-fermented) and black (fermented) tea Put, Spd, Spm, and Tym are detectable. The presence of other BAs in higher concentrations indicates a low tea quality, indicating unsatisfactory technological or hygienic conditions. Fermentation of plant juices such as cabbage juice leads to the formation of exceptionally high amounts of BAs. Thus, with regard to health claims related to these juices, control of the content of BAs is necessary. In conclusion, determination of BAs in food and beverages is still a topic for quality control.

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Determination of amino acids and biogenic amines in oriental fermented food and beverages for quality assessment

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Introduction

Soy sauce is one of the many oriental fermented foods commonly consumed in Asian countries. The traditional product is made by fermentation of soybeans, with or without the addition of rice or wheat, using the koji moulds *Aspergillus oryzae* or *Aspergillus soyae*, the yeast *Saccharomyces rouxii*, and lactic acid bacteria such as *Pediococcus halophilus* and *Streptococcus faecalis*. The main function of the koji mould is the digestion of protein and starch in the raw materials by microbial enzymes. The moulds show high activities of proteinases, exopeptidases such as carboxy and (leucine)amino peptidases, and amylases [1-5] .

It has been shown that D-amino acids (AAs) are common constituents of fermented foods. It is assumed that their origin is a result of the action of microorganisms i.e. bacteria, yeasts, and moulds. Further, treatment of proteinaeous food or food proteins under certain technological or experimental conditions causes racemization of protein or peptide-bonded L-AAs [6-8] .

Biogenic amines (BAs) are, in part, toxic substances. The major pathway of BA formation in fermented foods is the decarboxylation of free AAs mainly by microbial enzymatic activity. Studies have shown that BAs in fermented soy products are possibly formed by the lactic microflora active during fermentation [9-10] .

We investigated the pattern and amounts of free D-AAs and BAs in soy sauce for quality assessment using capillary gas chromatography (GC) with flame ionization detector (FID) for D-AA analysis and reversed-phase high-performance liquid chromatography (HPLC) with diode array detector (DAD) for BA determination.

Experimental

Chemicals and solvents

All chemicals and solvents were of analytical grade. Dichlormethane (DCM), 2-propanol (2-prpOH), acetyl chloride (AcCl), HCl (32 %) (all from Merck, Darmstadt, Germany), trifluoroacetic acid anhydride (TFAA), 2,6-di-*tert*. butyl-*p*-cresol (BHT).

Standards

AA Standard H (Pierce, Rockford, IL, USA) consisting of Gly and the L-enantiomers of Ala, Val, Thr, Ile, Pro, Ser, Leu, Asp, Met, Glu, Phe, Tyr, Lys, Trp, Arg, His (2.5 mM each) and L-Cystine (1.25 mM). AAs are abbreviated according to three-letter-nomenclature; special AAs are abbreviated as follows: γ-amino butyric acid (GABA), ornithine (Orn) (all from Fluka); L-norleucine (L-Nle) (Merck) was used as internal standard (IS). BAs were purchased from Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO, USA), respectively. Derivatizing reagents and chemicals used for preparation of samples were from Fluka (Neu-Ulm, Germany) and Aldrich (Steinheim, Germany).

Origin, preparation and derivatization of food samples for analysis

Soy sauces (n=8) imported from Japan, Taiwan and China, were purchased from local retail outlets. Preparation and derivatization of standards and samples were carried out as described in refs. [11-13] .

Capillary gas chromatography

For GC a Model GC-14A with FID and an integrator Model C-R3A (Shimadzu, Kyoto, Japan) were used. Derivatized AAs were analyzed on a 25 m x 0.25 mm I.D. Chirasil-L-Val column (Chrompack, Middelburg, The Netherlands). For chromatographic conditions see ref. [13] .

High performance liquid chromatography

For HPLC analysis a HP-1100 series comprising Model G1322A degasser, G1311A pump with low-pressure gradient former, G1313A autosampler, G1316A column thermostat, G1314A DAD with 13 μl flow cell, and software HP ChemStation for LC (Rev. A. 04. 02) were used (Hewlett-Packard, Waldbronn, Germany). Derivatized BAs were analyzed on a 250 x 4 mm I.D. Superspher 60 RP-18e column, 4μm (Merck) at 35°C.

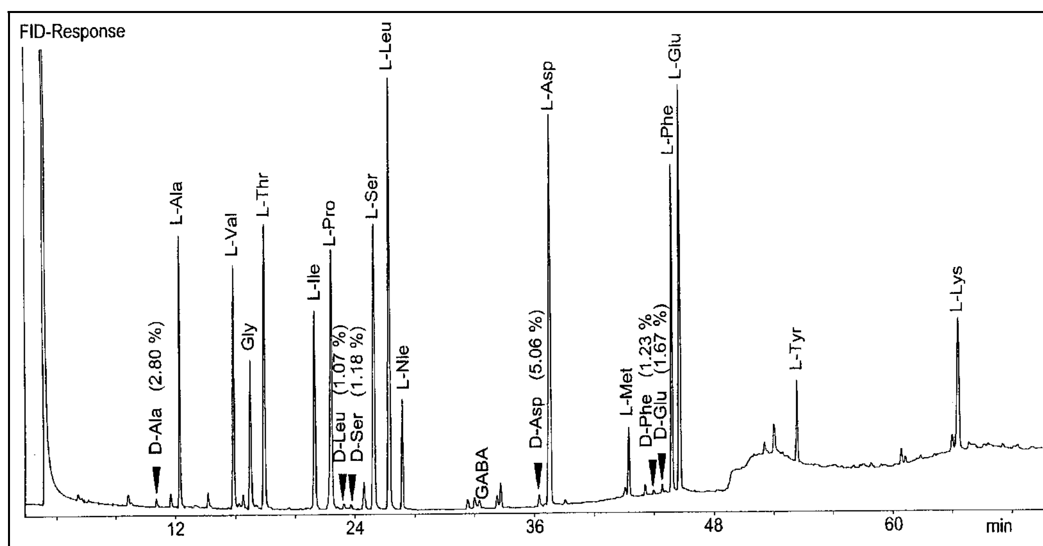

Fig. 1: GC/FID of soy sauce no. 4; AAs separated on Chirasil-L-Val as TFA-AA-2-propyl esters (for abbreviations see Experimental)

As a result of the derivatization procedures of AAs Asn and Gln, if present, were hydrolyzed to Asp and Glu, respectively. Therefore, the results shown are sums of the amounts of (Asp+Asn) and (Glu+Gln) (Fig. 1). Absolute amounts of D-Ala ranged from 29 mg/l to 217 mg/l, of D-Asp from 24 mg/l to 429 mg/l and of D-Glu from 18 mg/l to 362 mg/l corresponding to relative amounts of 2.3 -7.6 % of D-Ala, 1.9 - 9.5 % of D-Asp and 0.7 - 1.8 % of D-Glu. Remarkably, high amounts of D-Pro (249 mg/l) were found in one sample. This can be explained by the fact that these D-AAs are among the constituents of the peptide moieties of the peptidoglycans that form the bacterial cell walls [6] . In one sample only small amounts of D-Glu (0.5 %) but no D-Ala and D-Asp were found. The

chromatogram of this soy sauce is shown in Fig. 2. It is concluded that the soy sauce was not fermented by microorganisms. This is supported by very high amounts of Gly detected in this sample. Gly is used as sweetener in some Asian countries. Further, the sample contained high amounts of L-Glu (approx. 9000 mg/l) indicating the addition of monosodium glutamate as flavour enhancer. Results and Discussion

D-Amino acids in soy sauce

Significant amounts of D-AAs were detected in all soy sauces. This is attributed to the activity of the microorganisms during fermentation. D-Ala, D-Asp and D-Glu were most abundant among the D-AAs and with one exception they determined in all samples investigated. A chromatogram of a selected soy sauce is shown in Fig. 1.

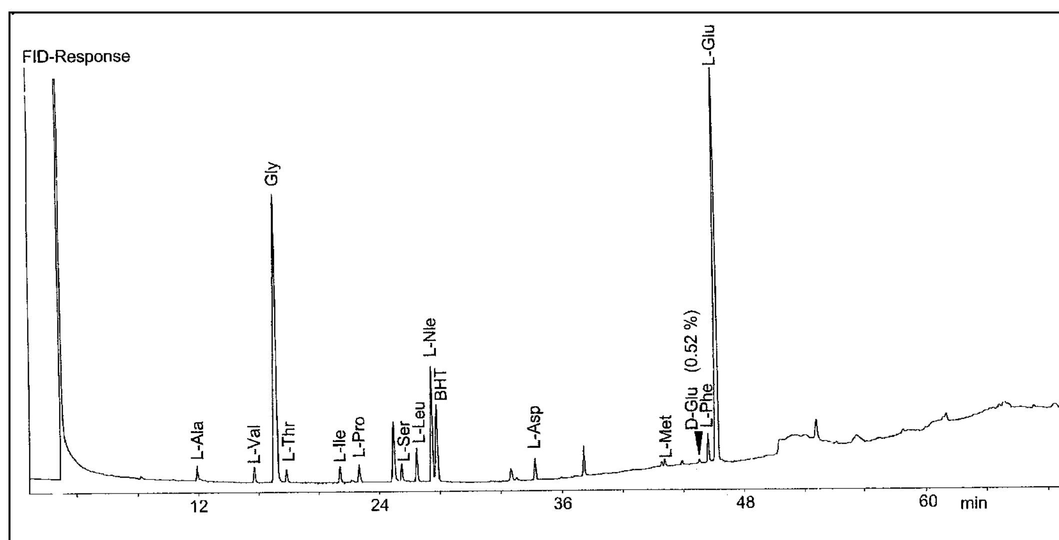

Fig. 2: GC/FID of soy sauce no. 8 suspected not to be produced by microbial fermentation; AAs separated on Chirasil-L-Val as TFA-AA-2-propyl esters (for abbreviations see Experimental)

Biogenic amines in soy sauce

The absolute amounts of BAs in selected soy sauce determined by HPLC after derivatization with PNZ-Cl are in Tab. 1. A chromatogram of soy sauce no. 4 is shown in Fig. 3. Tym, Seo and Him are the BAs with highest amounts in the soy sauces investigated. With a few exception, the polyamines Put, Spd and Spm were detected in each sample. These polyamines occur naturally in plant tissues [14]. The only sample without any of the polyamines is soy sauce no. 8. In this sample only Him (12.8 mg/l), Seo (45.5 mg/l) and Tym (39.4 mg/l) were detected. Consequently, soy sauce no. 8 was not manufactured with plant products.

Table 1: Amounts (mg/l) of biogenic amines in soy sauces no.1-8

BA	no. 1 - no. 7	no. 8
Putrescine (Put)	2.3 - 41.2	n.d.
Tryptamine (Trm)	n.d. - 22.7	n.d.
2-Phenylethylamine (Pea)	12.5 - 55.9	n.d.
Octopamine (Ocp)	15.7 - 47.7	n.d.
Histamine (Him)	5.6 - 418.9	18.6
Serotonin (Seo)	15.1 - 648.5	45.5
Tyramine (Tym)	1.8 - 646.9	39.4
Spermidine (Spd)	6.5 - 24.7	n.d.
Spermine (Spm)	7.6 - 19.8	n.d.

n.d. not detected

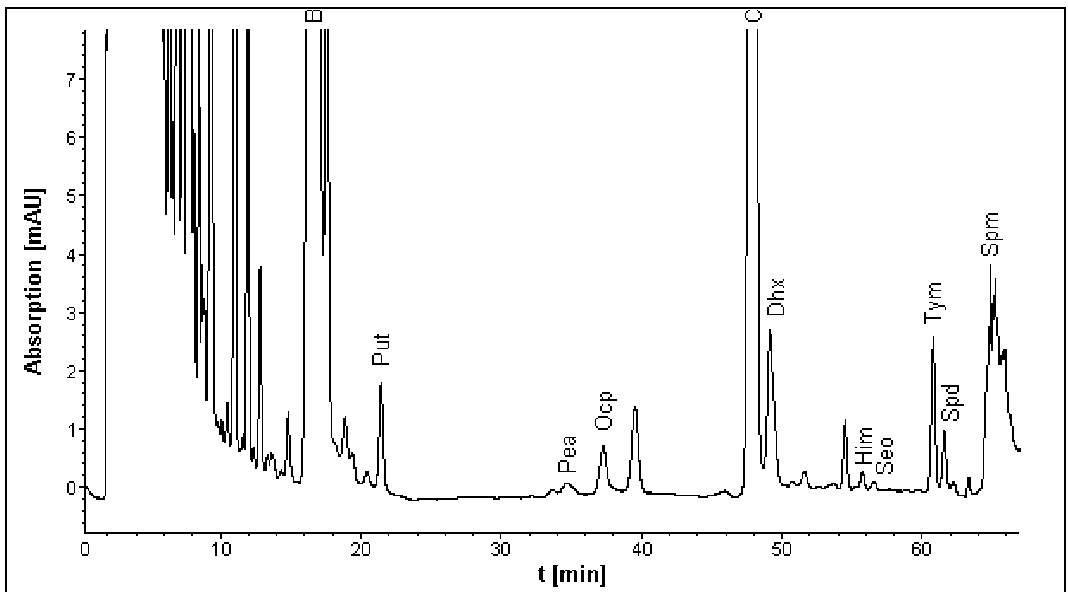

Fig 3: HPLC-chromatogram of soy sauce no.4 after derivatization of BAs with PNZ-Cl

Conclusions

From the results It is concluded that GC and HPLC procedures are highly suitable methods for quality assessment of oriental fermented food and beverages. The presence of D-AAs such as D-Ala, D-Asp and D-Glu indicates natural fermentation by microorganisms. Additionally, the use of flavour enhancers (monosodium glutamate) and sweeteners (Glycin) can be detected by AA analysis. The origin of raw materials could be assumed by analysis of BAs in pattern and amounts. BAs, in particular, increase in amount during microbial fermentation and spoilage. The lack of polyamines e.g. indicates that one of the soy sauces was not produced from plant materials. Furthermore, the amounts and ratios of BAs can serve as indicators for food quality via the “biogenic amine index“ (BAI) [15].

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Reducing sugars, vitamin C and phenol compounds in potato tubers of various cultivars in Poland

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Introduction

The development of potato processing in the world and Poland places an obligation of high quality on potato producers. Potatoes for direct consumption as well as processing for foods should have suitable morphological traits and chemical composition. The latter one determines a flesh colour and susceptibility to darkening of cooked tubers and fried products. As the researches have showed [2,3,5] reducing sugars and phenol compounds contents are of the greatest weight in this respect. Vitamin C is another considerable component of a tuber.

A belief is held that most of this vitamin in a Polishman`s diet comes from a potato. A cultivar decides about a tuber chemical composition, yet the effect of the environmental and agrotechnical factors is also important [1,4]. On the other hand, new cultivars of higher utility parameters have been introduced and the old ones withdrawn. Hence, a necessity to carry out studies on a wide range of varieties cultivated in the same climatic, soil and agrotechnical conditions.

Materials and methods

The studies were based on the results of a field experiment conducted in 3 years` cycles on the soil developed from high loamy sand. The soil showed a medium to high phosphorus content, a high potassium content, whereas its reaction was from light acid to neutral.

The potatoes were fertilized with manure in dose of 250 dt ha^{-1} and mineral fertilizers in amounts of: 100 kgN, 100 kg P_2O_5, 150 kg K_2O ha^{-1}.

The seeding material of the tested varieties was at the degree of superelite.

The experiment comprised 34 following Polish cultivars: Aster, Atol, Beryl, Bliza, Bogna, Brda, Bronka, Bryza, Bzura, Certa, Ceza, Cisa, Dryf, Duet, Elida, Elipsa, Fala, Fauna, Fregata, Frezja, Heban, Irys, Ja•min, Lotos, Mila, Orlik, Perkoz, Pilica, Pola, Ronda, Ruta, Sokó•, Stobrawa, Tarpan and 3 Dutch ones: Diamant, Eskort and Premieur.

All earliness groups were used in the experiment.

The analyses on the fresh material of 50 tubers from each potato cultivar were made immediately after the harvest. Reducing sugars were determined by the colorimetric method with the dinitrosalicylic acid (DNS). Vitamin C was established by means of Tillman`s method and phenol compounds after Mopson and Swain`s method. Each analysis was repeated three times for each variation. The content of a particular tuber component was in g/100g fresh matter (reducing sugars) and in mg/100 g f.m. (vitamin C and phenol compounds)

The results obtained were statistically worked out by means of the analyses of variance while their verification was made according to Tukey`s test.

Results

Reducing sugars content

In the potato tubers examined reducing sugars changed from 0,22 to 0,48 g in 100 g of fresh mass (Tab.1). The influence of a cultivar factor was on the order of the effect of weather conditions in the experimental years. Analysis on the reducing sugars content in the tubers regarding a connection with a cultivar earliness did not show any significant dependence. In each earliness group there appear some varieties with medium or high content of this component. Only in a group of very early and early cultivars a reducing sugars content was placed at the lower levels compared to the other groups. In a 3 years` cycle only five cultivars i.e. Irys, Jasmin, Lotos, Perkoz and Ceza did not exceed a boundary of 0,3 mg reducing sugars content that determines the bulbs usability for chips production. In the tubers of the other cultivars the compounds content did not surpass 0,5 mg boundary value required for French fries production.

Comparing the reducing sugars content in the Polish and Dutch tubers from the same earliness groups and cultivated in the identical conditions it was found out that the tubers of Dutch cvs Diamant and Premieur demonstrated a greater deal of this content as against analogical Polish varieties.

Tab.1: Content of reducing sugars, vitamin C and phenol compounds in tubers of various potato cvs grown in Poland.

earliness group varieties	vitamin C mg/100g f.m.		phenol comp. mg/100g f.m.		red. sugars g/100g f.m.	
	sphere	**middle**	**sphere**	**middle**	**sphere**	**middle**
very early						
Aster	18,6 -23,9	21	25,8 - 34,6	31	0,22 - 0,35	0,29
Frezja	15,8 - 16,9	16,2	23,5 - 26,4	24,5	0,28 - 0,33	0,3
Irys	14,0 - 16,8	15,8	20,3 - 27,2	24,2	0,25 - 0,30	0,28
Orlik	17,6 - 20,1	18,6	20,2 - 23,4	22,3	0,29 - 0,35	0,32
Ruta	15,3 - 16,5	16	21,3 - 24,5	23,6	0,25 - 0,32	0,3
early						
Duet	14,2 - 16,8	15,5	30,4 - 35,3	33,2	0,33 - 0,35	0,34
Elipsa	14,6 - 16,9	16	23,5 - 26,3	24	0,33 - 0,35	0,34
Jasmin	13,7 - 15,8	14,8	25,5 - 30,2	27,2	0,25 - 0,30	0,28
Lotos	18,2 - 23,7	19,6	30,0 - 42,5	34,2	0,26 - 0,30	0,28
Perkoz	18,3 - 21,1	19,5	23,6 - 28,2	25,7	0,23 - 0,27	0,26
Premieur	13,2 - 14,8	14	21,2 - 26,0	23,4	0,34 - 0,40	0,36
middle early						
Beryl	18,2 - 18,7	18,4	30,7 - 37,2	33	0,29 - 0,38	0,35
Bliza	16,0 - 18,5	17,6	35,8 - 43,0	38,2	0,33 - 0,37	0,34
Elida	14,0 - 18,8	15,9	32,2 - 38,1	34	0,33 - 0,35	0,34
Fauna	18,5 - 22,5	20,2	34,5 - 42,1	38,3	0,33 - 0,43	0,37
Mila	19,8 - 24,1	20	32,5 - 39,5	36	0,28 - 0,43	0,39
Pola	18,5 - 21,2	19,7	16,5 - 20,3	18,3	0,28 - 0,44	0,36
Ronda	17,2 - 19,7	18,8	37,9 - 45,2	42,3	0,29 - 0,40	0,38
Eskort	15,4 - 18,5	17,2	21,3 - 27,4	25,2	0,26 - 0,36	0,29

middle late						
Atol	18,2 - 19,7	18,7	34,8 - 43,2	37,5	0,24 - 0,32	0,29
Bogna	13,4 - 16,7	15,8	33,5 - 38,2	36,3	0,28 - 0,35	0,31
Brda	18,3 - 21,2	19,6	36,4 - 42,8	40	0,42 - 0,45	0,43
Bryza	19,4 - 23,5	21,6	38,3 - 45,0	42,2	0,29 - 0,45	0,41
Certa	17,4 - 21,1	18,9	37,2 - 44,5	40	0,25 - 0,35	0,29
Cisa	19,8 - 25,7	22,8	35,7 - 45,0	37,8	0,32 - 0,37	0,35
Fala	25,2 - 26,9	26,1	30,8 - 36,4	34	0,36 - 0,48	0,43
Foka	18,5 - 21,4	20,4	30,7 - 37,8	33	0,26 - 0,32	0,28
Sokós	20,3 - 25,5	23,9	23,4 - 27,6	25,3	0,26 - 0,42	0,35
Diamant	18,8 - 22,6	20,5	32,0 - 38,6	36,3	0,36 - 0,46	0,43
late						
Bronka	15,4 - 18,3	16,8	24,6 - 33,5	30,5	0,29 - 0,39	0,37
Bzura	18,5 - 23,5	21,5	33,2 - 38,8	35,4	0,29 - 0,36	0,33
Ceza	17,1 - 22,4	20,3	28,5 - 36,8	33,4	0,26 - 0,29	0,28
Dryf	17,8 - 23,7	19,9	40,3 - 45,2	42,2	0,41 - 0,43	0,42
Heban	13,2 - 16,8	15,2	38,6 - 45,8	42	0,22 - 0,35	0,29
Pilica	15,2 - 19,7	16,7	34,0 - 42,8	37,5	0,31 - 0,40	0,39
Stobrawa	13,2 - 18,5	16	34,1 - 42,6	38	0,33 - 0,45	0,4
Tarpan	16,0 - 22,5	20,6	24,5 - 32,8	28,2	0,31 - 0,40	0,37
$LSD_{0.05}$		1,88		6,1		0,07

Vitamin C

In the 37 potato cvs cultivated in the same climatic and soil conditions this vitamin occurred in concentration 13,2-23,5 mg in 100g of fresh mass tubers (Tab.1). The analysis of results did not demonstrate any significant differences in the component content conditioned by a vegetative period length of a cultivar. Only in the tubers from of medium and late varieties groups there were recorded a significant higher content of vitamin C. The tubers of Fala cv had the highest vit. C content (25,2-26,9 mg), while the lower contents were noted for the tubers of the Dutch early Premieur cv.

Phenol compounds

Their content in the tubers under investigation ranged from 24,5 - 45,8 mg in 100 g of fresh mass (Tab.1). The greatest number of phenol compounds was noted in the tubers of the following cultivars: Ronda, Bryza, Heban, Certa and Brda numbered among medium late and late ones. In comparison with these cultivars the tubers of early and medium early cvs contained significantly less number of phenol compounds.

Conclusions

1. The reducing sugars content in the tubers examined did not exceed 0,5 g in 100 g of fresh mass permissible for French fries production and in the tubers of Irys, Jasmin, Lotos, Perkoz and Ceza 0,3 g permissible for chips.

2. The tubers under investigation showed a low/medium vitamin C content and its greater concentration was recorded in the tubers of Fala and Sokól cvs, while in the tubers of a Dutch Premieur one -very low concentration.

3. Phenol compounds content was placed at the medium and high level. The tubers of the late cultivars showed a higher accumulation of these compounds as compared to the very early and early varieties.

4. The tubers of Premieur and Eskort cvs contained less amount of vitamin and phenol compounds, whereas Diamant and Premieur more reducing sugars than tubers of the Polish varieties numbered among the same earliness groups.

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Reducing sugars, vitamin C and phenol compounds in potato tubers cultivated with new synthetic growth regulators application

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Introduction

In 1992 in the west European countries there were 849 preparations registered and used as plant growth regulators of natural and synthetic origin [4]. Some of them demonstrate a wide range of operation on many plant species, while the others act selectively against one species only. It refers mainly to the synthetic regulators.

A considerable part of these preparations (44,4%) was recommended to be used in the floriculture. As to potato cultivation only 5,1% of the preparations could be used there. It suggests that new growth regulators for potatoes should be intensively worked on nowadays.

On account of this the present authors carried out a 3 years` cycle of researches with new synthetic growth regulators i.e. Poteitin made in Ukraine and Mival in Russia. These preparations were applied in cultivation of 34 potato varieties popular in Poland and 3 Dutch ones.

Poteitin is a complex of N oxide of 2,6 dimethylpyridine with succinic acid registered in Ukraine in 1993. It is a selective regulator recommended for potato cultivation only [1,2,3].

Mival is a silico-organic biostimulator of a wide operating spectrum for various plant species. Its active substance is made by 1-chloromethylsilatrane. It is classified as untoxical and clean ecologically. LD = 2 - 2,5 g per 1kg of white mouse body weight.

This preparation decomposes to SO_2 , CO_2 , H_2O, $CO(NH_2)_2$ and $C_2H_5NH_2$ [5] in the soil and plant.

Materials and methods

The studies were based on the results of a field experiment conducted in 3 years` cycles on the soil developed from high loamy sand. The soil showed a medium to high phosphorus content, a high potassium content, whereas its reaction was from light acid to neutral.

The potatoes were fertilized with manure in dose of 250 dt ha^{-1} and mineral fertilizers in amounts of: 100 kgN, 100 kg P_2O_5, 150 kg K_2O ha^{-1}.

The seeding material of the tested varieties was at the degree of superelite.

The growth regulators were used for seed-potato spray in form of Poteitin water solutions

10 mg dm^{-3} concentration and Mival 500 mg dm^{-3}. The preparation consumption was 300 mg of Poteitin and 15 g of Mival in 30 dm^3 water per 1t of seed-potatoes. The control tubers were sprayed the distilled water.

The experiment comprised 34 following Polish cultivars: Aster, Atol, Beryl, Bliza, Bogna, Brda, Bronka, Bryza, Bzura, Certa, Ceza, Cisa, Dryf, Duet, Elida, Elipsa, Fala, Fauna, Fregata, Frezja,

Heban, Irys, Jasmin, Lotos, Mila, Orlik, Perkoz, Pilica, Pola, Ronda, Ruta, Sokól, Stobrawa, Tarpan and 3 Dutch ones: Diamant, Eskort and Premieur.

All earlinerss groups were used in the experiment.

The analyses on the fresh material of 50 tubers from each potato cultivar were made immediately after the harvest. Reducing sugars were determined by the colorimetric method with the dinitrosalicylic acid (DNS). Vitamin C was established by means of Tillman`s method and phenol compounds after Mopson and Swain`s method. Each analysis was repeated three times for each variation. The content of a particular tuber component was in g/100g fresh matter (reducing sugars) and in mg/100 g f.m. (vitamin C and phenol compounds)

The results obtained were statistically worked out by means of the analyses of variance while their verification was made according to Tukey`s test.

Results and discussion

Reducing sugars content

The potato tubers cultivated with Mival and Poteitin showed a reducing sugars concentration ranging from 0,24 mg in 100 g of fresh mass in Ceza cv. to 0,45 mg in Bryza one. Analysing the regulator influence on this element concentration it was found out that Poteitin has statistically decreased significantly this concentration (by 10%-17%) in the tubers of only 4 later cultivars (Fauna, Fala, Eskort, Ceza),where as it increased in the tubers of 4 other varieties of short vegetative period (Aster, Perkoz, Frezja, Beryl). Yet, for the other cultivars the changes resulting from Poteitin use were statistically insignificant (Fig. 1.).

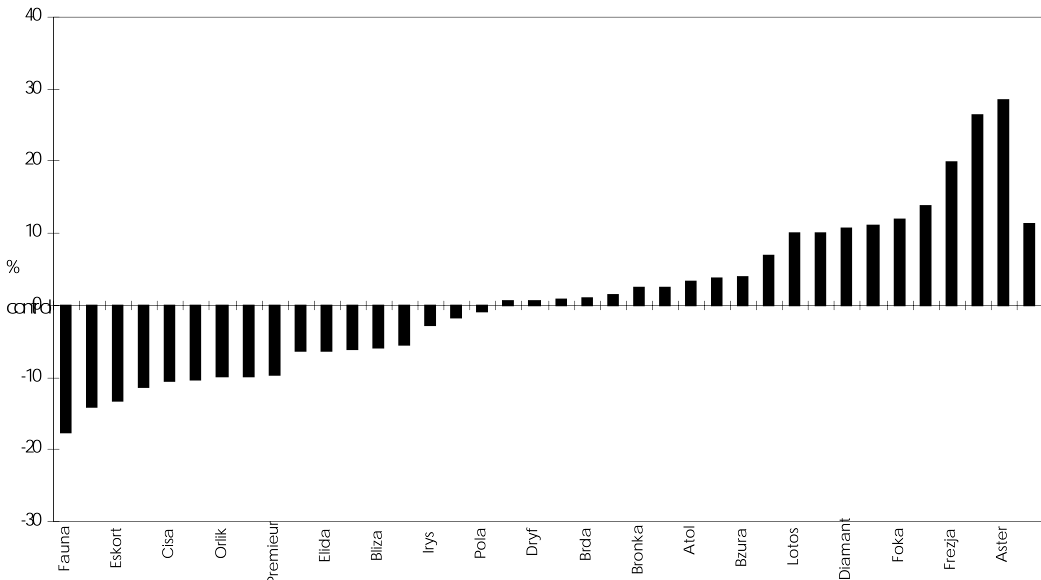

Fig. 1: Influence of Poteitin on the content of reducing sugars in potato tubers of different varieties (% of control).

Mival changed reducing sugars content in tubers to even smaller extent. The changes of the element proved to be statistically significant in the tubers of only 6 cultivars. In one cultivar -Fala there was a drop of concentration recorded and for five others (Atol, Aster, Heban, Pilica and Foka) a concentration growth (Fig. 2.). The reducing sugars content increase may limit the use of tubers of these cultivars for chips produce.

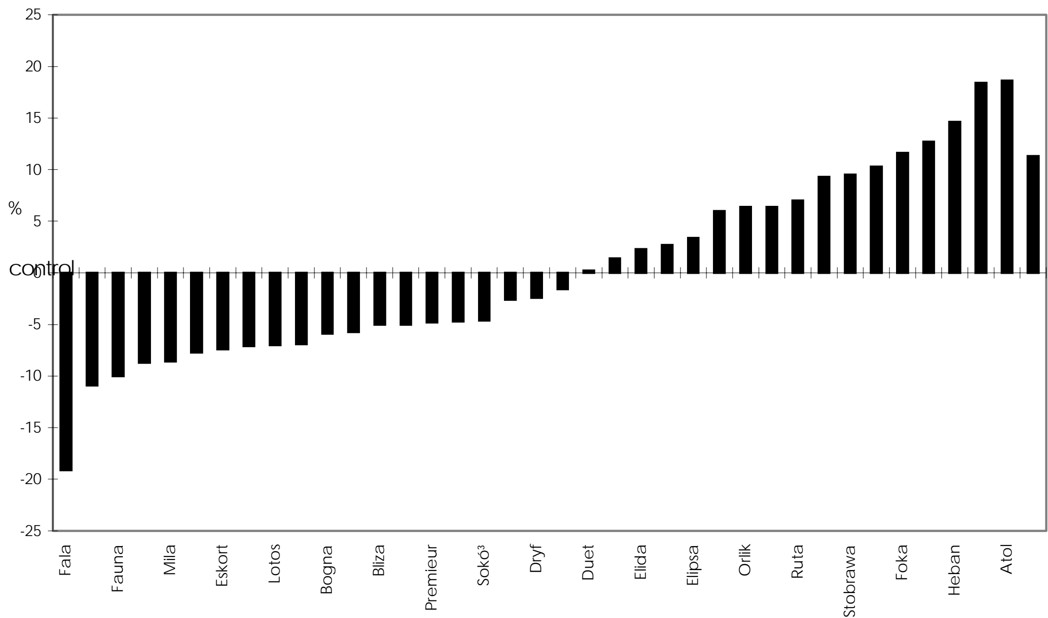

Fig. 2: Influence of Mival on the content of reducing sugars in potato tubers of different varieties (% of control)

Vitamin C content

The experiments of the present authors showed that Vit. C content determined as sum of the ascorbic and dehydroascorbic acid changed from 16,1 mg in the Elipsa tubers to 26,2 mg in 100 g of Diamanta fresh mass tubers. Poteitin used for seed-potato spray caused an increase of Vit. C content in the tubers of most cultivars and in some of them even by 20%-54% as compared to the control tubers (Fig. 3.). The most favourable changes of this element induced by Poteitin were stated in the tubers of Ruta, Lotos, Elida.

A significant decrease of Vit. C concentration occurred in the tubers of only two cultivars, that is Sokól and Aster.

The other regulator- Mival had an equally beneficial effect on Vit. C content as it also increased its concentration. A statistically significant growth of Vit. C content in the tubers was noted in twenty of cultivars studied, yet in eight varieties the changes were by 16%-45% as compared to this vitamin content in the control(Fig. 4.).

In six other cultivars Mival decreased vit. C content and the greatest drop was recorded for Sokól cv. (29,5%).

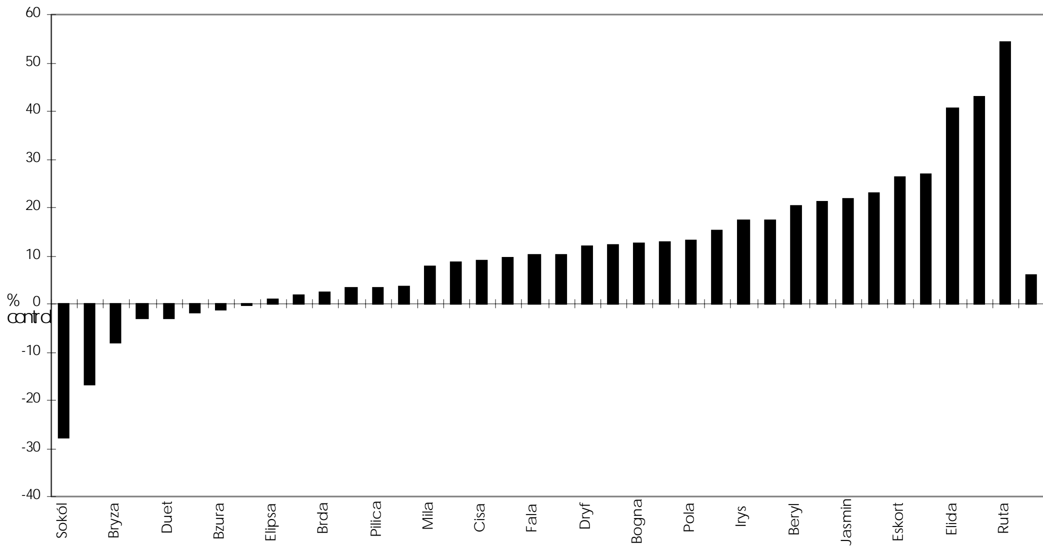

Fig. 3: Influence of Poteitin on the content of vitamin C in potato tubers of different varieties (% of control).

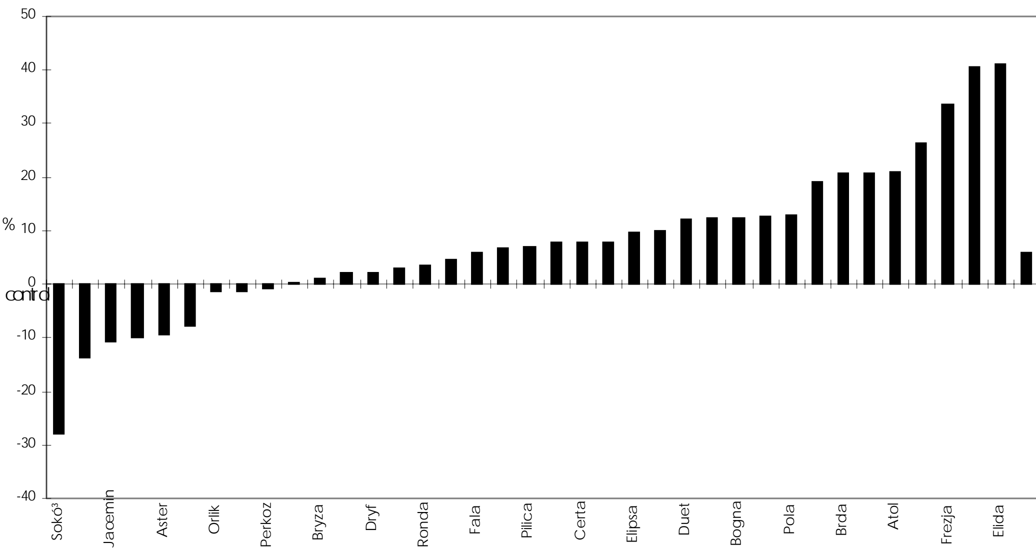

Fig. 4: Influence of Mival on the content of vitamin C in potato tubers of different varieties (% of control)

Phenol compounds content

The potato tubers with growth regulators ue showed a decrease of phenol compounds content by 11,9 mg for Pola cv. to 56,2mg in 100g of Diamanta tubers fresh mass. Out of both growth regulators used, Poteitin proved to change phenol compounds content to a greater degree. This regulator increased their content in the tubers of twenty cultivars, and in fifteen of them a growth by

20%-50% was noted as against the control. In the tubers of other eight varieties there was recorded a drop of 10%-20% of the phenol compounds, that is significant statistically(Fig. 5.).

The effect of Mival on the compounds concentration in potato tubers was smaller than that of Poteitin. This regulator has significantly increased phenol compounds in the tubers of 12 cultivars, still only in seven of them an increase was over 20%. In the tubers of 12 other cultivars there was a significant decrease of phenol compounds content due to Mival use(Fig. 6.). Both, an increase of the compounds content and their decrease did not depend on the vegetative period length. Such a response of the cultivars to the regulators use was an individual trait of a cultivar.

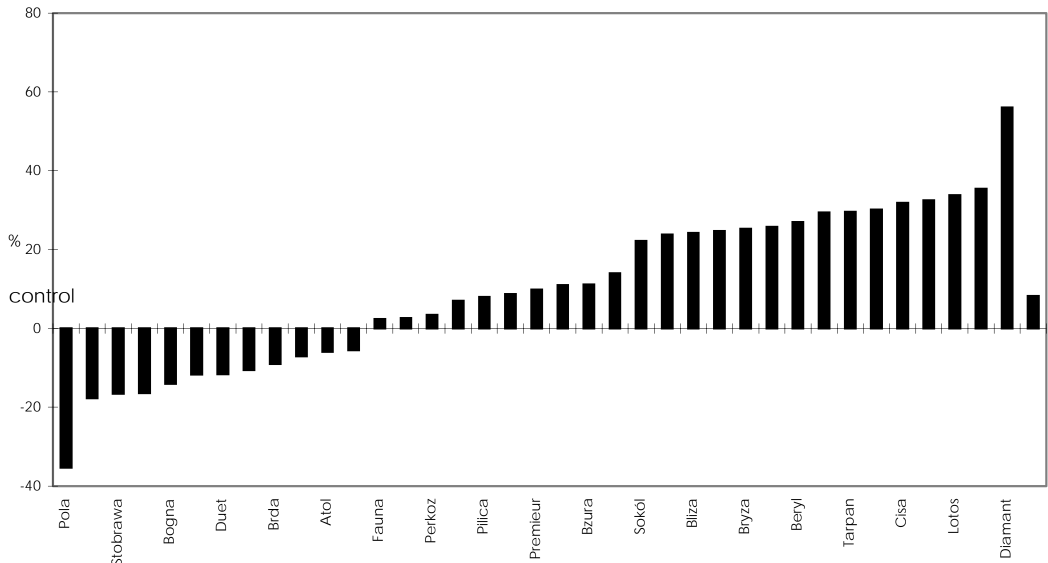

Fig. 5: Influence of Poteitin on the content of phenol compounds in potato tubers of different varieties (% of control).

Assessment of the effect of the synthetic growth regulators produced in Russia and Ukraine on the chemical composition changes in potato tubers showed that the changes were conditioned by cultivar character of plant and not by the vegetative period length.

The studies on Poteitin use for potato cultivation were conducted by the Ukrainian researchers [2, 4] and on Mival by Russian ones [1, 5] and the investigations covered only the regulators influence on the crop growth or starch content in tubers. Evaluation of the other elements content was not made, hence it is difficult to explain various directions of the changes of the same component content in different cultivars.

The agrotechnical assessment of Mival and Poteitin was performed by Sawicka [3]. Poteitin increased the tubers crop by 7,2% and Mival 9,4% on average. Generally, the preparations also increased starch content in the tubers.

Considering all the effects of changes caused by the regulators used it may be held that these preparations can be used for the cultivation of some potato varieties in Poland.

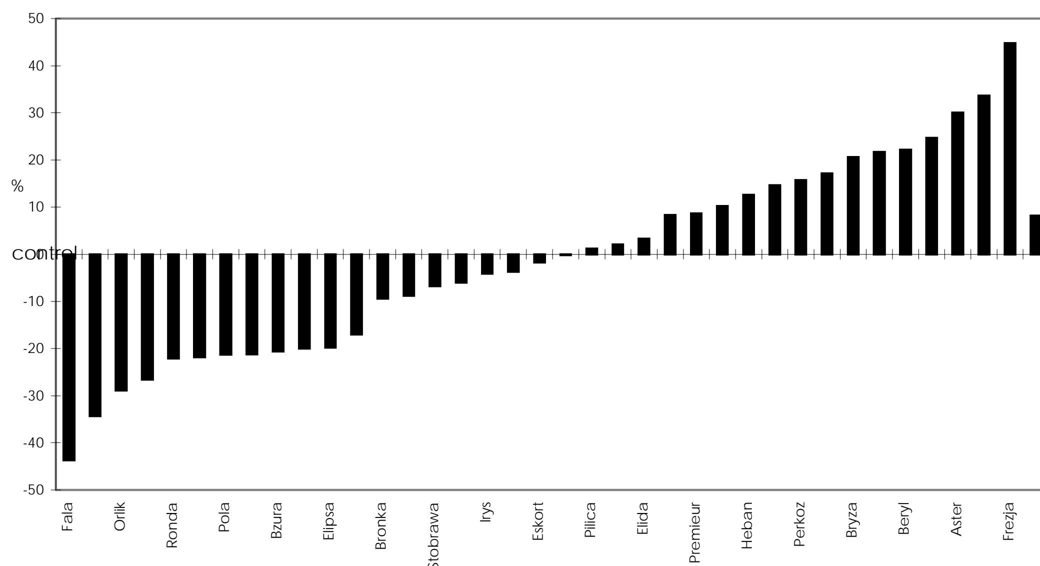

Fig. 6: Influence of Mival on the content of phenol compounds in potato tubers of different varietie (% of control)

Conclusions

The synthetic growth regulators Poteitin and Mival applied for 37 potato cvs gave the following results:

- an increase of vit. C and phenol compounds contents
- some groups of cvs responded with unfavourable changes in the chemical composition - drop of vit. C and phenol compounds and an increase of reducing sugars content
- this preparations may be recommended for cultivation of most potato cvs in Poland, that is, Poteitin
- particularly for the following cultivars: Bronka, Ceza, Cisa, Diamant, Elida, Fala, Lotos, Perkoz, Pilica. Mival - for Atol, Brda, Elida, Eskort, Frezja, Irys, Lotos, Stobrawa.

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The effect of industrial emissions in the Stalowa Wola region on heavy metals content in a potato tuber

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Introduction

Atmosphere pollution with dusts containing heavy metals leads to an increase of their content in soil, water and plants. Soils and plants found within the range of municipal, communicative and, first of all, industrial emissions proved to be particularly endangered with pollution. In Poland air pollution is caused by the power industry, fuel combustion and metallurgical industry to a great extent [1].

One of the main sources of heavy metals penetration into human organism is food contaminated with them. A steady increase of general contamination level of agricultural products with heavy metals accounts for controlling these metals content in the food of plant origin. An excess of these elements in the environment, particularly in food, may cause many diseases like cardiovascular one, kidney, skeletal system, developmental anomaly of children, mutagenic and teratogenic changes as well as neoplasm diseases [10].

Considering the facts mentioned above a heavy metals content was examined in potatoes cultivated in the area affected by great industrial works such like, power plants, thermal-electric power station, metallurgical complex with container and electrolytic steelworks as well as iron foundry. All of them emit a great quantity of dusts. Among the emitters there were also plants of sulphur industry and a sulphur mine.

In the tubers under investigation there was determined a content of elements toxic for human like Cd, Pb, as well as those necessary for man and plants to develop at the vegetative period. Their deficiency results in some disorders of life functions in human organism, yet on the other hand, their excessive consumption may endanger his health (Cu, Zn, Fe, Mn, Co, Ni).

The region where the examined potatoes were cultivated is located near Stalowa Wola [Fig.1] and according to the Chief Central Statistical Office (GUS) 1995, this region was placed on the 25th-26th position in Poland regarding area and population, in respect of gas emitted - 7th position, while dusts emission - 9th place. This region is especially endangered with pollution produced by the following seven great industrial plants located there:

1. Power Plant Stalowa Wola
2. Steel Plant Stalowa Wola
3. Thermal-Electric Power Station Sandomierz
4. Pilkington-Sandoglass Sandomierz
5. Thermal-Electric Power Station Tarnobrzeg
6. Sulphur Industrial Works ”Siarkopol” Tarnobrzeg
7. Communication service Plant Gorzyce

Quantity of pollutant emission from these plants in 1996 is presented in Tab.1

Table. 1: Contribution of greater industrial plants to actual emission of pollutants in 1996 [thousands tones/year] [7]

Plant	SO_2	NO_x	En. dusts	Industrial dusts	Other	Total
Power Plant Stalowa Wola	11475	5186	1623	-	233	18787
Steel Plant Stalowa Wola	22	166	1	134	298	621
Thermal-Electric Power Station Sandomierz	127	61	105	-	173	466
Pilkington-Sandoglass Sandomierz	226	1214	-	111	56	1607
Thermal-Electric Power Station Tarnobrzeg	379	78	302	-	27	786
Sulphur Industrial Works ”Siarkopol” Tarnobrzeg	1852	370	945	86	347	3600
Communication service Plant Gorzyce	308	78	192	134	131	711

The Provincial Inspectorate of Environment Protection [7] also examined the fall of lead and cadmium compounds in this area in 1996. Surpassing of some leadfall standards were recorded in a few places in Sandomierz and Stalowa Wola. Neither of both metals exceeded the acceptable standards at the rest of region studied.

The tubers analysed came from gatherings in 1996.

The collecting material points at the field were situated near Stalowa Wola. In the north-west a boundary was made by a route Stalowa Wola-Sandomierz and from the south-west - a route Stalowa Wola-Tarnobrzeg (Fig.1). From the northern part this region incurs pollution from Stalowa Wola and Sandomierz emitters, whereas from the southern part - the emitters from Stalowa Wola and Tarnobrzeg.

Materials and methods

In autumn 1996 twenty seven tuber samples from nine potato cultivars were gathered to be examined. Those were the following varieties: Atol, Bronka, Bryza, Ibis, Irys, Janka, Mila, Pola and Sokól numbered among various earliness groups. Each cultivar was represented by 50 tubers of different size. The tubers were divided into three parts in dependence on a form the potatoes are usually consumed by man and animals. That is why whole tubers, flesh and peeling were analysed separately.

The experimental material was washed, ground and dried. Then it was mineralized in a lab oven at 150-450 C temperature, the ash dissolved in hydrochloric acid (1:1 - HCl : H_2O) and afterwards evaporated to dryness. The residue was dissolved 0,1 M HCl and after hot filtering the solution was complemented till a required volume. In the solution there was determined a concentration of Cu, Zn, Mn, Fe after ASA method. However, Cd, Pb, Ni, Co content was analysed according to ASA method, yet first, they got complexized by pyrolidinoditiocarbaminian (APDC) and transferred to the organic phase of methyl isobutyl ketone (MIBK) to increase their concentration in the solution. The determinations were repeated three times. The statistical evaluation of the results obtained was made according to a variance analysis method, while significance of differences by Tukey`s test.

Fig. 1: Distribution of experimental points in the region

Results

Copper

A copper content in the tubers examined changed within wide range of 3,5 - 15,9 mg in `kg of dry mass (Tab.2). Higher concentrations of copper were found in the tubers gathered in north western part of the area investigated. Generally, those were the values higher than 4.0mg/1kg - a content acceptable for potato[6]. In the tuber samples coming from the south western region of Stalowa Wola, copper concentrations were lower in general, still they also exceeded the acceptable standard. Alike, a high copper content was stated by Jasiewicz [3] in the potato tubers from Cracow allotment gardens.

Comparing copper accumulation in tubers of different potato cultivars it was found that Sokól cv. grown together with other varieties accumulated more copper in tubers (14,8 mg) than for instance, Janka cv. (Fig.2.)

Zinc

This element concentration in potato tubers from the Stalowa Wola region changed in the range 20,0 - 56,7 mg in 1kg dry mass (Tab.2). A little higher zinc concentration was recorded in tubers gathered from north western part of the region.

A high content of this element was recorded in Sokól cv. (56,7mg), medium in Mila, Bronka, Bryza and Pola, while low in Janka (Fig.2).

A zink content in all the tubers examined exceeded a standard acceptable for potatoes - 10mgxkg d.m [6]. Zinc amount recorded by the present authors was roughly the same as that found in the tubers from Cracow allotment gardens by Krelowska-Kulas [5].

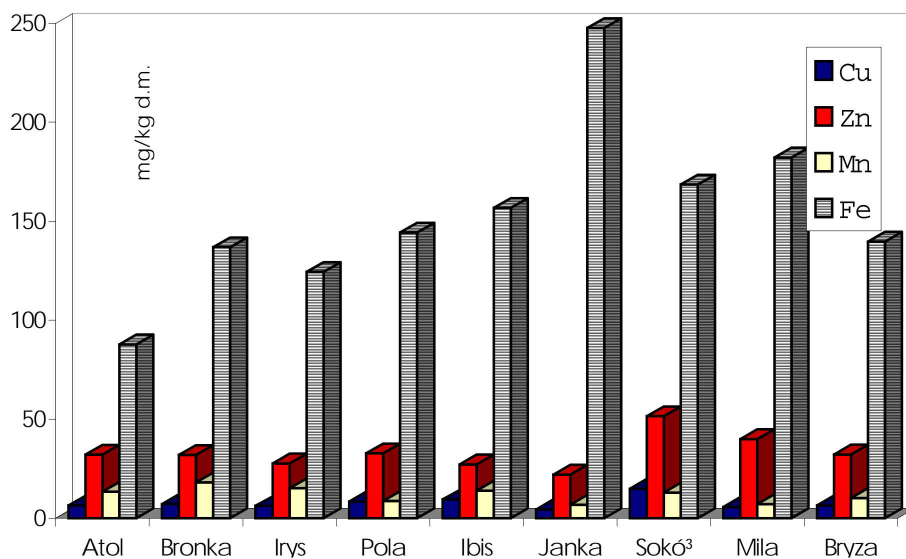

Fig. 2: Cu, Zn, Mn, Fe content in the tubers of different potato cultivars [mgxkg^{-1} d.m.]

Manganese

Mean contents of this element for most of the tubers analysed are contained in the range of values given in the literature, that is 3,6 - 15,0 mg in 1kg dry mass [2,4]. Yet, in some tubers the values were twice higher (1,5 -2) and average 24,8 - 33,3 mg x kg^{-1} d.m. (Tab.2). However, there was no any record of manganese toxic value surpassing in any sample as human and plant tolerance to this element is fairly high and manganese intoxication is rather sporadic [4].

Iron

Iron occurred in the greatest amount [Tab.2] among all the elements determined in the tubers studied. Sometimes the concentrations recorded exceeded the values most frequently presented in literature for a few times. Undoubtedly, this situation results from vicinity of the industrial works, mainly Stalowa Wola, emitting serious quantity of dusts (Tab.1) with great share of iron. It primarily refers to the steel plant and power plant [1,2,4]. Out of the cultivars examined over the research time, Janka cv. was distinguished by high accumulation of iron (x=247,5mg). Actually iron exceeded the acceptable standards in all the tuber varieties, except for Atol (Fig.2).

Alike some other cultivars, iron demonstrated some regularity as well, namely the tubers gathered from the north western part of the region showed more iron quantity than those from south western ones. That can evidence a more considerable influence of the emitters not only from Stalowa Wola but Sandomierz as well polluting the agricultural land.

Iron distribution in tubers was quite differentiated, its average content in peeling (281,9mg) was around twice as large as compared to whole tubers (x=180,3mg) and quadruple higher than in edible part of tuber (Fig.3).

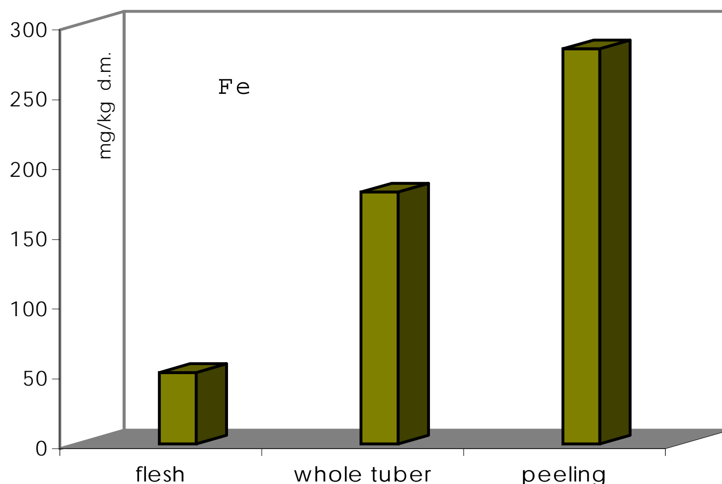

Fig. 3: Iron distribution in various parts of potato tuber [$mgxkg^{-1}$ f.m]

Cadmium

A cadmium content in the potato tubers studied placed within the values 0,0005 - 0,027 mg in 1kg of fresh mass (Tab.2). Among the cultivars examined the highest quantity of this element was accumulated in the tubers of Sokól, Pola and Janka cvs (Fig.5). Cadmium was not distributed uniformly in the tubers.

The peeling contained this element twice as large as compared to whole tuber and flesh (Fig.4). All the cadmium concentrations determined by the present authors were at a lower level than this acceptable (0,08 mg in 1kg fresh mass) for food.

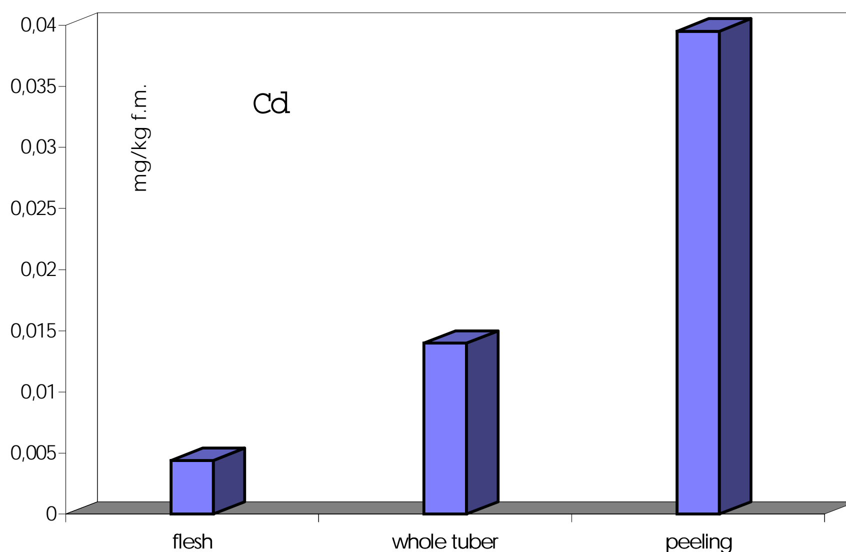

Fig. 4: Cadmium distribution in various parts of potato tuber [mg x kg^{-1}f.m.]

Lead

It occurred in slight concentrations [0,00510 - 0,0050 mg 1kg f.m.] (Tab.2) in the potato tubers examined. Irys and Mila showed greater susceptibility to lead accumulated in tubers as against other varieties cultivated in the same conditions (Fig.5).Evidently higher lead content was established in peeling than in other parts of tuber (Fig.6).

A level of elements particularly harmful for man (Cd, Pb) was determined in the tubers and turned out to be low. There was no record of surpassing an acceptable concentration by any of these metals [6].

The values obtained were comparable to these elements concentrations presented by the other authors [1,4,8,9,10] for tubers coming from unpolluted area - for lead the values were 0,0017 mg and for cadmium 0,0028 mg.

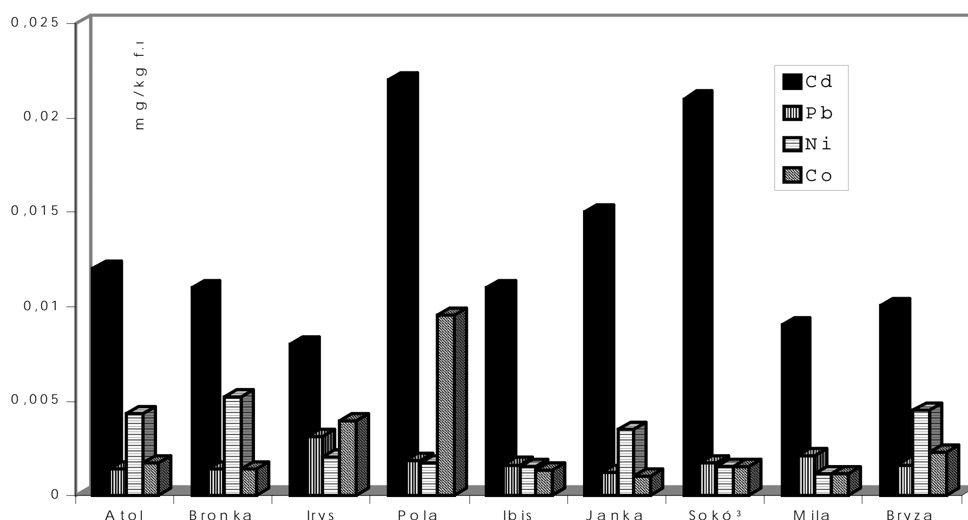

Fig. 5: Cd, Pb, Ni, Co content in the tubers of different potato cultivars [mgxkg^{-1} f.m.]

Cobalt

Cobalt concentrations determined in the potato tubers should be numbered among low values (0,0010 - 0,0155 mg kg f.m.) (Tab.2). Higher content of cobalt appeared in the tubers of Pola and Irys cvs, lower one in Bronka, Sokól and Mila and the lowest in Janka (Fig.5). The cobalt distribution was almost even that was seen in the full section of potato (Fig.6).

A place of tuber sampling was not significant for cobalt accumulation in the tubers.

Nickel

The potato bulbs analysed by the present authors showed very low nickel concentrations (0,0010-0,0100 mg) (Tab.2). Out of all the tubers examined slightly more nickel quantity was recorded in the tubers of Atol, Bronka and Bryza (Fig.5). Nickel distribution in a tuber was similar to that of other elements - more quantity in peeling and much less in the flesh (Fig.6). Cobalt and nickel belong to the elements potentially indispensable for plants and quite significant in man and animal`s diet. In the tubers examined by the present authors both elements occurred in low and very low quantities and they placed at the concentration level of the tubers from unpolluted region [4,9].

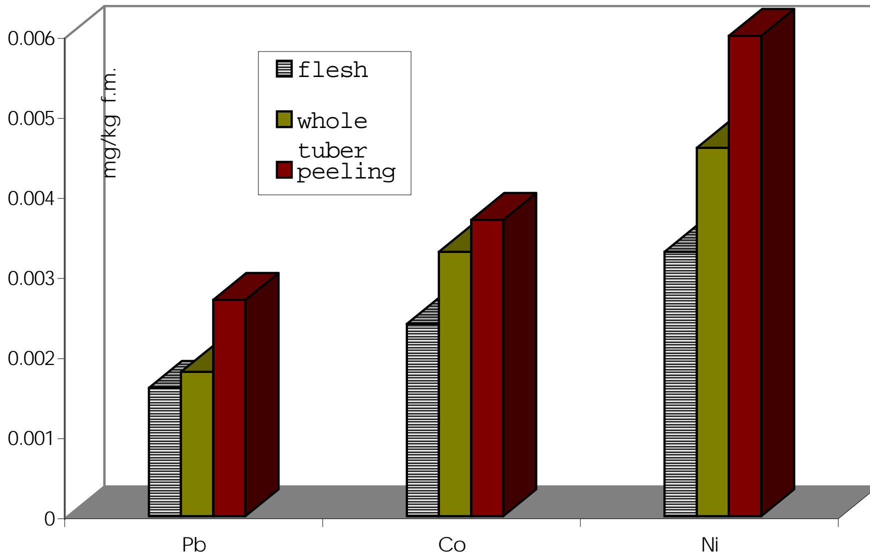

Fig. 6: Pb, Co, Ni distribution in various parts of potato tuber [mg x kg^{-1} f.m.]

Table 2

varieties	Cu	Zn	Mn	Fe	Co	Cd	Pb	Ni
	mg kg^{-1}d. m.				mg kg^{-1}f. m.			
Atol	3,8-10,7	24,3-44,4	4,8-24,8	46,8-160,4	0,0010-0,0020	0,007-0,020	0,0010-0,0020	0,0010-0,0100
Bronka	3,9-10,7	20,4-41,3	5,5-33,3	70,4-230,9	0,0010-0,0390	0,009-0,022	0,0010-0,0021	0,0030-0,0080
Bryza	4,3-8,7	28,9-35,8	10,1-10,5	124,5-154,0	0,0020-0,0027	0,010-0,012	0,0010-0,0022	0,0040-0,0050
Ibis	5,5-13,3	22,2-40,8	5,8-22,2	83,9-215,4	0,0010-0,0018	0,011-0,0014	0,0010-0,0018	0,0010-0,0030
Irys	4,5-7,0	24,2-29,0	8,0-22,2	53,6-160,2	0,0019-0,0064	0,005-0,014	0,0010-0,0050	0,0011-0,0030
Janka	4,5-4,6	20,9-23,3	4,9-8,8	223,5-271,6	0,0010-0,0012	0,015-0,016	0,0011-0,0013	0,0031-0,0039
Mila	4,1-7,4	40,1-40,3	6,3-8,2	182,1-184,6	0,0010-0,0012	0,008-0,010	0,0020-0,0022	0,0010-0,0013
Pola	3,5-14,5	23,3-40,5	5,5-21,5	77,1-185,5	0,0011-0,0016	0,007-0,027	0,0010-0,0025	0,0010-0,0030
Sokól	14,2-15,9	46,5-56,7	10,7-15,3	167,1-170,0	0,0014-0,0016	0,015-0,028	0,0014-0,0021	0,0014-0,0016
X	7,5	32,0	12,5	140,7	0,0028	0,0116	0,0017	0,0045
$LSD_{0,05}$ varieties)	7,8	24,1	-	71,6	0,0007	0,0012	-	-

Conclusions

1. Content of Cu, Zn, Mn and Fe in the tubers from the industrial region of Stalowa Wola maintained the level of high and very high concentrations that generally exceeded the standards acceptable for foods.
2. Content of toxic metals Cd and Pb in the tubers under investigation was situated within a range of low concentrations correlating with those in the tubers from unpolluted region.
3. Concentration of all the metals was considerably higher in peeling than in tuber flesh.
4. Pollution of the region examined was affected to a great extent by the emitters located at the north-western part of it.
5. The elements essential for plants and significant for man and animals, that is cobalt and nickel appeared in very small quantities in the tubers studied.
6. An increase of accumulation ability of the determined heavy metals was observed in Sokól, Bronka, Janka, Pola and Mila cvs that should not been cultivated in this region.

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Oxidative stability of ω3-rich oil from *Camelina sativa*

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Abstract

Camelina sativa oil contains 35-40% α-linolenic acid (ALA, C18:3ω3) and is therefore a potentially rich food source of ω3 fatty acids (ω3FAs). Consumption of ω3FAs has been associated with a decreased risk of coronary heart disease. However, polyunsaturated fatty acids such as ALA are susceptible to lipid oxidation. Oxidative stability of camelina oil was compared with olive, sunflower, sesame, corn, linseed, rapeseed and fish oils using the Schaal Oven Test at 65°C. Peroxide values (PV), conjugated dienes (CD) and trienes (CT), ρ-anisidine values (AnV) and TBARS were measured. Camelina oil was found to be more stable than linseed and fish oils, but less stable than rapeseed, corn, sesame, sunflower and olive oils ($p<0.05$). Storage stability of a camelina oil spread was compared with an otherwise identical sunflower oil spread. Samples were assessed by a 10-member sensory analysis panel on a scale of 1-10. Appearance and odour scores did not deteriorate during the 16 weeks. Flavour and overall acceptability scores for the camelina spread were lower than those for the sunflower spread, but were still acceptable at week16. The camelina spread had higher PV, AnV and TBARS values than the sunflower spread throughout the 16 weeks ($p<0.05$).

Introduction

Camelina sativa is a member of the Cruciferae (Brassicaceae) family and is also known as false flax or gold-of-pleasure (Budin, Breene and Putnam 1995). It is a summer annual oilseed crop with seed oil yields of >440gkg^{-1}DM (Eades and Crowley 1994). Current interest in camelina oil is due to its high content of α-linolenic acid (35-40%).

α-Linolenic acid (ALA; C18:3ω3) is an ω3 essential fatty acid (ω3FA). ω3FAs which must be supplied by the diet as they cannot be synthesised in the body. The main sources of ω3FAs are fish oils, linseed, walnut, soybean and rapeseed oils (Tab. 1).

Fish oils contain two longer chain ω3FAs eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6ω3) whereas plant oils contain ALA. Consumption of ω3FAs are associated with a reduction in platelet aggregation, serum cholesterol / triglycerides resulting in a reduced risk of coronary heart disease. Relief of rheumatoid arthritis symptoms anti-tumorigenic properties have also been attributed to ingestion of ω3FAs. (Erasmus 1993; Sanders and Roshanai 1983; Thomas and Holub, 1994; Dyerberg and Bang 1979; de Lorgeril et al 1994; Sanders and Younger 1981)

Camelina oil has a greater content of ω3FAs than any plant oil currently available to consumers (Fig. 1) with the exception of linseed oil which is considered to be unsuitable for food use due to its susceptibility to degradation by lipid oxidation. Lipids become rancid and develop of off-flavours and odours as a result of lipid oxidation, limiting shelf life of oils and products containing them. Lipid oxidation occurs at the double bonds or points of unsaturation of a fatty acid. The rate of oxidation of fatty acids depends on their degree of unsaturation. The relative rate of oxidation of oleic (C18:1ω9),

linoleic (C18:2ω6) and linolenic (C18:3ω3) acids are 1:40-50:100 on the basis of oxygen uptake. (Heish and Kinsella 1989) This indicates that camelina oil would be prone to degradation by lipid oxidation due to its high content of α-linolenic acid.

This study compares the stability of camelina oil with that of fish, linseed, rapeseed, corn, sesame, sunflower and olive oils. Storage stability of a camelina oil-based spread (53% camelina oil) was compared with an otherwise identical sunflower oil spread.

Tab.1: Fatty acid profiles of various plant oils

				%			
Oil	C16:0	C18:0	C18:1	C18:2ω6	C18:3ω3	C20:1	C22.1
Camelina	5.5	2.3	2.3	16.3(13-20)	38.9(35-40)	15.9	2.3
Linseed	6.5	4.5	20.0	17.0	60.0	-	0.9
Canola	6.2	0.0	61.0	20.0	10.0	-	-
Walnut	-	-	-	60	10	-	-
Olive	-	11.0	73.0	10.0	-	-	-
Sunflower	5.0	2.0	28.0	63.0	-	0.1	-
Sesame	10.0	5.5	39.5	43.0	-	-	-
Rapeseed	7.0	0.0	67.0	17.0	10.0	-	-
Corn	10.0	4.0	29.0	55.0	-	-	-
Soybean	11.2	4.1	21.7	54.0	7.0	0.2	0.1

Materials and methods

Materials

Cold pressed camelina, linseed and rapeseed oils were obtained from Oak Park Research Centre, Carlow, Ireland. Fish oil was obtained from Moorepark Research Centre, Fermoy, Co. Cork, Ireland. Cold pressed corn, sesame, sunflower and olive oils were obtained from a health food store in Limerick, Ireland. Camelina oil was refined at the University of Reading, U.K. Camelina and sunflower spreads were formulated and packaged by Golden Vale, Charleville, Co. Cork. The spreads were packaged into plastic tubs with lids and a heat sealed aluminium foil diaphragm to cover the product.

Methods

Oxidative stability of the oils was evaluated using the Schaal Oven Test for 16 days @ 65°C. Oils were protected from light during heating. Duplicate samples of each oil were removed from two replicate beakers for analysis after various time periods. Peroxide value (PV), ρ-anisidine value (AnV), conjugated dienes (CD) and trienes (CT) were determined using IUPAC standard methods (Paqout 1979). TBARS method by Eskin and Frenkel, 1976 was used.

Tubs of each spread were stored at 4°C and 8°C for 16 weeks. Two tubs were removed fortnightly and duplicate samples were analysed using PV, AnV, TBARS, CD and CT. Sensory analysis of the spreads were carried out every fortnight by a 10-member panel at the University of Limerick. Freshly opened tubs of spread were scored for appearance, odour, flavour and overall acceptability on a scale of 1-10 (1=unacceptable, 10=excellent). Fatty acid analysis of the oils by GC was carried out at Oak Park Research Centre. Data were analysed using analysis of variance (ANOVA) and t-test. Significance was accepted at $p<0.05\%$.

Results and discussion

Accelerated storage tests such as the Schaal Oven Test are used to indicate the resistance of an oil to lipid oxidation. Peroxide value measures the formation of hydroperoxides, primary oxidation products. Peroxides are flavourless but decompose into aldehydes and ketones, secondary oxidation products, many of which have pronounced off-flavours and odours. ρ-Anisidine and TBARS values measure these secondary oxidation products. Conjugated dienes and trienes are formed when linoleic and linolenic acids are oxidised to form hydroperoxides and the double bonds in the oil become conjugated. Primary oxidation products and CD have maximum absorption at 232nm. Secondary oxidation products and CT at 268nm (Hamilton and Rossell 1986; Bowers 1992).

Oxidative stability of camelina oil compared with fish, linseed, rapeseed, sunflower, sesame, corn and olive oils

Rapeseed, sesame, sunflower, corn and olive oils had significantly higher PV than camelina oil during the test period. Camelina oil had significantly higher AnV, TBARS, CD and CT values than rapeseed, sesame, sunflower, corn and olive oils throughout the 16 days. This is probably due camelina oils high α-linolenic acid (ALA, C18:3ω3) content which makes it more susceptible to lipid oxidation than the other oils which are less highly unsaturated (Fig.1). The initial higher values for sesame, sunflower, corn and olive oils could be due to the time elapsed between cold pressing the oils and the test period. These oils were purchased at a high street store and there was no record available of when the oils had been pressed so some oxidation could have taken place prior to testing.

Camelina oil had significantly lower PV, AnV, TBARS, CD and CT than fish and linseed oils throughout the 16 day test period. This indicates that camelina oil is more resistant to degradation by lipid oxidation than fish and linseed oils. Fish oil contains eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:63ω3) which are superunsaturated long chain fatty acids. EPA and DHA contain 5 and 6 double bonds respectively, whereas the α-linolenic acid (ALA, C18:3ω3) in linseed and camelina oil contains three double bonds. The rate of lipid oxidation is greatly increased with increasing number of double bonds making EPA and DHA more susceptible to lipid oxidation than ALA. Linseed oil contains 60% ALA and is oxidised faster than camelina oil which contains 35-40% ALA.

The camelina spread had significantly higher PV, AnV, TBARS, CD and CT values than the sunflower spread throughout the 16 weeks storage period. This shows that the camelina spread is more susceptible to lipid oxidation than the sunflower spread. This is probably because camelina oil in the spread is more susceptible to degradation by lipid oxidation than sunflower oil in the sunflower spread. Sensory analysis of the camelina and the sunflower spreads show that appearance and odour scores for both spreads did not deteriorate significantly during the 16 week storage at 4°C

and 8°C. The camelina spread had a distinctive , but not objectionable flavour, which may have masked the onset of off-flavours making sensory evaluation of flavour difficult. Flavour and overall acceptability scores were lower for the camelina oil spread than for the sunflower spread from week 2 on at both 4°C and 8°C, but were still acceptable at week 16.

Conclusions

Camelina oil was found to be more stable than fish and linseed oils and less stable than rapeseed, corn, sesame, sunflower and olive oils. The camelina spread found to be more susceptible to lipid oxidation then the sunflower spread. Flavour and overall acceptability scores for the camelina spread were lower than those for the sunflower spread but were still acceptable at week 16. Camelina oil would be a more appealing and convenient source of ω3 fatty acids than fish oil.

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Selected probiotic dairy products in Germany - microbiological and physico-chemical parameters

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Probiotic dairy products (yoghurt-mild, yoghurt drinks etc.) are recently in the focus of the consumer and are more successful than classical products. From the beginning of the application of these bacteria, their effectiveness was most important. This depends on the quantity of probiotic bacteria which are present in the product at the time of consumption. A definite quantity has not been established so far, but most of the investigators consider 10^6 cfu/ml (± 0,5 lg cycles) as a minimum for an effect in the intestine of the consumer. However, this effect depends additionally on the quantity of the product which is consumed (e.g. 100 g versus 250 g). Official investigations determined only low numbers of probiotic bacteria in such products or even no bacteria could be detected. Therefore a controlled investigation should be performed in order to analyze the current microbiological quality of probiotic dairy products.

Within an investigation period of four months in total 16 products of 11 manufacturers could be analyzed directly after production, in the middle and at the end of the „best before end" date. The samples derived from different locations of a production charge. The analysis was performed in three parallel procedures. After the transport at 4°C the samples were microbiologically analyzed with selective and elective solid media for lactobacilli (LaS, LSD, MRS) and bifidobacteria (DP-medium). In specific cases a species identification was performed.

The products contained probiotic cultures of the *L. acidophilus*- and the *L. casei*-group as well as from the genus *Bifidobacterium*. Products with strains from the *L. acidophilus*-group showed quantities (geometric mean) of lg 4.89 to lg 8.34 cfu/ml directly after production. Most of the products showed lg 6.5 to 7.5 cfu/ml. At the end of the „best before end" date the quantities decreased in single cases more than 1.5 lg-cycles. Within the *L. casei*-group the differences were minor than in the *L. acidophilus*-group and the respective quantities were lg 6.85 to 8.59 cfu/ml at the beginning of the „best before end" date and decrease at the end of this date less than 1 lg-cycle. Bifidobacteria showed lower quantities directly after production (lg 4.05 to 7.53 cfu/ml) with most products having lg 6.0 to 7.5 cfu/ml. At the end of the consuming period their quantities significantly decreased to ca. lg 5.0 cfu/ml, one product showing even 7.31 cfu/ml, but another with only 1.10 cfu/ml.

As could be shown the content of viable probiotic cultures depends strongly on the species applied. Cultures containing *L. casei*-group strains showed constantly cfu/ml of more than lg 6.0, but strains of the *L. acidophilus*-group showed higher variability. A disadvantage could be seen for products, which contained more than one probiotic culture. Bifidobacteria were most sensitive to storage periods and decreased most dramatically. However, the results confirm that it is possible to produce cultures which contain a sufficient number of viable probiotic bacteria over the whole consumption period. Products with strains of the *L. acidophilus*-group and from the genus *Bifidobacterium* are of special interest, as these microorganisms do not belong to the normal microflora of food, whereas strains of the *L. casei*-group are a common part of the microflora of many foods.

The microbiological investigations were supported by determination of selected physico-chemical parameters: pH-value, lactic acid, carbohydrates. The quantitative determination were performed with enzymatic methods (Amtliche Sammlung §35 LMBG). The content was determined of inulin/oligofructose with HPLC by acid hydrolyzes from selected samples.

The pH-value ranged in all samples from 3.6 – 4.6. The reason is for the variation the different probiotic cultures in the dairy products. Nevertheless the pH-value was stable in the individual product in the period of the investigation.

Lactic acid is typical of dairy products. The concentration ranged in all samples from 0.4 – 0.90g/100g for L-lactic acid and 0.01 – 0.2g/100g for D – lactic acid. These values are described in the literature for yoghurt. The concentration was stable from L-/D - lactic acid in the individual products at the end of the „ best before end“ date.

The contents were unchanged of glucose, fructose and saccharose in the determined dairy products in the period of investigation.

The results show that the metabolism is reduced of microorganism under these experimental conditions.

The concentration was determined of inulin/oligofructose once in selected products. Inulin/oligofructose shows bifidogenic properties and is not metabolized by the bacteria in the human gut. The determined values - 0.5 - 1.6 - were corresponded the declaration of the producer.

The sensory quality is in the focus of the consumer by these products. The sensory quality was determined by using the standardized DLG – scheme with five auditors. At the end of the „best before end“ date two products had the attribute „superior quality“, three products „very good quality“ and ten products „ good quality“.

Some products showed a decreased flavour in the storage period.

In the investigations could be shown that the probiotic dairy products have a good physico-chemical and sensory quality of the same value of „classical“ dairy products.

Improvement of sensory and toxicological characteristics by N-Acetylcysteine using a canned sausage model system

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Summary

Heterocyclic Aromatic Amines (HAA) were produced in meat and meat products by thermal processes. Several of these compounds showed a carcinogenic effect. The formation of these compounds based on the Maillard reaction. The results of model experiments using canned sausages showed that sulfur containing amino acids, such as N-acetylcysteine (NAC) can reduce the formation of HAA and the Maillard reaction. Only the β-carbolines Norharman and Harman were found. The HAA were formed in low concentrations in the control samples without NAC (IQ 0.3 ng/g) at heating times 120 and 140 min. The addition of NAC had especially a positive effect of colour and flavour of canned products in the sensory testing.

Introduction

Heterocyclic aromatic amines (HAA), which are powerful mutagens, are formed in meat, fish and meat products subjected to thermal processes. These substances are produced at the surface of fried, broiled or cooked meat or fish. Since their discovery about 20 heterocyclic amines have been identified and quantified in cooked foods (FELTON and KNIZE, 1990). The structure of HAA are characterized by the fact that most of them had an exocyclic amino group and a methyl group in a ortho position. This type of structure was similar to the powerful synthetic chemical carcinogen 2',3-dimethyl-4-aminobiphenyl. On the basis of results from long-term animal-studies on rodents and non-human primates (ADAMSON et al., 1990), several of HAA have been classified as probably carcinogenic (IARC, 1993). For these reasons, the IARC recommends the reducing of human exposure to these substances. The formation of these compounds based on the Maillard reaction. Creatine or creatinine and the Maillard reaction products such as pyrazines, pyridines and aldehydes are assumed to condense to IQ-compounds. Sulfur containing amino acids such as cysteine, N-acetylcysteine and the tripeptide glutathione inhibit the Maillard reaction and the action of mutagens, carcinogens and other toxic compounds by direct interaction (FRIEDMAN and MOLNAR, 1990). These antioxidant and antitoxic effects are due to several mechanisms including the ability to act as reducing agents, scavengers of reactive oxygen, strong nucleophiles that can trap electrophilic compounds and intermediates, precursors for intercellular reduced glutathione, and inducers of cellular detoxification (FRIEDMAN and MOLNAR, 1990).

The aim of this study was examine the possibility to reduced the formation of HAA by using the additive N-acetylcysteine. In addition to the products were investigated of acceptability by sensory tests and colour measurements.

Materials and methods

Preparation of canned sausages

First, we prepared a batter of finely comminuted sausage consisting of 25 % coarsely defatted pork, 25 % coarsely defatted beef, 30 % pork backfat and 20 % ice. The meat and fat were chopped together in a chopper (Type K64 DC, Seydelmann, Aalen, D). Nitrite curing salt (17g/kg), sodium ascorbate (0.5 g/kg), spices (white pepper 1 g/kg, coriander 1 g/kg, nutmeg 0.5 g/kg, cardamom 0.5 g/kg) and half of the ice were added and the mixture was chopped until the temperature reached 12°C. Then the remaining half of the ice was added.

Trial 1: The formulation was a batter of finely comminuted sausage.

Trial 2: In 6-kg-batch, 0.15 % NAC and 0.25 % phosphate was added to the batter. The mixture was chopped in a little chopper (Type UMC 12, Stephan, Hameln, D).

Each mixture was filled in cans with 250 g and the cans were heated separately for different times at 121 °C sterilization temperature. The following heating times were used: 40, 60, 80, 100,120 and 140 min.

Determination of HAA

The method of HPLC analysis based on the method described by GROSS and GRÜTER (1992).

Sensory tests

The task of the sensory panellists was to evaluate the cans with different heating times and additive of NAC/Phosphate for colour and flavour using the following 6 score scale:

6 = Like extremely	3 = Dislike slightly
5 = Like very much	2 = Dislike moderately
4 = Like moderately	1 = Dislike extremely

Colour measurement

The colour of the canned sausages products was measured with a Minolta Chroma Meter CR 200 with CIE-LAB-system (DIN 6174). The values are means of the L*, a*, b*- co-ordinates taken at 3 points on the surface.

Results and discussion

In the above experiments the core temperatures did not exceed 120 °C, nevertheless HAA were formed. The HAA compound IQ (2-amino-3-methylimidazo[4,5-f]quinoline) were found at very low concentrations (0.33 and 0.25 ng/g) in the batches without additives and at the heating times of 120 and 140 min, respectively. Batches which contained NAC and Phosphate IQ-compounds could not be detected.

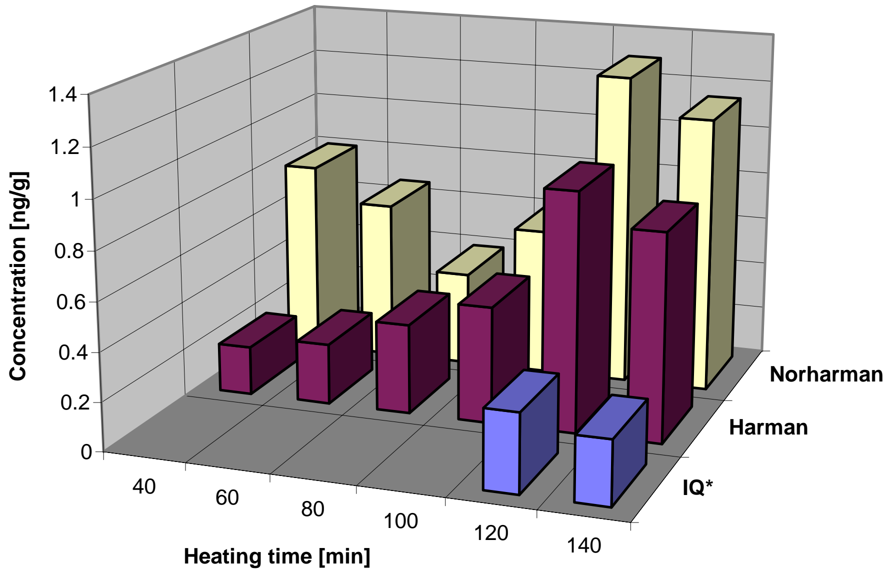
The compound NAC can reduce the formation of Maillard products and HAA-compounds. All analysed samples containing the β-carbolines Norharman and Harman, but samples with the combination of additives NAC/Phosphate have lower concentrations of Harman and Norharman (Fig.1). Norharman and Harman are not mutagenic with reference to *Salmonella typhimurium* TA 98 and TA 100, but Norharman enhances the mutagenic activity of Trp-P-1 and Trp-P2 (SUGIMURA et al., 1982).

The addition of NAC had a positive effect on the colour of the canned sausages. The lightness indicator L* value was higher and the b* value was lower, i.e. the yellow colour was less intensive and the colour was lighter compared to the control sample at the same heating times (Fig.2). Our sensory tests showed that the addition of NAC improves the sensory characteristics of the canned sausages. The testers preferred the products with the addition of NAC and Phosphate. Especially the colour was positively influenced, but also the taste was better evaluated than the taste of control samples above all at heating times of 60, 80 and 100 min (Fig. 3 and Fig. 4). But the difference was not significantly. We use the combination of NAC and Phosphate because an acid taste could be compensated by the addition of 0.25 % diphosphate (HILMES, 1996). FRIEDMAN and MOLNAR (1990) reported that unlike cysteine, NAC produced minimal or no off-flavour at concentrations that inhibit browning in protein-containing foods. The sensory results of canned products are not directly transferable to grilled or fried meat products since a intensive brown colour of the products is wanted here.

In commercial canned products, the sterilisation temperatures were normally lower and the heating times shorter than in this study (HILMES, 1996). Therefore the concentration of HAA is generally lower than in fried or grilled food (FELTON and KNIZE, 1990).

Conclusions

The addition of the combination of NAC and phosphate has especially a positive effect of colour and flavour of the canned sausages and can inhibit the formation of Maillard reaction products and also HAA. But in comparison to grilled or fried meat products the HAA were formed in very low concentrations in canned meat products



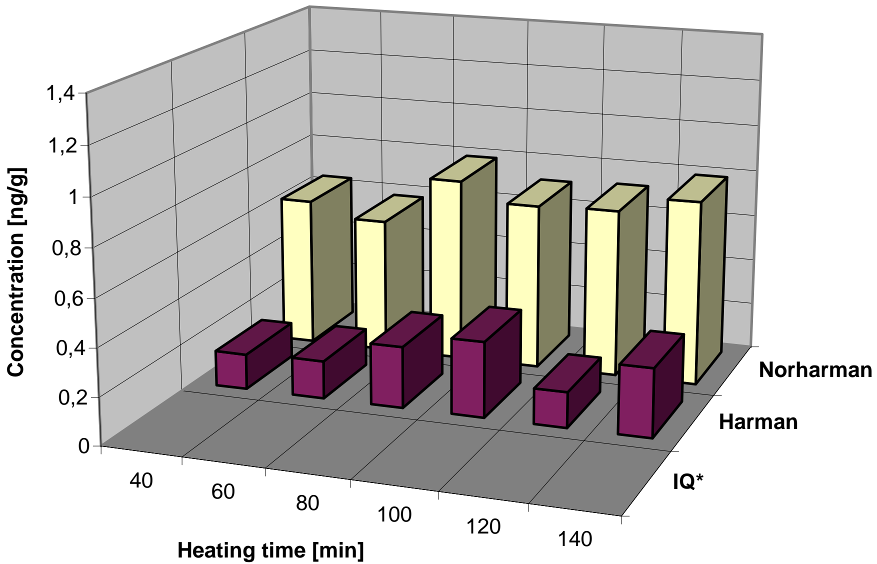
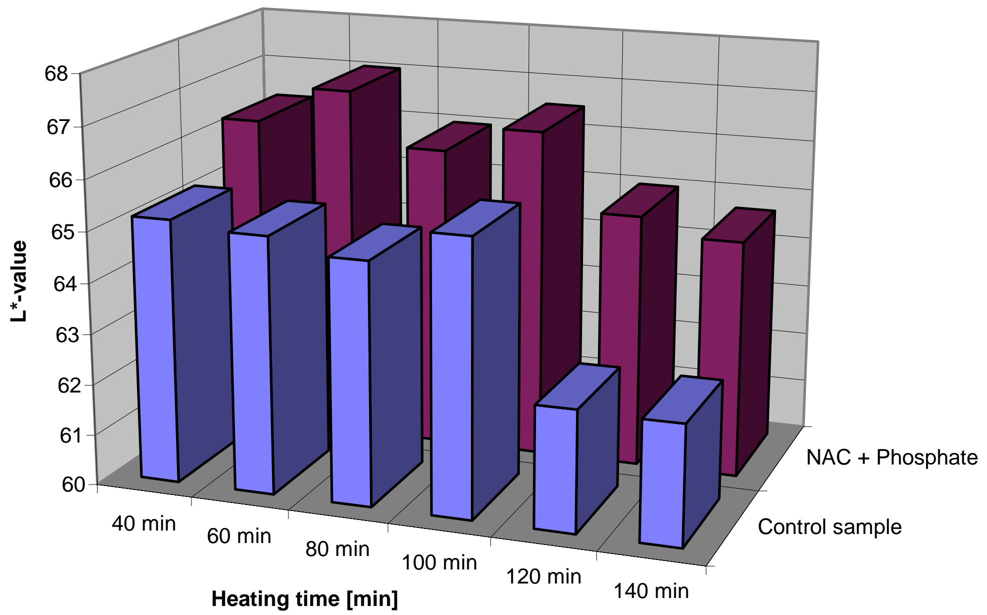


Fig. 1: Concentration of Heterocyclic Aromatic Amines in cans without and with the additive of NAC (0.15 %) and Phosphate (0.25 %) at different heating times



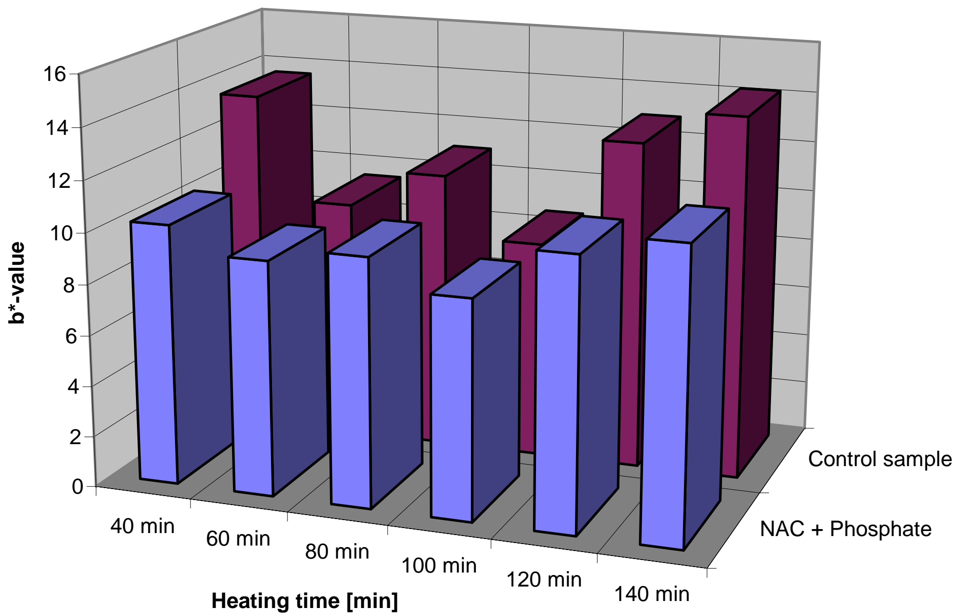
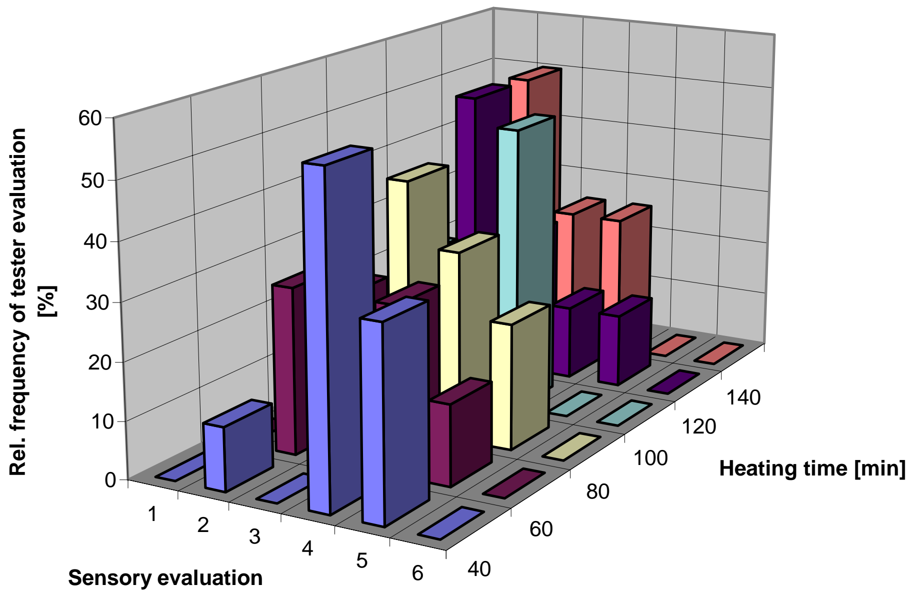


Fig. 2: Colour measurement of b*- and L*-value



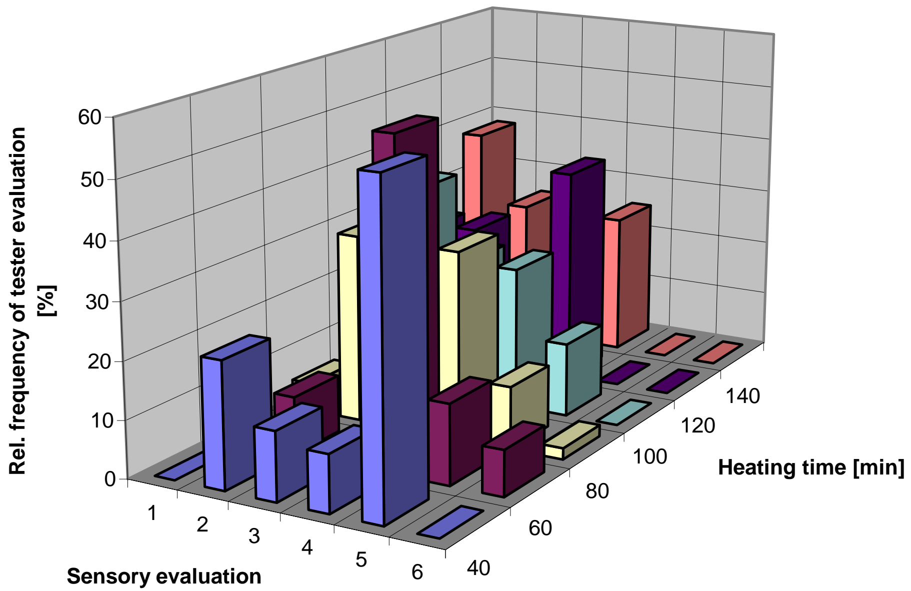
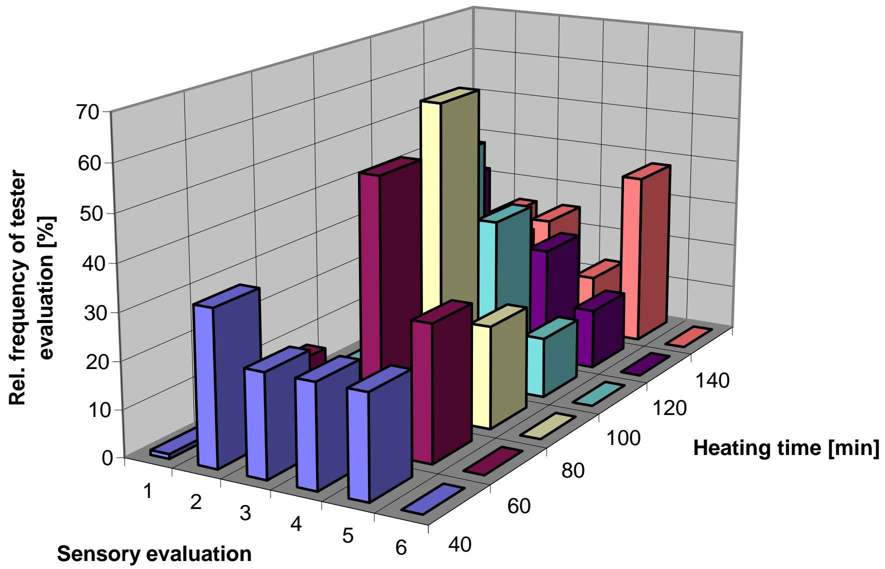
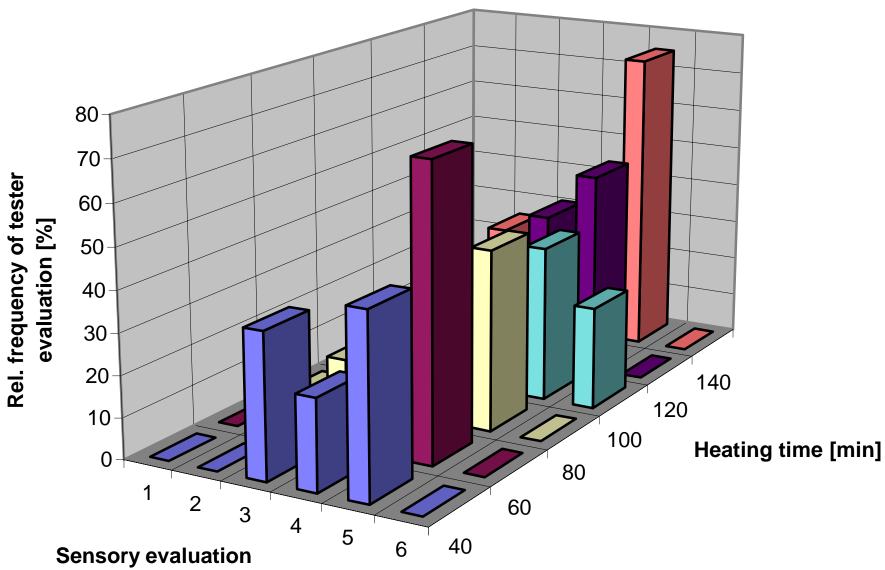


Fig. 3: Relative frequency of tester evaluation (taste/flavour) for the canned sausages at different heating times





6 = Like extremely	4 = Like moderately	2 = Dislike moderately
5 = Like very much	3 = Dislike slightly	1 = Dislike extremely

Fig. 4: Relative frequency of tester evaluation (colour) for the canned sausages at different heating times

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Carry over of additional vitamin E amounts from feeds into food of animal origin

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Introduction

Supplementation of high vitamin E levels is very common in animal production in many countries. Vitamin E demand of domestic animals varied between 10 and 20 mg kg^{-1} DM of the feed (GfE 1986, 1987, 1995, 1999). Supplementation of 50 to 100 mg kg^{-1} DM are used in many farms (e.g. BOSSOW, 1995). In some cases higher dosages of vitamin E are added to 1 kg feed under farm conditions. There are many reasons to give high vitamin E levels to domestic animals:

- to improve animal performances,
- to increase animal health,
- to improve the antioxidative state of the animal,
- to improve the quality of food of animal origin,
- to increase the vitamin E content of food of animal origin in order to improve the vitamin E intake of man.

There exist evidence that vitamin E supplementation above the physiological requirements does mostly not improve animal performances as demonstrated in many experiments (e.g. JAKOBSEN et al., 1997, SCHWARZ et al., 1997, SÜNDER et al., 1997a,b, VEMMER et al.,1997).

An increased antioxidative potential in consequence of higher vitamin E intake may improve animal health as measured in improved immunological reactions, decreased mastitis etc. (e.g. HOGAN et al., 1993, MC DOWELL et al., 1996, NOCKELS et al., 1993).

Parameters of quality of foods of animal origin as colour, oxidative stability, tenderness, storage properties etc. may be improved by abundant vitamin E supply as shown by many authors and summarised by ANGELO (1992), FLACHOWSKY et al. (1997b), LEONHARDT et al. (1997), MC DOWELL et al. (1996) recently.

Vitamin E supplementation of feed increased vitamin E content of food of animal origin and may improve vitamin intake of man. This way could be important because of suboptimal vitamin E intake of the population (man: 95%; woman: 84%) compared to the recommendation of the DGE (DGE 1991, 1996). Therefore in some cases vitamin E supplementation of animal feed is recommended to produce better food in order to increase vitamin E supply of human population.

Nothing is known about the carry over of supplemented vitamin E levels from feed into food of animal origin or about the efficiency of such ways to improve vitamin E intake of man. The objective of the present report is to analyse the carry over of additional vitamin E levels into food of animal origin. Mainly data from our research group have been considered in the study.

Materials and methods

Feeding experiments were carried out with dairy cows, bulls, fattening pigs, broilers and layers. Different diets contained various levels of vitamin E or they were supplemented with high vitamin E dosages before slaughtering. More details of experimental design are described by authors mentioned in tables 1 to 4.

Vitamin E determination in feeds and foods of animal origin was done by HPLC as described by MATTEY et al. (1991). Carry over of vitamin E from feed into food was calculated in the following way:

- determination of vitamin E content of edible products of animal origin (milk, meat, fat, eggs, liver etc.),
- determination of weight of edible products of animal origin,
- calculation of vitamin E levels in the edible products (e.p.),
- total carry over = $\frac{\text{vitamin E in e.p.}}{\text{vitamin E intake}} \times 100$
- carry over of vitamin E supplement

$$= \frac{\text{vit. E in e.p. with supplement. - vit. E in e.p. without supplement.}}{\text{supplemented vitamin E amount}} \times 100$$

Results and discussion

Vitamin E content of foods

Vitamin E supplementation increased vitamin E content of foods of animal origin as shown in tables 1 to 4. The lowest effect were observed in dairy milk (Table 1).

Feeding of silage or hay (preservates) effected lower vitamin E content of milk than forage feeding. Oilseeds like rapeseed may increase the vitamin E content of seeds and the better vitamin E absorption together with fats (COHN, 1993, KAYDEN and TRABER, 1993).

Similar responses of abundant vitamin E levels were measured in beef and pork (Table 2). Short term vitamin E supplementation of bulls (1 g per animal per day beginning 21 days before slaughtering) increased tocopherol concentration of muscle from 1.0 to 1.1 mg kg^{-1} (KUHN et al., 1997). Probably the period of vitamin E application was to short for bulls. Longer application (0.6 or 2 g per animal per day for 120 days) increased vitamin E concentration of muscle to >3 mg kg^{-1} (SCHWARZ et al., 1997).

In four experiments with fattening pigs various vitamin E levels were supplemented before slaughtering (Table 2). In the fifth experiments 0, 100 and 200 mg vitamin E were added per kg feed and compared with 1.2 g vitamin E supplementation per day during the last 21 days before slaughtering.

Table 1: Vitamin E content of dairy milk depending on feeding and vitamin E supplementation of cows by various authors

Author	Feeding (roughage), Vitamin E supplementation (g per animal per day)	Vitamin E content of milk	
		mg l^{-1}	µg g^{-1} fat
JAHREIS	Without rapeseed	1.1	25
et al. (1993)	+ 1kg rapeseed per animal per day	1.6	41
NICHOLSON	Corn silage	max. 1.1	max. 28
and	Corn silage (+7 g)	2.0	50
ST. LAURENT (1991)	Alfalfa silage	1.3	max. 32
	Alfalfa silage (+ 3 g)	2.0	50
NICHOLSON	Alfalfa silage	0.6	15
et al. (1991)	Alfalfa silage (+ 3 g)	1.0	26
SCHEIDE	Silage (+ 1 g)	1.3	35
et al. (1995)	Fresh grass (+ 1 g)	1.8	45
ST. LAURENT	Forages (+0.7 g)	0.7	17
et al. (1990)	Forages (+1 g vit. E)	1.0	25

Similar vitamin E intake (~25 g) was achieved of pigs consuming 100 mg vitamin E per kg feed or 1.2 g vitamin E per day during the last 21 days. The results show adequate vitamin E levels in muscle and fat (Table 2, ROSENBAUER et al., 1998). In consequence of those data results after short term vitamin E application seems to be representative and could be considered for carry over calculations.

Broilers showed a higher response of vitamin E supplementation (Table 3) than bulls and pigs. 100 mg vitamin E per kg feed increased vitamin E concentration in thigh muscle from 1.1 to 4.9 and in breast muscle from 2.6 to 7.2 mg kg^{-1}.

Considerable increase of vitamin E content was measured in eggs (Table 4). High dosages of vitamin E to broilers and layers (Tables 3 and 4) were offered to study toxicological effects of the vitamin (SÜNDER et al., 1998).

Table 2: Vitamin E content in selected food from pigs on vitamin E supplementation by various authors

Author	Feeding, Vitamin E supplementation	Vitamin E content (mg kg^{-1}) Liver	Muscle	Backfat
Berk	Control[1)]	3.8	3.9	7.0
et al. (1998)	+ 0.5 g vit. E per day[2)]	5.6	6.2	9.8
	+ 1 g vit. E per day[2)]	7.0	7.8	14.0
Flachowsky	Control[1)]	3.7	1.7	84.8
et al. (1997a)	+ 1 g vit. E per day[2)]	29.6	2.3	91.3
	+ 5% false flax expeller	8.1	2.0	52.0
	+ 1 g vit. E per day[2)]	27.6	3.0	82.3
	+10% false flax expeller	4.4	1.9	60.7
	+ 1 g vit. E per day[2)]	19.0	2.0	72.4
Flachowsky	Control[1)]	5.4	1.8	12.5
et al. (1993)	+ 1 g vit. E per day[2)]	50.3	3.5	30.0
	+ 10% rapeseed	5.3	2.8	13.4
	+ 1 g vit. E per day[2)]	49.1	3.8	31.1
	+ 20% fullfat soybean	6.2	2.1	11.1
	+ 1 g vit. E per day[2)]	35.6	3.7	32.1
Gottschalk	Control[1)]	0.5	0.5	7.0
et al. (1994)	+ 1 g vit. E per day, 7 days before slaughtering	9.2	0.5	6.8
	+ 1 g vit. E per day, 14 days before slaughtering.	5.9	0.9	10.6
	+ 1 g vit. E per day, 21 days before slaughtering	8.6	1.1	12.8
Rosenbauer	Control[1)]	n. d.	2.4	9.4
et al. (1998)	+ 100 mg vit E per kg feed	n. d.	4.9	19.8
	+ 200 mg vit E per kg feed	n. d.	5.6	24.8
	+ 1.2 g vit. E per day[2)] (adequate to 100 mg per kg feed)	n. d.	4.5	19.9

[1)] Vitamin E content of control mixtures varied between 15 and 20 mg per kg
[2)] Vitamin E supplementation started 21 days before slaughtering

Table 3: Vitamin E content of food from broilers depending on vitamin E supplementation (SÜNDER and FLACHOWSKY, 1997)

Vitamin E supplementation (mg kg^{-1} feed)	Vitamin E content (mg kg^{-1})		
		Muscle	
	Liver	Thigh	Breast
Control (20 mg per kg)	3.0	1.1	2.6
+ 100	11.7	4.9	7.2
+ 1 000	44.9	31.8	34.5
+ 10 000	158	90.5	88.7
+ 20 000	332	122	122

Table 4: Vitamin E content of eggs and carry over of vitamin E into eggs depending on vitamin E supplementation (SÜNDER et al., 1997a)

Vitamin E supplementation (mg kg^{-1} feed)	Vitamin E content		Carry over of vitamin E (% of supplementation into eggs)
	μg g^{-1} yolk	mg per egg	
Control (20 mg kg^{-1})	72	1.1	-
100	247	3.7	25
1000	1418	19.8	16
10 000	2600	35.3	3
20 000	3201	42.5	2

Carry over of vitamin E

Calculations of carry over of vitamin E from feed into food of animal origin are simple for milk and eggs. The products are mainly total used as food in both cases. Vitamin E is stored in some organs and tissues (e.g. some interior organs, fat, blood etc.) of the slaughtering body, but they are not always used as food for human nutrition.

Under consideration of data shown in tables 1 to 4 and some references carry over of additional vitamin E levels from feed into food of animal origin is shown in Table 5. The highest carry over values were calculated for eggs (Table 5) decreasing with higher vitamin E supplementation (Table 4).

In comparison to eggs the carry over of added vitamin E from feeds into milk or meat is very low (0.2 to 2%; Table 5). Of course it seems possible to improve the vitamin E supply of man via food of animal origin, but the efficiency is very low except eggs.

Higher vitamin E supplementation may improve some parameters of quality of food of animal origin, but its contributions for human nutrition is negligible. Furthermore the higher costs of vitamin E supplementation are mostly not repaid to the farmers.

Table 5: Influence of vitamin E supplementation on vitamin E content of food and carry over of vitamin E into food of animal origin (average from own experiments and added by references for milk and beef)

Food of animal origin	Vitamin E supply (mg kg^{-1} DM or supplementation)	Vitamin E content of food (mg l^{-1} or mg kg^{-1})	Carry over of vitamin E supplementation into food (% of supplementation)
Milk	50	1.2	-
	+ 1 g d^{-1}	1.8	1.2
	+ 3 g d^{-1}	2.0	0.5
Beef	40	2.0	-
	+ 1 g d^{-1}	4.0	0.2
Pork	20	2.0	-
	+ 100 mg kg^{-1} feed	4.0	0.5
Chicken	20	2.0	-
	+ 100 mg kg^{-1} feed	6.0	2.0
Egg	20	20	-
	+ 100 mg kg^{-1} feed	70	25

Summary

Some experiments with dairy cows, bulls, fattening pigs, broilers and layers were carried out to study the effects of abundant vitamin E levels on vitamin E content of foods of animal origin and to calculate the carry over of vitamin E into food.

Vitamin E supply to dairy cows increased vitamin E content of milk to small portions (e. g. from 1.2 to 1.8 mg kg^{-1} milk, when 1 g per animal per day was supplemented). An increase of the vitamin E supply to five times of the recommendations results in an increase of vitamin E concentration in meat by a factor of 2 and in eggs by a factor of 4. Apart from eggs the highest vitamin E concentrations were measured in liver and depot fat.

The highest carry over of additional vitamin E into foods of animal origin was measured for eggs (16 or 25% when 1000 or 100 mg vitamin E per kg feed were supplemented). It was below 2% for all other foods.

In consequence of the low carry over additional vitamin E amounts in animal nutrition to improve vitamin E supply of men could not be recommended except eggs.

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Determination of oligosaccharides in soy bean products using an evaporative light-scattering detector

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Introduction

Legumes are important sources of protein but they contain also antinutritional factors. Among these factors are thermostable, galactose-containing oligosaccharides such as stachyose and raffinose. They are not digestible by the enzymes of the gastrointestinal tract but are metabolized by intestinal microorganisms. These saccharides might cause wellness problems such as flatus, abdominal rumbling and diarrhoea [1]. Other saccharides are also present in soy beans or are added as sweeting agents to soy bean products.

Oligosaccharides can be separated on aminopropyl silica phases using high-performance liquid chromatography (HPLC). Sensitive detection of sugars is difficult because they show only weak absorption in UV. Therefore, usually a refraction index detector is used, however, no gradient elution can be applied in this case.

Here we describe a method for the quantitative determination of saccharides. They are separated on aminopropyl silica using gradient elution and determined by employing an evaporative light-scattering detector (ELSD).

Experimental

The saccharides fructose, glucose, sucrose, maltose, lactose, mellibiose, mellicitose and raffinose were purchased from Fluka (Buchs, Switzerland), stachyose was from Acros (Neuss, Germany), acetonitrile (MeCN) and ethanol (EtOH) were of the gradient grade and purchased from Merck (Darmstadt, Germany). Bidistilled water was prepared using a quartz distil.

Soy bean products (see Tab. 2) were purchased in local retail outlets.

Aliquots of soy bean products were suspended in 80% EtOH (v/v) and a mellibiose solution (2.5 mM) was added as internal standard. The mixtures were refluxed for 1 h at 75 °C and filtered. Defatting was achieved by passing through Sep-Pack® cartridges (Waters) previously conditioned with 80% EtOH. Eluates were evaporated to dryness *in vacuo*, the remaining residues were dissolved in 80% EtOH (v/v) and subjected to analysis by HPLC.

For HPLC a LaChrom System (Merck-Hitachi, Tokyo, Japan) was used comprising organizer D 7000, pump D 7100, autosampler L 7250 and oven L 7300; column LichroSpher® 100 NH_2 (5 µm) 250 x 4 mm I.D. with LichroCart RP18 guard column; flowrate 1 ml/min; temperature 35 °C; eluent A MeCN/ H_2O 75/25 (v/v); eluent B H_2O; gradient program 0-5 min. 0% B,10 min 5% B, 18 min 7% B, 22 min 15% B, 35 min 15% B.

The ELSD used was a PL-EMD 960 (Polymer Laboratories Ltd., Shropshire, UK).

Results and discussion

The response of the ELSD is affected by the nebulization gas flow rate which is controlled by the inlet gas pressure and the temperature of the evaporator tube. These factors are responsible for changes in the nebulization process and the particle size distribution [2].

Compressed air was used as nebulizer gas. Optimal detection parameters were a nebulization gas flow of 3.5 l/min, 206 kPa gas-pressure and an evaporation temperature of 65 °C (Fig. 1) [3]. A gas flow rate below 3.5 l/min resulted in baseline drift and peak tailing.

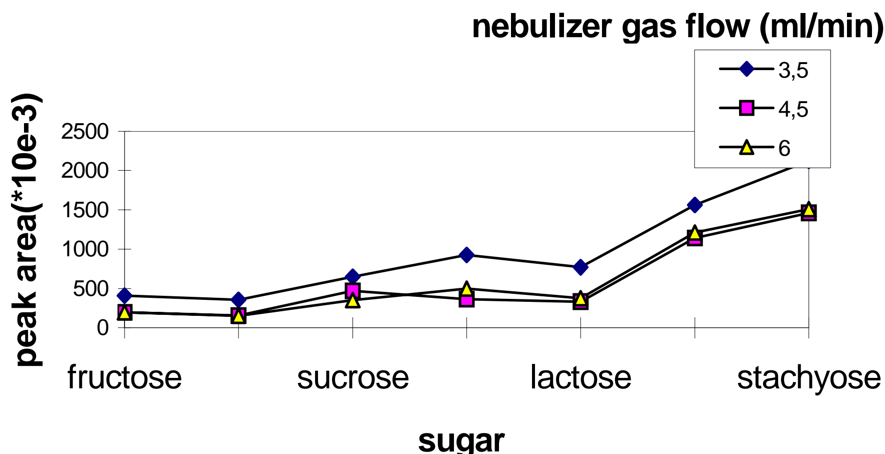

Fig. 1: Correlation of nebulizer gas flow and peak areas of saccharides in a standard mixture (1mM)

The response of the ELSD is not linear. At equimolar concentrations the detected response increases on increasing molecular weights (Fig.2). The drift of the baseline results from the amounting of water in the course of gradient elution. The detection limit is approximately 200 pmol for all saccharides.

The logarithmic calibration curves show an excellent correlation between concentration and peak area (Table 1).

Table 1: Calibration curves of sugars (n = 3)

Sugar	retention time (min.)	r^2	curve coefficients [1)] (A_0)	(A_1)	(A_2)
fructose	7,4	0,9987	2,009e-001	4,165e-006	-4,606e-013
glucose	8,6	0,9987	2,009e-001	4,165e-006	-4,606e-013
sucrose	13,1	0,9988	4,513e-001	2,937e-006	-1,175e-013
maltose	15,4	0,9991	3,132e-001	4,040e-006	-2,041e-013
lactose	17,2	0,9989	3,214e-001	4,650e-006	-2,711e-013
raffinose	23,2	0,9977	4,420e-001	-3,017e-007	1,880e-013
stachyose	30,2	0,9992	5,904e-001	3,010e-014	-4,439e-014

[1)]coefficients of $y= A_0 +A_1*x +A_2*x^2$; y = peak area, x = conc. (mM); r^2 = correlation factor

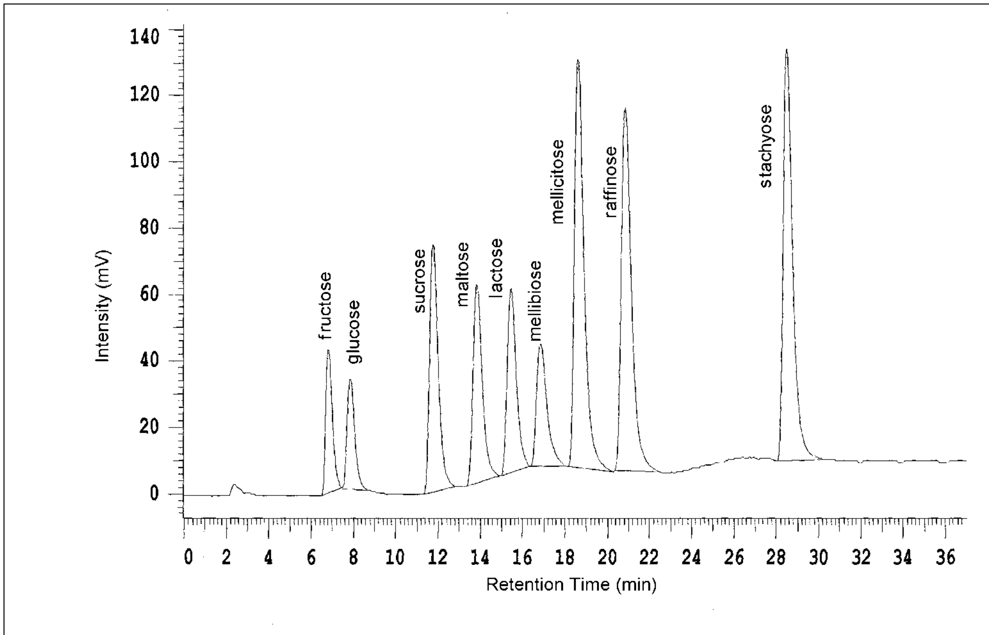

Fig. 2: HPLC of a standard of sugars (2mM); for conditions see Experimental

Sucrose is the most abundant sugar in all soy bean products (Fig. 3). However, relative high amounts of stachyose and raffinose could also be detected in all soy bean products. Soy bean drink 1 did not contain sucrose, in contrast to soy bean drink 2 which was declared to contain sucrose.

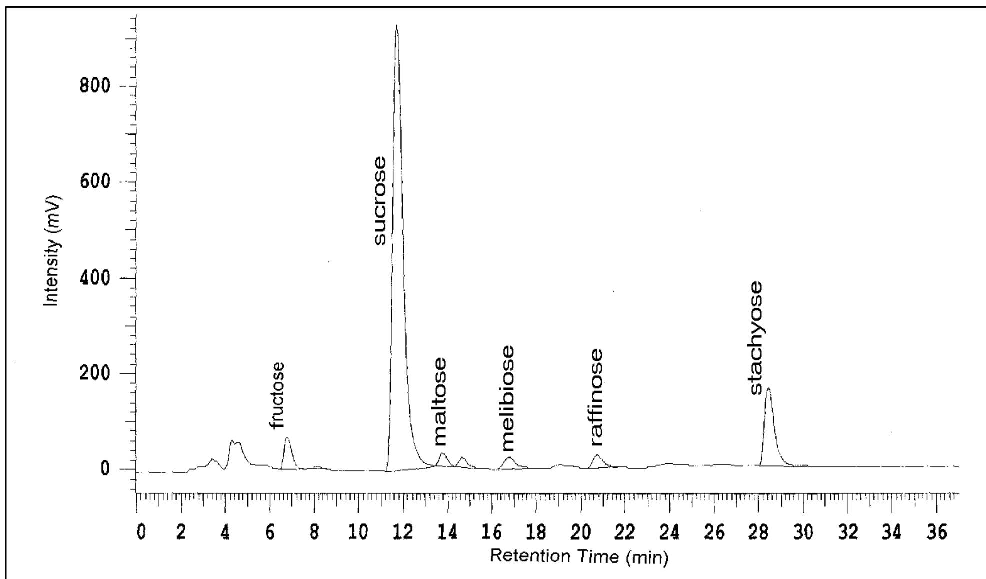

Fig. 3: Chromatogram of soy bean powder

Table 2: Amounts of saccharides (g/kg or g/l) in soy bean products

Saccharide	Soy bean		Soy bean powder		Instant tofumix		Soy bean drink 1		Soy bean drink 2	
	x	SD	x	SD	x	SD	x	SD	x	SD
fructose	4.74	0.128	5.64	0.074	2.45	0.007	3.0	0.006	0.28	0.002
glucose	0.41	0.041	n.d.		0.37	0.107	0.14	0.006	n.d.	
sucrose	44.11	0.138	55.43	0.047	39.11	0.115	n.d.		4.98	0.021
maltose	1.92	0.081	0.76	0.045	0.485	0.019	n.d.		0.12	0.003
raffinose	3.2	0.005	4.0	0.036	3.3	0.008	2.1	0.004	1.8	0.004
stachyose	15.33	0.131	22.7	0.267	13.25	0.058	1.39	0.158	2.77	0.039

x = average, SD = standard deviation, (n = 3)

The data show that HPLC and use of an ELSD are highly suitable methods for the determination of oligosaccharides in food. Simple sample preparation and short analysis time are of advantage.

The method is well suited for the fast and reliable determination of stachyose and raffinose in soy bean products.

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Analysis of vitelline membrane proteins of fresh and stored eggs via HPLC

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Summary

The outer layer of the vitelline membrane of hen's eggs was investigated with respect to changes in dependence upon storage temperature and duration of storage. At a temperature of 20°C the amount of VMO1 and VMO2 in the salt soluble fraction decreased during storage, whereas eggs stored under refrigerated conditions did not show significant changes. Membrane weight and protein amount of the salt soluble fraction seemed not to be influenced during storage while the lysozyme percentage in the salt soluble fraction increased. This leads to the conclusion that the deterioration of the vitelline membrane during storage may be related to disintegration of the proteins VMO1 and VMO2 from the membrane.

Introduction

The outer layer of the vitelline membrane formed after ovulation in the upper part of the oviduct consists of sublayers, which are composed of fibrils. It consists of ovomucin, lysozyme and at least two other proteins - the vitelline membrane outer proteins VMO1 and VMO2 [1, 2]. The vitelline membrane deteriorates during storage. Investigations on the protein composition revealed a loss of VMO1 as well as the formation of a lysozyme dimer in the outer layer [3]. To investigate changes in the components of the outer layer of the vitelline membrane during storage our studies focused on lysozyme, VMO1 and VMO2.

Materials and methods

Hen's eggs (grade A, weight 60 - 65 g) were stored up to 30 days at temperatures of 5, 10 and 20°C at a relative humidity of 60 %. The vitelline membrane was separated and the proteins lysozyme, VMO1 and VMO2 were extracted by concentrated NaCl-solution. The proteins of the salt soluble fraction were fractionated by HPLC on a RP-C_4-column and subsequently quantified.

Results and discussion

The salt soluble fraction was separated into 7 distinct peaks via HPLC (Figure 1). The molecular weights of the substance peaks eluted at retention times of 8.8 min., 15.7 min. and 18.1 min. were determined by means of SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE). According to the areas of the peaks lysozyme, VMO1 and VMO2 a relation of 80:15:5 was calculated.

In the case of vitelline membranes from fresh eggs the lysozyme content was about 55 % of the protein content in total (Figure 2). During storage of 30 days it increased up to 70%. The determined content of lysozyme showed a high variation during storage. The differences were only significant during storage at 20°C.

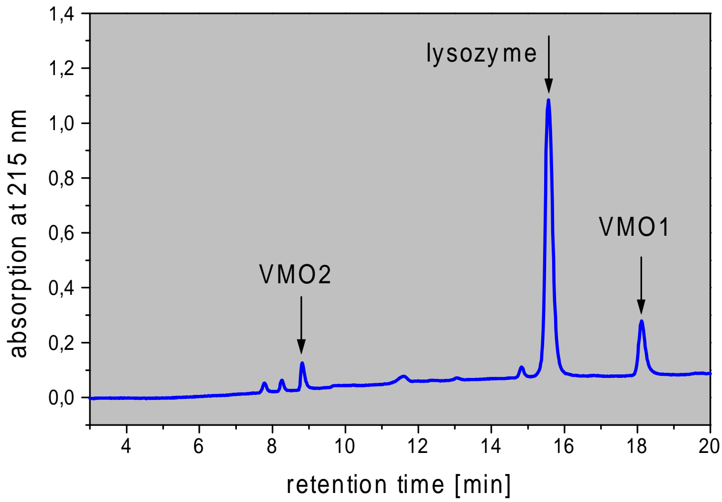

Figure 1: Separation of the salt soluble fraction of the vitelline membrane by HPLC on a RP-C_4-column.

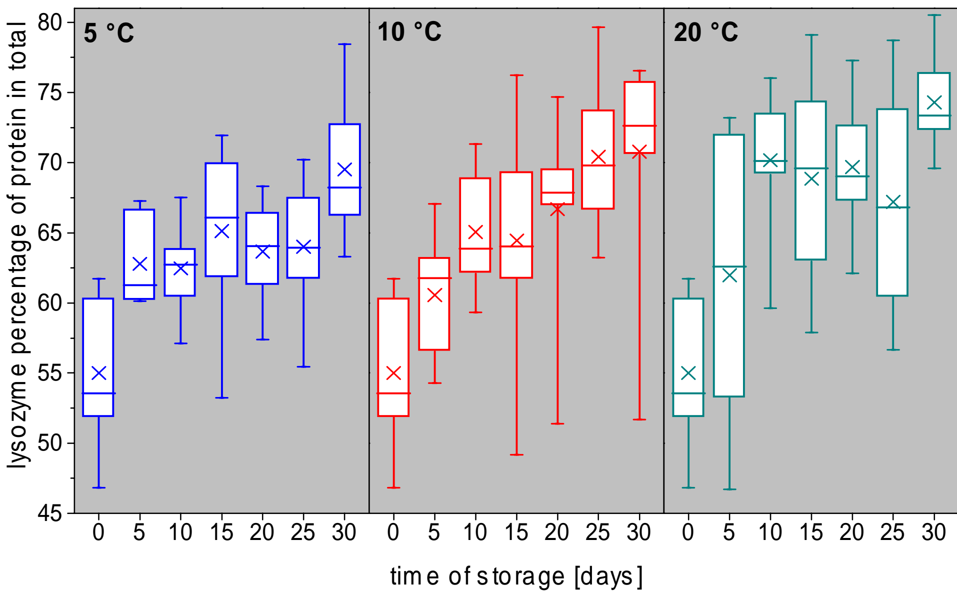

Figure 2: Changes of the lysozyme content during storage at different temperatures.

Under refrigerated conditions (5 and 10 °C) no significant decrease could be observed for the VMO1 content. Whereas at a storage temperature of 20°C a slight but significant decrease appeared (Figure 3). The percental peak area of VMO1 related to the total peak area of the three above mentioned proteins shows a decline from about 15% to 5% during 20 days of storage. The value for the VMO2 content decreases already at storage temperatures of 5°C and 10°C from about 5% to 3% within a period of 10 days and remains constant (Figure 3). Storage for 20 days at 20°C causes a continuous decline of the VMO2 content to about 1%.

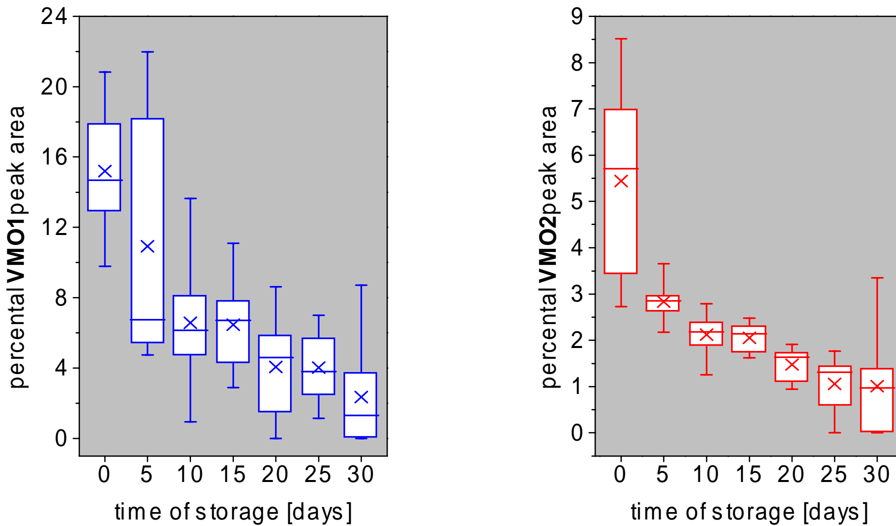

Figure 3: Changes of the peak areas of VMO1 and VMO2 during storage at 20°C.

It can be concluded that the dissociation of salt soluble proteins during storage is responsible for the disintegration of the membrane structure, which leads to a reduction of the membrane strength. The ratio of the salt soluble proteins changed throughout storage because of the different individual diffusion rates. As the changes during storage at room temperature were significantly higher than under refrigerated conditions, the membrane proteins might serve as an indicator for the applied storage conditions.

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Correlations of anticarcinogenic conjugated linoleic acid with other C18 fatty acids in German bovine milk fat

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Abstract

Based on different studies certain conjugated linoleic acids (CLA), in particular the isomer *cis* Δ9,*trans* Δ11-C18:2 (c9t11), are reported to probably have antioxidative, antiatherogenic and especially anticarcinogenic properties. On the other hand, *trans*-C18:1 as well as several *trans*-C18:2 isomers partly are related to negative physiological effects, as e.g. premature atherosclerosis. Knowledge of the variation of c9t11 in milk fat associated with feeding as well as of its formation in the rumen of cows is important to exert influence on the CLA content in milk fat. In different feeding trials it could be demonstrated that the CLA content in milk fat can be increased considerably by means of feeding. But in all cases this also increased the contents of undesirable *trans*-C18:1 and *trans*-C18:2 fatty acids. The high correlation of C18:3 with CLA ($r = 0.71$) and the smaller inverse correlation of C18:2 with CLA ($r = -0.40$) suggest the possibility of CLA formation from linoleic acid.

1 Introduction

Due to their probably antioxidative, anticarcinogenic and antiatherogenic properties conjugated linoleic acids (CLA) are of importance [1-6]. Bovine milk fats exhibit higher CLA contents than vegetable fats by far [7]. Having a proportion of at least 90 %, *cis* Δ9,*trans* Δ11-C18:2 (c9t11) represents the predominant CLA isomer in bovine milk fat [7]. Knowledge of the variation of this isomer associated with feeding as well as of its formation in the rumen of cows possibly could allow to exert influence on the CLA content in milk fat. On the other hand, with respect to a possible increase of the CLA contents in milk fat, correlations with further *trans*-C18:2 but also *trans*-C18:1 isomers are of significance, as these, in contrast to CLA, frequently are reported to have negative physiological properties.

In recent years publications on dietary or epidemiological studies caused a Worldwide discussion about potential risks associated with an elevated consumption of *trans* fatty acids (excluding CLA). In this context debates on premature atherosclerosis and cardiovascular diseases were prominent which particularly is supposed to concern *trans*-C18:1 fatty acids [see reviews 8-12]. Moreover, *trans*-C18:2 (excluding CLA) and in particular the isomer t9t12 are regarded as competitive inhibitors for the conversion of linoleic acid to arachidonic acid, partially blocking formation of C20:5 n-3 [13,14]. Thus, the present study is to focus on the relations and correlations of CLA contents with the contents of other *trans* fatty acids as well as linoleic and linolenic acid associated with feeding.

2 Materials and methods

Ca. 2000 bovine milk fats were analysed by gas chromatography (GC) to establish the influence of typical feeding conditions on the content of c9t11. From 1756 milk fats the contents of CLA c9t11, *trans*-C18:1 isomers (sum of t4, t5 to t16) and the *trans*-C18:2 isomers (excluding CLA; sum of t9t12, c9t13, t8c12, t8c13, c9t12, t9c12, t11c15) were calculated by triglyceride formulae derived in a

way described earlier [15]. With this method - based on triglyceride analysis (EU reference method [16]) and calibration with the butterfat reference material CRM 519 prepared in the EU funded BCR project MAT1-CT92007 [17], the different *trans* fatty acids could be determined with high precision.

Furthermore, from 100 of the 2000 milk fat samples, which exhibited a representative range of variation in fat composition, total fatty acids including CLA c9t11 and all *trans*-C18:1 isomers were determined by Ag-TLC/GC of FAME without the use of formulae.

In a further EU funded project (VO-EWG 1001/90-11.3) another trial with a two periods cross-over design was performed to investigate, among other things, the influence of technical treatment of the fed rape-seed (00-sort) on the contents of CLA c9t11 and *trans*-C18:1 in milk fat. Other parameters from this study concerning fat composition or milk fat properties have been published earlier by Frede et al. [18]. In all variants of feeding equal amounts of fat-free wholemeal from extracted rape and extracted soya (3 kg dry matter) as well as variable amounts of wheat were given. The wheat was replaced with rape fat and different manufacturing types being equivalent in net energy. Concentrate variants: NC: negative control without rape fat; 550 g rape oil; 1300 g wholemeal from rape-seed, corresponding to 550 g rape oil; 1450 g whole rape-seed pellets, corresponding to 620 g rape oil. As basic diet all animals obtained maize silage (4.5 kg per day) and whole plant silage from winter wheat ad libitum. Each of these feeding variants was performed with 6 cows and repeated later with another herd of 6 cows. Thus, the investigations were based on 12 cows.

Additionally, milk fat samples were collected weekly during 1 year from 4 great milk collection areas in Germany. These samples were analysed for *trans*-C18:1 fatty acids, CLA (c9t11), linoleic and linolenic acid.

The total fatty acid composition covering ca. 70 fatty acids in the range of C4 to C24 was determined by gas chromatographic analysis of the methyl esters on a 25-m capillary column (CP-Wax 58 CB, equivalent to FFAP, i.d. = 0.25 mm, df = 0.20 μm). On the other hand, after trans-esterification into methyl esters and argentation thin-layer chromatography (Ag-TLC) *trans*-C18:1 as well as C18:2 isomers (excluding CLA) were analysed gas chromatographically on a highly polar 100-m capillary column (CP-Sil 88, i.d. = 0.25 mm, df. = 0.20 μm) at 175°C (*trans*-C18:1) and 150°C (C18:2), respectively. Further details on the calibration and quantitation as well as the Ag-TLC fractionation have been published elsewhere [19,20]. For the GC analysis of CLA isomers CP-Sil 88 capillary columns of 100 and 50 m length were used. Temperature programming was done from 125°C to 240°C with 2°C/min and from 50°C to 240°C with 5°C/min, respectively.

For the identification of *cis*- and *trans*-C18:1 isomers as well as the C18:2 isomers, FAME standards c6, c7, c9, c11, c12, c13, c15, t6, t7, t9, t11, t12, t13, t15 and t9t12, c9t12, t9c12, c9c12 obtained from Sigma (St. Louis, Missouri, USA) were used. All data are given in g/100 g free fatty acids.

Gas chromatographic analyses of triglycerides were performed combining triglycerides with identical acyl-C number and, thus, quantitating C24 to C54. Analytical conditions correspond to those used in the EU-project MAT1-CT92007 [17] and described in another publication [19].

3 Results and discussion

Fig.1 shows chromatograms of two bovine milk fats with the corresponding Ag-TLC fractions as well as of an Ag-TLC fraction from a typical margarine. These partial chromatograms exhibit the range of linoleic acid isomers (excluding CLA) that in case of the unfractionated milk fats interferes with some

cis-/*trans*-C18:1 isomers (c11, c12, c13, c14, c15 and t16) as well as C19. Identification of the peaks was achieved by several standards and particularly on the basis of the findings of Ratnayake and Pelletier [21] as well as of Ulberth and Henninger [22] as described earlier [20].

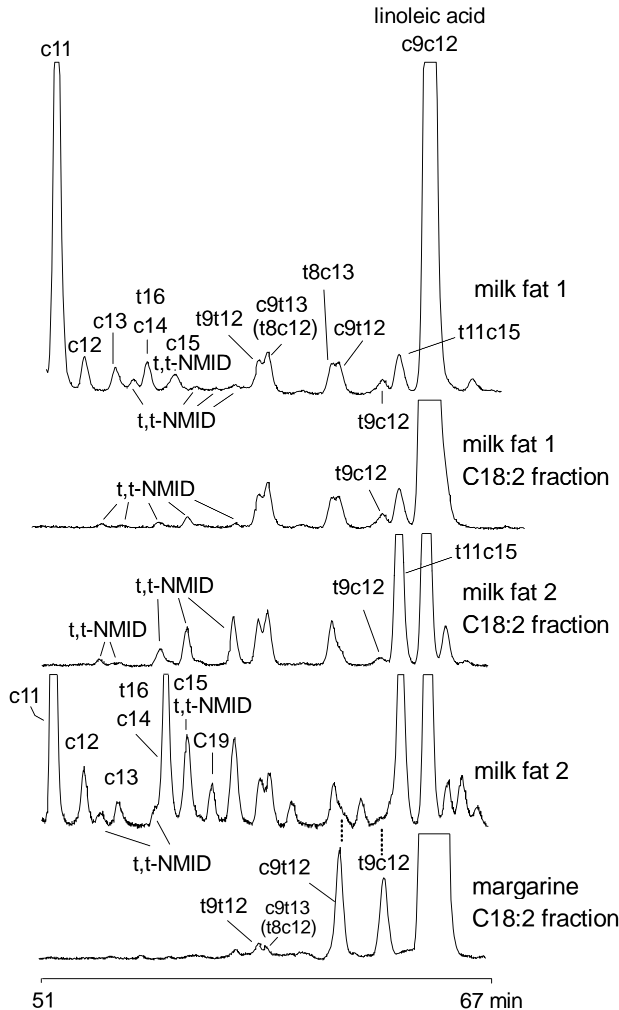

Fig.1: Partial gas chromatograms of two different milk fats and the corresponding Ag-TLC fractions containing C18:2 FAME in comparison to an Ag-TLC fraction from margarine obtained on a CP-Sil 88 capillary column (100 m x 0.25 mm). t = *trans*-bond; c = *cis*-bond; t, t-NMID = unknown non methylene interrupted *trans, trans* dienes; t8c13: tentative identification.

It follows from Fig.1 that the main *trans*-C18:2 isomers in bovine milk fats are t9t12, c9t13+t8c12, t8c13+c9t12 and t11c15. However, the identification of t8c12 and t8c13 has to be considered as tentative. Compared with that margarine contains the main isomers t9t12+c9t13+t8c12, c9t12 and t9c12. Thus, there is only little agreement between milk fat and margarine. In contrast to margarine particularly t9c12 occurs only in trace amounts in milk fat, whereas the main isomer t11c15 of milk fat is not present in margarine. However, the comparison of milk fat 1 and 2 shows that t11c15 contents exhibit a wide variation.

In contrast to these *trans*-C18:2 isomers the group of CLA elutes distinctly later (Fig. 2). Among the conjugated linoleic acids in particular the c9t11 isomer is considered important in terms of anticarcinogenic activity [23]. At the same time c9t11 is the CLA isomer with the highest content in bovine milk fat. Seven further CLA isomers as e.g. t9c11, c10t12 or t10c12 as well as $\Delta 8\Delta 10$ and $\Delta 11\Delta 13$ with all possible *cis* and *trans* configurations were detected in milk fat from French cheese products [24]. In human adipose tissue besides c9t11 the CLA isomers t9t11, c9c11 and t9c11 were found [25].

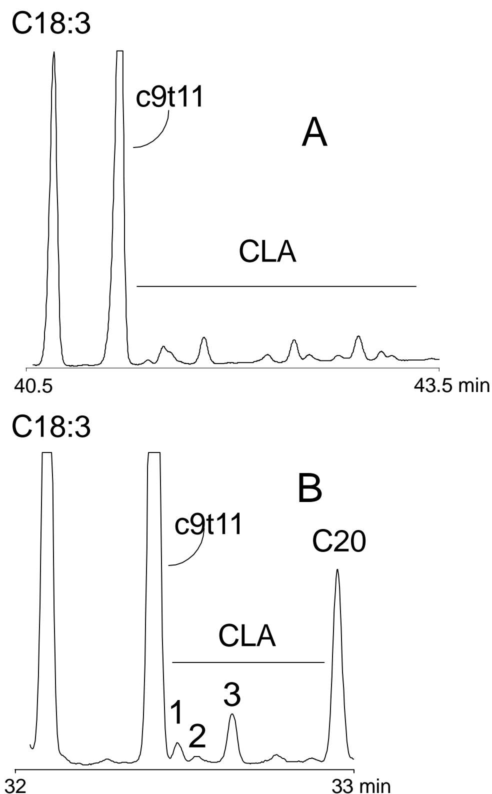

Fig.2: Gas chromatographic patterns of conjugated C18:2 isomers (CLA) eluted on a CP-Sil 88 capillary columns of 100 m length (A: oven programmed from 125°C to 240°C with 2°C/min) and 50 m length (B: oven programmed from 50°C to 240°C with 5°C/min) typically for bovine milk fat

The range of CLA in bovine milk fat obtained after gas chromatographic analysis on a 100-m column as well as on a 50-m column using different temperature programs is presented in Fig.2. With the shorter column C20 eluted within the rear end of CLA, whereas the analytical conditions of the longer column caused the elution of C20 before C18:3 avoiding the interference with CLA isomers. Despite the identical cyanopropyl polysiloxane phase the different column length and temperature programming obviously results in a variable resolution of CLA isomers. The distribution of Fig.2 B particularly corresponds to the pattern of CLA peaks found by Lavillonnière et al. [24] for milk fat from cheese who identified the peaks 1, 2 and 3 as combinations of the CLA t9,c11+c10t12, t10c12 and c8c10+c9c11+c10c12+c11c13. Moreover, small amounts of c8t10 are reported to be included in the c9t11 peak. Compared with that, the longer column (Fig. 2 A) possibly exhibits a further splitting of these numerous peaks. However, also according to Adlof and Lamm [26] peak 1 in Fig.2 B is t9c11. Further, Fritsche et al. [25] identified the corresponding peak as t9c11 in fat from human tissue as we did in our studies on fat from human milk. Apparently, the gas chromatographic conditions used by us at least separate c9t11 from t9c11. However, an overlap with a rather small amount of c8t10 can not be excluded, but possibly this isomer only results from the biohydrogenation in cheese [24].

Information on gas chromatographic conditions, Ag-TLC fractionation and identification of *trans*-C18:1 positional isomers in bovine milk fat have been given by us in several publications [19,27-29] and are not to be explained in detail here. In particular, we pointed out that the literature frequently provides too low *trans*-C18:1 contents, mainly because *trans* isomers partly were masked by *cis* isomers [28,30].

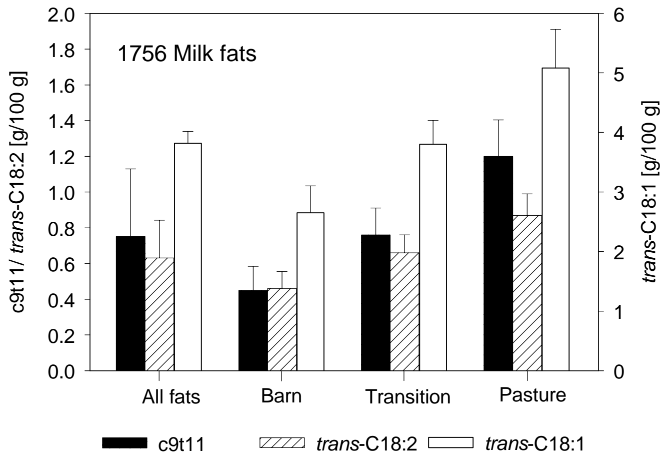

Fig.3: Contents and standard deviation of CLA, *trans*-C18:1 and *trans*-C18:2 (excluding CLA) at typical *barn* feeding (n = 927), during the *transition* period (n = 236), at *pasture* feeding (n = 593) and on average over *all fats* (n = 1756)

Our analyses of 1756 milk fats resulted in an average c9t11 content of 0.75% (range: 0.10-1.89 %). The mean content increased from barn feeding in winter (n=927) to the transition period in spring and late autumn (n = 236) and further to pasture in summer (n = 593) from 0.45% over 0.76% up to 1.20% (Fig.3). The average contents of *trans*-C18:2 (excluding CLA; sum of t9t12, c9t13, t8c12, t8c13, c9t12, t9c12, t11c15) and *trans*-C18:1 (sum of t4, t5 to t16) amounted to 0.63 % and 3.62 %, respectively. Further, the 3 feeding periods exhibited *trans*-C18:2 contents of 0.46, 0.66 and 0.87 % and *trans*-C18:1 contents of 2.65, 3.80 and 5.08 %.

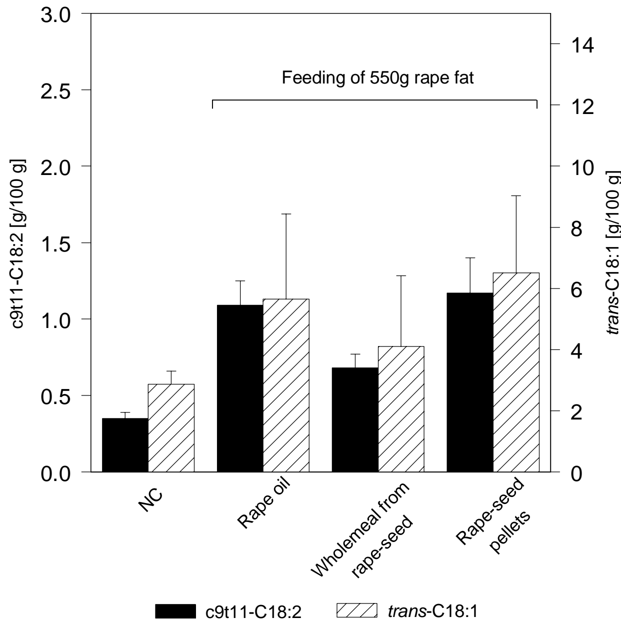

Fig.4: Influence of the technical treatment of fed rape seed or rape oil on CLA (c9t11) and *trans*-C18:1 contents in milk fat.

Further studies with defined feedings of cows exhibited particularly high CLA contents (up to 3 times higher compared to the basic diet NC) after feeding of 550 g rape oil or rape-seed pellets (Fig.4), whereas less high but still elevated CLA contents resulted from feeding of wholemeal from rape-seed. Regarding *trans*-C18:1 (sum of t4, t5 to t16) the four feeding variants shown in Fig.4 led to contents of 2.87, 5.65, 4.10 and 6.51 %.

Our previous studies [29] demonstrated that the changes in CLA contents associated with feeding correspond to changes in the *trans*-C18:1 content affecting almost exclusively vaccenic acid (t11), however. As can be seen from Fig.5 that is based upon 100 milk fats selected for a great variation in composition a strong linear correlation in particular is found between t11 and c9t11 with an extremely high correlation coefficient of r = 0.98 [c9t11 = 0.0476565 + 0.437086 • t11]. A reason for this finding may be that c9t11 is a precursor of in-vivo synthesis of vaccenic acid. In milk fat from different cheeses Lavillonnière et al. [24] found a correlation coefficient between CLA (c9t11) and vaccenic acid of r = 0.96. Regarding this, also Jiang et a. [31] established a correlation with r = 0.78 from feeding trials with milk cows.

Another high correlation with r = 0.95 follows from Fig.5 for CLA and total *trans*-C18:1 (sum of all positional isomers).

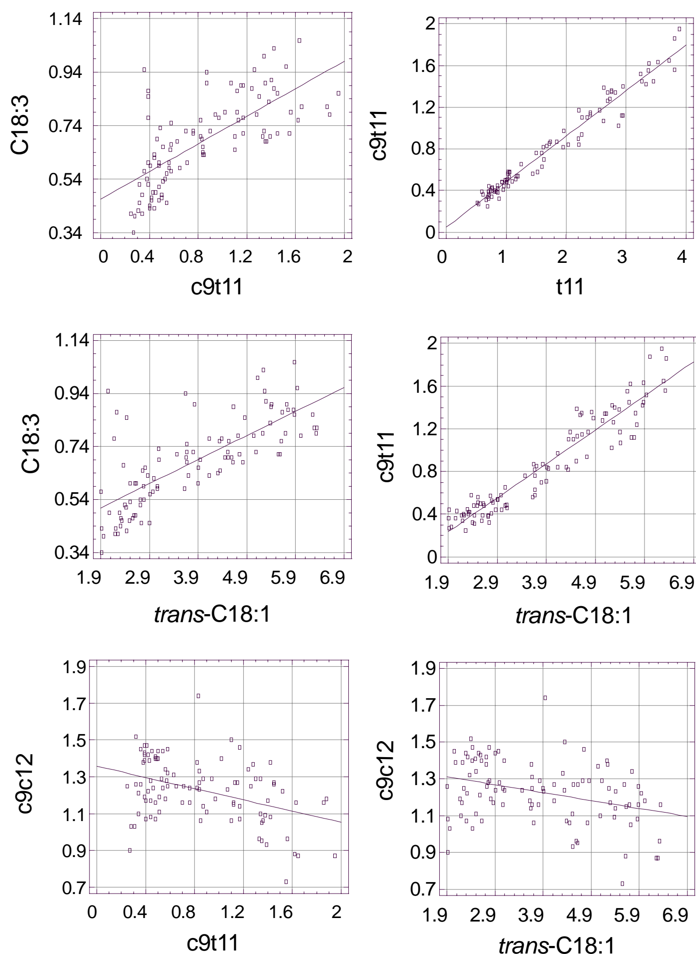

Fig.5: Relations between the contents of conjugated linoleic acid (c9t11), linolenic acid (c9c12c15), *trans*-C18:1 (all positional isomers), vaccenic acid (t11) and linoleic acid (c9c12)

The statistical evaluation of the correlations shown in Fig.5 leads to the following coefficients r:

C18:3 - CLA	:	r = 0.71
C18:3 - *trans*-C18:1	:	r = 0.74
CLA - t11	:	r = 0.98
CLA - *trans*-C18:1	:	r = 0.95
c9c12 - CLA	:	r = - 0.40
c9c12 - *trans*-C18:1	:	r = - 0.34

Fig.6 shows the results obtained weekly for the contents of C18:3 (c9c12c15), *trans*-C18:1, C18:2 (c9c12) and the CLA c9t11 in milk fat from a large German milk collection area during one year. The qualitative course of data points is nearly identical for C18:3, *trans*-C18:1 and CLA. So, linolenic acid contents are higher in summer compared to winter by 50 %, whereas *trans*-C18:1 isomers and CLA contents exhibit a corresponding seasonal rise by two and three times, repectively. Thus, here as well the desirable increase in CLA contents achievable by feeding is associated with a distinct but undesirable increase in *trans*-C18:1 contents, as already follows from Figs.3 and 4.

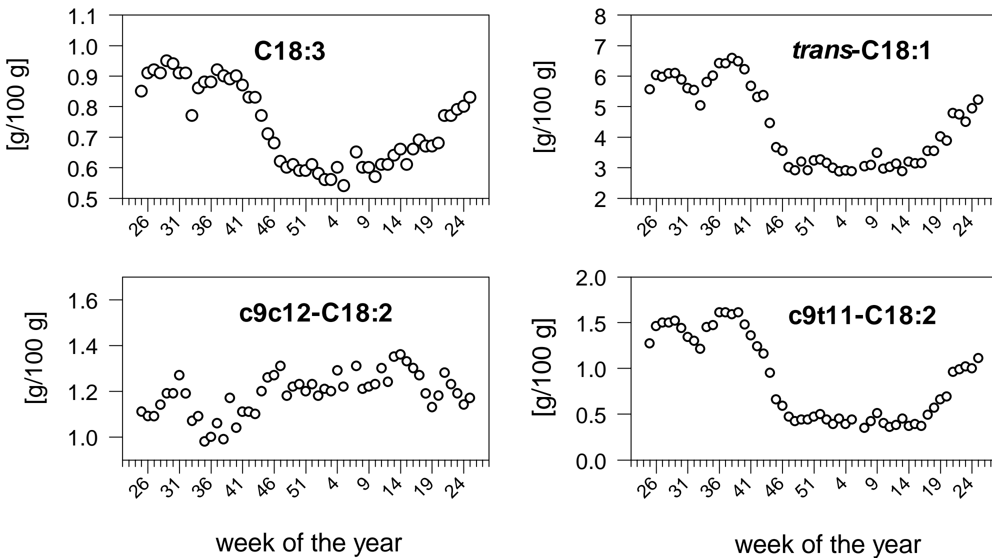

Fig.6: Weekly variation in the contents of C18:3, *trans*-C18:1, C18:2 and CLA c9t11 in milk fat from a large collection area

Further, Fig.6 shows a very good correlation between C18:3 (c9c12c15) and the CLA c9t11 in contrast to C18:2 (c9c12). In Fig.6 there seems to be a small inverse correlation between C18:2 and c9t11 also expressed by the correlation coefficient. On the other hand, in the studies of Lavillonnière et al. [24] the CLA contents in milk fat from different kinds of cheese exhibited a positive correlation (r = 0.57) with C18:2 (c9c12), whereas Lin et al. [32] found an inverse relation again.

In several publications the first step in the biohydrogenation pathway of linoleic acid is reported to be the formation of c9t11 effected by the rumen bacterium Butyrivibrio fibrisolvens. In a second step hydrogenation of the CLA leads to vaccenic acid (t11) [33-36]. When C18:3 was used in biohydrogenation trials it was at first isomerized to c9t11c15 according to Kepler and Tove [37] or to c9c11c15 or c9c13c15 according to Wilde and Dawson [38]. The latter authors found that this trienoic acid was further hydrogenated to a non-conjugated octadecadienoic acid containing at least one *trans* double bond. Regarding the conjugated trienoic acid c9t11c15 Kepler and Tove [37] described a further hydrogenation step to a non-conjugated *cis*,*trans*-dienoic acid but no monoenoic acid. Shorland et al. [39] studied the biohydrogenation of linolenic acid by sheep rumen contents. They found that a Δ11,15-C18:2 acid was the major resulting dienoic acid. This possibly could explain the comparatively high average t11c15-C18:2 content (see Fig.1) of 0.3 % (n = 100) found in our studies corresponding to ca. 27 % of the mean linoleic acid content. Regarding the C18:3 contents being relatively low in summer milk fat as compared to the high amount ingested from the pasture, the question arises how this fatty acid is metabolized in the rumen. In grass samples from different locations Prodöhl [40] found 38.7-68.5 % C18:3 (mean: 50.8 %), 13.4-32.3 % C18:2 (mean: 25.6 %) and 1.4-4.9 % C18:1 (mean: 3.6 %), whereas summer milk fat only exhibits ca. 1% C18:3 (see Fig. 6). Lavillonnière et al. [24] did not rule out the possibility of CLA formation as intermediates at least in the biohydrogenation of C18:3 in cheese, as was also described by Viviani [41] in studies concerning the metabolism of long chain fatty acids in the rumen. Lavillonnière et al. [24] found a positive relation between the α-linolenic acid content and the CLA content in cheeses (r = 0.74).

Because of the high correlation of C18:3 with CLA and the minor correlation of C18:2 with CLA found in our studies and because of the elevated CLA and *trans*-C18:1 contents in milk fat resulting during the extremely high ingestion of C18:3 from pasture, we also do not exclude that CLA and *trans*-C18:1 isomers in bovine milk fat derive to a considerable extent from C18:3. It should be emphasized that feeding trials performed by Wolff et al. [42] exhibited a high correlation of C18:3 with CLA in bovine milk fats as well but in accordance with our studies also no corresponding correlation of C18:2 (c9c12) with CLA (c9t11).

4 Conclusions

The content of the conjugated linoleic acid c9,t11-C18:2 in milk fat can be increased considerably by means of feeding. As shown by all the different feeding trials, this also strongly increases the contents of undesirable *trans*-C18:1 and *trans*-C18:2 fatty acids. A high correlation of C18:3 in pasture and milk fat with CLA as well as a minor correlation of C18:2 with CLA suggest the possibility of a CLA formation from linolenic acid.

Acknowledgement

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Modulation of lipids of brain, heart and liver by dietary rapeseed oil as compared to olive oil and high-oleic sunflower oil

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Abstract

The level of the cardioprotective ω3 docosahexaenoic acid was steeply increased in total lipids, phosphatidylethanolamines and phosphatidylcholines of heart and liver, but not of brain, of rats after feeding rapeseed and olive oils as compared to high-oleic sunflower oil. The concentration of arachidonic acid (ω6), a precursor of "inflammatory" eicosanoids, was lowered in cardiac lipids after feeding rapeseed oil. The changes in the levels of the ω3 and ω6 fatty acids are attributed to a relatively high proportion of α-linolenic acid (ω3) in rapeseed oil and phenolic constituents of olive oil. Dietary rapeseed and olive oils seem to be beneficial to health for the prevention of arteriosclerosis.

Introduction

The incidence of coronary heart disease in the Mediterranean countries is relatively low which is attributed, in part, to the high amount of the monounsaturated oleic acid (*cis*-9-octadecenoic acid) present in olive oil in Mediterranean-type diet (Nestle, 1995). It has been shown that oleic acid can be as effective as polyunsaturated fatty acids in lowering low density lipoprotein cholesterol of blood plasma (Gardner and Kraemer, 1995; Mattson and Grundy, 1985; Riccardi and Rivellese, 1993). The mechanism by which oleic acid lowers the incidence of coronary heart disease is not known, so far. The influence of minor components of plant oils, such as phenolics and flavonoids, on fatty acid metabolism has been taken into consideration to explain the physiological effects of olive oil (Petroni et al., 1994).

The present study was undertaken to establish whether the beneficial effects of dietary olive oil are solely due to the high levels of oleic acid. In order to achieve this, oils with high and intermediate levels of oleic acid were compared with olive oil. We report here the effects of dietary 'double-zero' medium-oleic rapeseed oil (RAP), olive oil (OLI) and high-oleic sunflower oil (HOS) on fatty acid alterations in total lipids, phosphatidylethanolamines and phosphatidylcholines of heart, liver and brain of rats. In particular, the n-3 and n-6 long-chain polyunsaturated fatty acids (LC-PUFA) in the two phospholipid classes were examined to determine whether the levels of these fatty acids were modulated by the concentrations of the corresponding precursor fatty acids of diet or whether dietary oleic acid itself had some modulating effects on the fatty acid composition of the cellular phospholipids.

Materials and methods

Isocaloric pelleted diets (metabolizable energy 13.1 kJ/g) containing recommended levels of proteins, carbohydrates, vitamins and nutrient minerals as used in the Altromin standard diet for rats and one of the above experimental oils (120 g/kg diet) plus corn oil (20 g/kg diet) were prepared by Altromin International, Lage, Germany (Weber et al., 1995). Composition of the major constituent

fatty acids of the dietary triacylglycerols and the respective oils of the three groups was as follows: Rapeseed oil, RAP (7 and 6% 16:0, 54 and 60% 18:1 n-9, 28 and 22% 18:2 n-6, 7 and 8% 18:3 n-3), olive oil, OLI (14 and 12% 16:0, 66 and 74% 18:1 n-9, 17 and 11% 18:2 n-6, 0.5 and 0.6% 18:3 n-3); high-oleic sunflower oil, HOS (5 and 6% 16:0, 75 and 82% 18:1 n-9, 15 and 9% 18:2 n-6, <0.2 and <0.2% 18:3 n-3).

Weaned male Wistar rats (Lippische Versuchstierzucht, Extertal, Germany) weighing 85 - 90 g were caged individually and divided into groups of 10 animals. The rats were fed the diets containing rapeseed oil, olive oil or high-oleic sunflower oil for 10 wk. The rats were killed by subjecting them to ether narcosis followed by sectioning of the aorta. Heart, liver and brain were rapidly removed and kept frozen until the lipids were extracted. All procedures for the animal experiments were approved by the official commission for animal experimentation [Der Regierungspräsident Münster, permission no. 26.0834 (48/90)].

Procedures used for lipid extraction, fractionation of lipid classes by thin-layer chromatography, determination of fatty acid composition of total lipids and lipid classes by gas chromatography of methyl esters and statistical analysis are reported elsewhere (Weber and Mukherjee, 1998).

Results and discussion

The composition of the major constituent fatty acids in total lipids of hearts and livers of individual rats after the feeding of rapeseed oil (RAP), olive oil (OLI) or high-oleic sunflower oil (HOS) diets over a period of ten weeks is given in Table 1 (Weber and Mukherjee, 1998). These results show that the proportions of docosahexaenoic acid (DHA) in total lipids of heart were significantly higher ($P<0.01$) in OLI and RAP than in HOS. Moreover, DHA concentrations of total liver lipids were significantly higher for the groups RAP ($P<0.01$) and OLI ($P<0.05$) than HOS (Table 1).

Concentration of arachidonic acid (AA) in total lipids of heart of the RAP group was significantly lower than in OLI and HOS groups, whereas in the total lipids of liver the proportions of AA were essentially identical for the three groups (Table 1). The proportions of linoleoyl moieties were significantly higher in total lipids of heart and liver of the RAP group as compared to the other two groups (Table 1).

In the total lipids of brain no significant difference was observed between the three groups with respect to the levels of AA and DHA. However, the proportion of n-6-LC-PUFA (AA + 22:4 n-6 + 22:5 n-6) in total brain lipids was significantly lower for the RAP group than OLI and HOS groups.

The fatty acid compositions in the pooled samples of phosphatidylethanolamines (PE) of heart and liver of rats fed RAP, OLI and HOS diets are given in Table 2 (Weber and Mukherjee, 1998). The proportions of DHA in the PE fractions of heart of both RAP and OLI groups were increased by about 4.5- and 3-fold, respectively, as compared to the HOS group, whereas the proportions of AA in the PE of hearts were similar (20 - 25%) in all the three groups (Table 2).

The proportions of AA were similar (29 - 33%) in the pooled samples of PE of the livers of all three experimental groups, whereas a steep increase (by about fourfold) in the proportions of DHA was found in this lipid class isolated from the livers of both RAP and OLI groups as compared to PE of the HOS group (Table 2).

Table 1: Composition (%) of the major constituent fatty acids in total lipids of hearts and livers of individual rats after feeding rapeseed oil (RAP), olive oil (OLI) and high-oleic sunflower oil (HOS) for ten weeks*

Fatty acids	Fatty acid composition (%) of total lipids of							
	Heart				Liver			
	RAP	OLI	HOS	± SEM	RAP	OLI	HOS	± SEM
16:0	9.6[a]	9.1[a]	9.5[a]	1.4	14.8[b]	17.9[a]	17.5[a]	1.5
18:0	19.1[b]	21.7[a, b]	25.2[a]	3.9	11.1[b]	9.8[b]	15.5[a]	2.9
18:1 n-9	17.5[a]	12.1[a]	16.3[a]	5.5	27.7[b]	38.9[a]	31.9[b]	6.3
18:1 n-7	4.7[a]	4.2[a]	3.0[b]	0.6	1.5[a]	0.7[b]	1.7[a]	0.3
18:2 n-6	16.8[a]	12.6[b]	12.7[b]	1.7	18.6[a]	11.0[b]	8.8[c]	2.0
18:3 n-3	0.9	Tr	Tr[***]	0.2	1.8	Tr	Tr	0.3
20:4 n-6	16.5[b]	25.8[a]	22.8[a]	3.5	14.6[a]	13.9[a]	16.9[a]	3.5
22:5 n-6	0.2[c]	3.3[b]	4.5[a]	0.9	0.2[c]	0.6[b]	1.3[a]	0.3
22:6 n-3	9.3[a]	7.5[a]	2.8[b]	2.1	4.9[a]	2.8[b]	2.0[b]	1.0

*Values given are means ± pooled SEM (n=10). Values of fatty acids in the various feeding groups not carrying the same superscript are significantly different ($P < 0.01$).

The ratios of total n-3 LC-PUFA (predominantly DHA) to n-6 LC-PUFA (predominantly AA) in the PE of heart and liver of the HOS group were distinctly different from those of both OLI and RAP groups. Thus, in the PE of hearts the ratio of n-3 to n-6 was about 0.2 in the HOS group, 0.6 in the OLI group and 1.5 in the RAP group. The ratio of n-3 to n-6 was as low as 0.1 in the PE of livers of the HOS group, whereas in those of the RAP and OLI groups ratios of about 0.5 and 0.4, respectively, were observed.

In other organs and tissues, such as pooled samples of aorta, kidneys, lungs, spleen, stomach and jejunum as well as in blood higher proportions of DHA were found in the PE of rats fed the RAP and OLI diets than in those fed the HOS diet, however, the differences were much lower compared to those observed in heart and liver.

In the PE of brain, however, no significant difference was observed between the three groups with respect to the levels of AA, DHA as well as total n-6-LC-PUFA (AA + 22:4 n-6 + 22:5 n-6).

In the pooled samples of heart and liver lipids distinctly higher proportions of DHA were also found in the phosphatidylcholines (PC) of rats fed the RAP and OLI diets than in those fed the HOS diet (Table 3), however, the differences were much lower compared to those observed in PE of heart and liver of the animals of the corresponding experimental groups (Table 2) (Weber and Mukherjee, 1998).

The ratio of n-3 to n-6 LC-PUFA in the PC of heart and liver of the groups RAP and OLI was distinctly higher than that of the HOS group. The pattern of these ratios was similar to that observed for PE of the corresponding tissues, however, much less pronounced. The levels of AA in PC of

heart and liver were, however, distinctly higher for the OLI group as compared to RAP and HOS groups (Table 3) which did not reflect the levels of dietary 18:2 n-6 – a precursor of AA.

In the PC of brain no significant difference was observed between the three groups with respect to the levels of AA and total n-6-LC-PUFA (AA + 22:4 n-6 + 22:5 n-6). However, the proportion of DHA in PC of brain was significantly higher for the RAP group than the HOS group although there was no significant difference in the level of DHA in the PC of brain of the OLI and HOS groups.

Table 2: Fatty acid composition of phosphatidylethanolamines in hearts and livers of rats after feeding rapeseed oil (RAP), olive oil (OLI) and high-oleic sunflower oil (HOS) for ten weeks

Fatty acids	Fatty acid composition (%) of phosphatidylethanolamines of					
	Heart			Liver		
	RAP	OLI	HOS	RAP	OLI	HOS
16:0	4.7	4.8	7.0	10.9	12.4	18.9
18:0	25.3	23.4	33.3	26.5	25.4	32.2
18:1 n-9	5.8	6.1	8.6	4.4	4.8	5.4
18:1 n-7	2.1	1.8	1.1	2.0	1.7	0.8
18:2 n-6	2.8	1.7	2.0	4.4	2.3	2.6
20:4 n-6	19.9	24.5	24.9	30.2	32.5	29.1
22:4 n-6	1.3	3.1	2.7	0.1	1.1	1.4
22:5 n-6	0.2	9.7	11.7	Tr	2.5	4.4
22:5 n-3	6.1	1.8	0.5	2.5	1.2	0.3
22:6 n-3	31.7	23.0	7.4	17.1	14.8	3.9

In the RAP group the increase in DHA in the total lipids (Table 1) and PE (Table 2) of heart and liver can be easily explained by the presence of moderate proportions of α-linolenoyl moieties in the RAP diet which are finally converted by a sequence of elongation/ desaturation/retroconversion reactions to DHA (Bourre et al., 1993; Sprecher et al., 1995; Winters et al., 1994). However, in the OLI group, in which the diet contained only trace amounts of α-linolenic acid, some other constituents of the oil, such as phenolics and flavonoids (Petroni et al., 1994) seem to be responsible for the higher proportions of DHA found in the PE of heart and liver (Table 2).

It is of great interest that a large increase in DHA, which was found predominantly in PE after feeding of both the OLI and RAP diets (Table 2), was in heart, which is an organ affected particularly by atherosclerois. These findings seem to contribute to the proposed protective effects of olive oil as well as plant oils containing α-linolenic acid, such as rapeseed oil and soybean oil (Chan et al., 1991; Katan et al., 1995; Valsta et al., 1992), against atherosclerotic lesions, particularly in the heart.

Table 3: Fatty acid composition of phosphatidylcholines in hearts and livers of rats after feeding rapeseed oil (RAP), olive oil (OLI) and high-oleic sunflower oil (HOS) for ten weeks

Fatty acids	Fatty acid composition (%) of phosphatidylcholines of					
	Heart			Liver		
	RAP	OLI	HOS	RAP	OLI	HOS
16:0	12.4	10.3	12.4	18.6	16.1	19.2
18:0	27.2	28.7	32.6	23.2	24.9	28.2
18:1 n-9	6.3	5.8	7.7	5.4	6.4	7.2
18:1 n-7	4.5	3.2	2.9	2.7	2.2	1.3
18:2 n-6	7.2	3.7	3.6	9.7	4.6	4.5
20:4 n-6	31.3	40.4	32.4	31.1	36.1	31.5
22:4 n-6	0.5	1.3	1.2	Tr	0.4	0.4
22:5 n-6	Tr	1.5	2.1	Tr	1.3	1.7
22:5 n-3	3.4	1.0	0.4	0.9	0.6	Tr
22:6 n-3	6.1	3.4	1.1	6.1	5.8	1.8

It is envisaged that some of the health effects of olive oil upon cardiovascular diseases are due to improvement of heart function by DHA-induced changes of membrane structure (McLennan and Dallimore, 1995). DHA is known to be an important component of the structural lipids, e.g. PE, of cell membranes, predominantly of heart, liver, brain, and retina (Neuringer and Connor, 1986; Tahin et al., 1981).

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Cholesterol reduction in culture fluids by lactobacilli

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Introduction

One of the best known effects of probiotic products is the reduction of the concentration of plasma cholesterol. By radiotracer methods a lactobacillus salivarius strain has been examined regarding its cholesterol reducing and metabolizing effects. This strain before in tests at the Federal Research Centre for Nutrition in Karlsruhe had proved effective in reducing the cholesterol level in oxgall containing culture fluids.

Methods

The lactobacillus salivarius NCFB 1555 strain was subcultured twice in MRS broth before incubation in MRS with 0,6% oxgall and radiolabelled cholesterol(4-^{14}C) for 24h at 37°C.

The lactobacilli protoplasts have been prepared by the action of lysozyme and mutanolysin.

For HPLC analysis on cholesterol and metabolites the culture fluid and the microorganisms were extracted with diethyl ether. HPLC has been performed with an ALLTECH Alltima Silicia column (250x4,6mm), hexane/isopropanol (97/3) as eluent and a liquid flow cell as radioactivity detector.

Results

Investigation on chemical reactions: HPLC analysis showed no cholesterol metabolites. Also in the polar residues of the extraction and in the extracted microorganisms no relevant parts of the added radioactivity could be found. Thus the possibility remained, that cholesterol was chemically unmodified coprecipitated, bound or incorporated into the microorganisms.

Dissolving the cell wall: In case of an attachment of the chemically unchanged cholesterol to the surface of the lactobacilli it should be possible to remove the cell walls without a loss a viability. After the isolation of the protoplasts the activity was localized in the protoplasts (92-94%) and especially in the plasma membranes (96-98%). The addition of cholesterol during the enzymatic dissolution had the same result. Obviously cholesterol is attached to the lipophilic surface of the protoplasts instantly. The enrichment in this fraction seems to be caused primarily by the affinity of cholesterol to the lipid membrane of the protoplasts since the found mechanism of enrichment contrary to the experiments with intact lactobacilli works also in the absence of oxgall (fig.1). Without added oxgall the bound cholesterol activity is relatively small (about 10%). For that reason it is not probable that the binding to the plasma membrane is the main reason for the enrichment found with the intact lactobacilli.

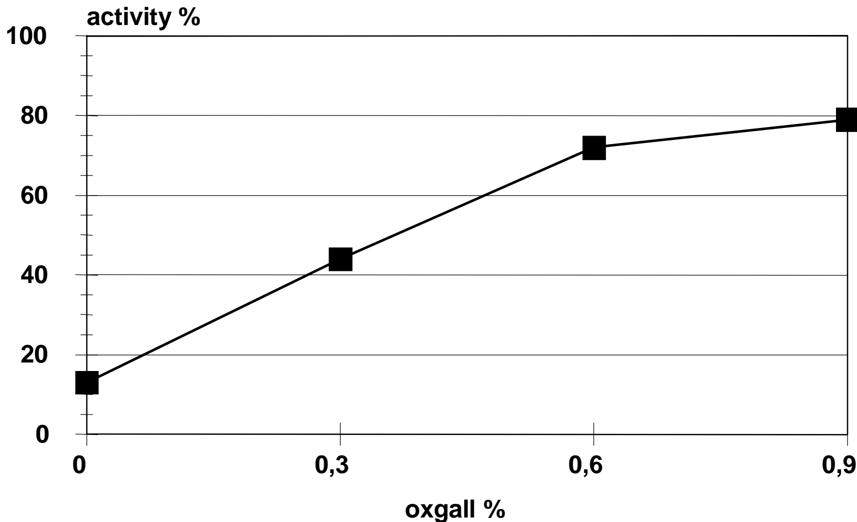

Fig. 1: Amount of bound cholesterol as a function of bile concentration

Experiments for dissolving the precipitated deconjugated bile acids and cholesterol respectively: Some strains of lactobacilli are able to cleave (deconjugate) the bile acids (which are conjugated with glycine or taurine) to free amino acids and free bile acids (BSH theory, Klaver et al. 1993). The latter coprecipitate in acid solution (as it is prepared by lactobacilli) together with cholesterol and it is possible, to redissolve them again in alkaline solution, as it could be confirmed in an experiment with deconjugated bile acids. In a corresponding experiment with microorganisms loaded with cholesterol even after 24h in a buffer solution of pH 9 not the whole amount of cholesterol could be dissolved (about 10-20% remain in the fraction of the microorganisms). For that reason a weak, surfacial fixation of cholesterol is not probable. There is the possibility too, that there are coexisting at the same time different possibilities of precipitation or fixation, what is suggested also by a microscopically perceptible inhomogenity (microorganisms, crystals, oily masses) of the precipitate. This solution experiment also points to a part of cholesterol being excreted together with the microorganisms, since there are similar conditions in the small intestine at pH 9.

Conclusion

A lactobacillus salivarius strain has been found to reduce the cholesterol concentration in culture fluids without chemical modification. The results confirm the hypothesis of the coprecitiation together with bile acids, but cannot be explained by it exclusively. The bound cholesterol dissolves in alkaline media incompletely (under conditions as in the small intestine). Thus it partially can be excreted and withdrawn from the organism respectively.

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Lipase-catalyzed synthesis of designer lipids with improved nutritional properties

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Abstract

Lipid designing using plant and microbial *sn*-1,3-specific triacylglycerol lipases has been employed for the preparation of structured lipids for functional foods and nutraceuticals. Human milk fat replacer containing palmitic acid, esterified at the *sn*-2 position of triacylglycerols and unsaturated fatty acids at the *sn*-1,3-positions have been prepared by transesterification of tripalmitin with fatty acids of rapeseed oil using a *sn*-3-regiospecific lipase as a biocatalyst present in an inexpensive crude papaya (*Carica papaya*) latex preparation. Structured triglycerides containing medium-chain fatty acids or ω3 and ω6 polyunsaturated fatty acids esterified at definite positions can be prepared using such a lipase from papaya latex, which may find good acceptance as compared to lipases from transgenic microorganisms.

Introduction

Designing of lipids using lipases, such as *sn*-1,3-specific triacylglycerol acylhydrolase (EC 3.1.1.3) as biocatalysts (Figure 1) has been employed for the preparation of structured lipids for use in functional foods and nutraceuticals (Mukherjee, 1998). Structured triglycerides containing palmitic (C16:0) acid, esterified predominantly at the *sn*-2-position and C18-unsaturated fatty acids at the *sn*-1,3-positions of triacylglycerols (Figure 2) are produced for use as human milk fat replacer by transesterification of tripalmitin with oleic acid or polyunsaturated fatty acids using *sn*-1,3-specific microbial lipases as biocatalysts (Kavanagh, 1997).

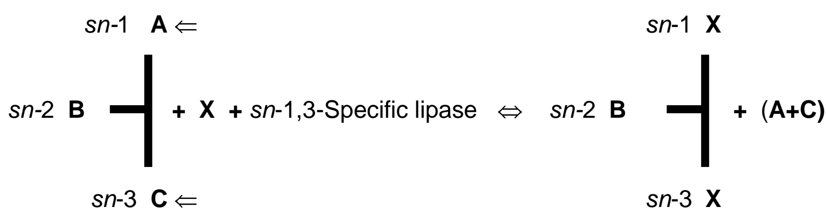

Figure 1: Interesterification of triacylglycerols using *sn*-1,3-specific triacylglycerol lipases: (A,B,C,X = Fatty acids / Acyl moieties).

Lipases from plants rather than those from transgenic microorganisms may find an easier acceptance as biocatalysts for the preparation of the above type of designer lipids for use in infant food formulations and nutraceuticals (Mukherjee, 1994). This communication reports the preparation of structured triacylglycerols resembling human milk fat by transesterification of tripalmitin with fatty acids of low-erucic rapeseed oil using lipase present in crude latex of papaya (*Carica papaya*) as biocatalyst; for comparison an immobilized microbial *sn*-1,3-specific lipase preparation (Lipozyme®) was studied.

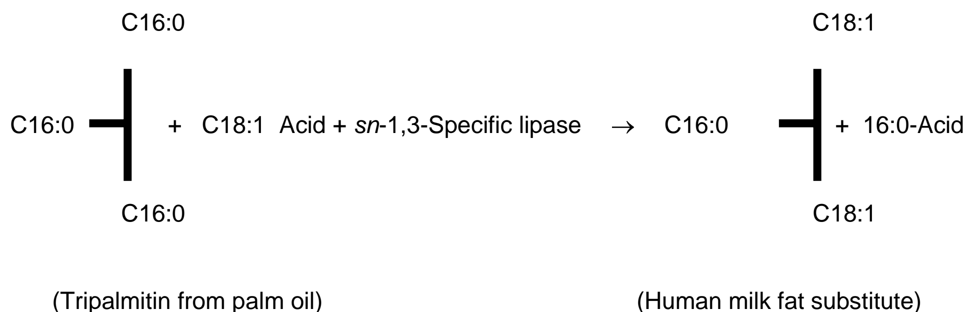

Figure 2: Interesterification of tripalmitin with oleic acid using *sn*-1,3-specific triacylglycerol lipase for the preparation of human milk fat substitute (Betapol).

Experimental

Tripalmitin (0.5 mmol) and rapeseed oil fatty acids (0.5 mmol) containing 10% palmitic (C16:0), 59% oleic (C18:1) and 20% linoleic (C18:2) together with 54 mg of either finely ground (<0.8 mm mesh) *Carica papaya* latex (Sigma) or immobilized lipase from *Rhizomucor miehei* (Lipozyme IM 20, Novo) were stirred under nitrogen at 60°C for various periods. The reaction products were centrifuged to separate the biocatalyst. The products were fractionated by thin-layer chromatography into fatty acids and triacylglycerols and subsequently analyzed by gas chromatography of their methyl esters (Mukherjee and Kiewitt, 1998). Aliquots of triacylglycerols were subjected to hydrolysis by porcine pancreatic lipase and the *sn*-2-acylglycerols formed were isolated by thin-layer chromatography, converted to methyl esters and analyzed by gas chromatography to determine the composition of the acyl moieties at the *sn*-2-position (Christie, 1980). Aliquots of triacylglycerols were also subjected to Grignard degradation and *sn*-1,3-diacylglycerols formed were isolated by thin-layer chromatography, converted to methyl esters and analyzed by gas chromatography to determine the composition of the acyl moieties at the *sn*-1,3-positions (Christie, 1980).

Results and discussion

Transesterification of tripalmitin with rapeseed oil fatty acids, catalyzed by papaya latex and Lipozyme, was found to result in incorporation of 10 to 25% C18:1 plus C18:2 acids into the triacylglycerols (Figure 3). Simultaneously, C16.0 moieties from tripalmitin were released as palmitic acid into the reaction products.

The proportion of C16:0 moieties at the *sn*-2-position of triacylglycerols was very slowly reduced during transesterification and little increase in the levels of C18:1 and C18:2 moieties occurred at this position. Thus, very little exchange of C18:1 and C18:2 acids against the C16:0 moieties at the *sn*-2-position of triacylglycerols occurred with both enzyme preparations which is in agreement with their known regioselectivity (Mukherjee, 1998; Foglia and Villeneuve, 1997; Villeneuve et al., 1995).

Transesterification led to reduction in the level of C16:0 and increase in the concentrations of C18:1 and C18:2 moieties at the *sn*-1,3-positions of the triacylglycerols at a much higher rate as compared to the changes in the concentration of these acyl moieties at the *sn*-2-position (Figure 4).

The data presented in Figures 3 and 4 show that transesterification of tripalmitin with fatty acids of low-erucic rapeseed oil using either papaya latex or Lipozyme as biocatalyst yielded triacylglycerols resembling those of human milk fat containing C16:0 moieties predominantly at the *sn*-2-position of the glycerol backbone and C18 unsaturated acyl moieties at the *sn*-1,3-positions (Figure 2).

Crude papaya latex containing papain is available commercially in bulk scale, it is inexpensive and is widely used in food and beverage industries. Papaya latex also exhibits lipolytic activity with a strong *sn*-3-selectivity (Villeneuve *et al*., 1995). The regiopreference of this enzyme has been utilized for the synthesis of structured triacylglycerols (Foglia and Villeneuve, 1997). Moreover, lipase in papaya latex exhibits a strong selectivity for specific unsaturated fatty acids, i.e. the ability to discriminate against fatty acids having a *cis* double bond at Δ^4 (*all-cis*-4,7,10,13,16,19-docosahexaenoic acid, DHA), Δ^6 (*all-cis*-6,9,12-octadecatrienoic acid, i.e. γ-linolenic acid, GLA and *all-cis*-6,9,12,15-octadecatetraenoic acid, i.e stearidonic acid) and Δ^8 (*all-cis*-8,11,14-eicosatrienoic acid, i.e. dihomo-γ-linolenic acid, DGLA) in esterification with *n*-butanol (Figure 5).

The data reported here show that crude papaya latex can be efficiently used as a biocatalyst for the preparation of designer lipids resembling human milk fat and possibly other structured triglycerides containing medium-chain or specific long-chain polyunsaturated fatty acyl moieties at definite positions of the glycerol backbone. For example fatty acids such as DHA and GLA with specific beneficial effects on health can be enriched from marine oils and borage oil, respectively, via kinetic resolution (selective hydrolysis or esterification) (Mukherjee, 1995) catalyzed by papaya latex. Subsequently, it should be possible to insert these fatty acids into the *sn*-1,3-positions of triacylglycerols by transesterification or esterification catalyzed by papaya latex to obtain designer lipids for use in functional foods.

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Non digestible oligosaccharides are promising functional foods

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1 What are they?

1.1 Oligosaccharides...

Oligosaccharides are a class of carbohydrates which is difficult to define solely on chemical or solely on physiological criteria. They have been chemically defined as carbohydrates with chain lengths (DP or degree of polymerisation) varying between DP 2-10 (Cummings & Englyst 1995).

The generally accepted dividing point between oligosaccharides and polysaccharides at present is considered to be carbohydrates with DP 10.

This distinction however is artificial, and does not make much sense from a physiological point of view: would DP 11 behave differently than DP 10?

This definition also does not take into account the physiological differences of the type of monosaccharide or type of bonds between those moieties.

On the other hand, is a classification based on physiological properties not really clear-cut, as one oligosaccharide affects the physiology of its consumer on more than one way. So there is not a single criterion for classification available.

It recently was proposed by Cummings et al. (1997) to define oligosaccharides based on molecular size, as chemistry finally determines function. Further subdivision of the oligosaccharides are based on chain length and type of composing monosaccharides.

1.2 ... need to be Non Digestible for having functional food properties

In order to be non-digestible, the oligosaccharides should resist the acid hydrolysis in the stomach, as well as the degradation by (pancreatic) catabolic enzymes in the small intestine. (Roberfroid et al. 1993)

The non-digestibility of the NDO in the upper intestinal tract is demonstrated by means of balance studies with ileostomised volunteers (Ellegård et al. 1997; Knudsen et al. 1995, Livesey 1993).

The NDO reach the large intestine, where they are subject to fermentation by the complex intestinal flora. This part of the gut is very much depleted in energy; the competition for the incoming source of energy by the different intestinal bacterial populations is such that systematically none of the NDO escapes fermentation.

The past decennium inulin and its partial hydrolysate oligofructose have been subject to intensive nutritional research. It has been demonstrated by means of in vitro experiments and confirmed by means of human nutrition studies that these ingredients are non digestible. In Europe these food ingredients are becoming common ingredients in foods with a nutritional plus. Other types of NDO at present are in an evaluation phase, but most still are not accepted for use in human nutrition in the EC.

1.3 Inulin and Oligofructose

Present paper will focus on inulin and oligofructose as most of the work both in Japan and in Europe was performed with these ingredients.

Inulin is a set of ß(2-1) linked linear fructan chains with different chain length. The chain length varies between 3 up to 65 in the case of chicory inulin. Inulin occurs in many foodplants (Van Loo et al 1995), but chicory is for technological reasons preferred for industrial production.

The longer chains of inulin can enzymatically be hydrolysed to shorter chains. This partial hydrolysate is called oligofructose. This ß(2-1) fructan contains chains with a length varying between 3 and 8 fructose moieties. Oligofructose also can be synthesised enzymatically from sucrose.

2 The potential health effects of the inulin and oligofructose

2.1 Functional food

As stated in §1.2 Functional Food is food that positively alters a physiological function in the body. A functional claim refers to (positive) consequences of interactions between a food component and a specific genomic, biochemical, cellular or physiological function, without direct reference to any health benefit or disease prevention (Roberfroid 1996).

2.1.1 Low caloric food ingredients

As they are carbohydrates which are not hydrolysed into their hexose moieties in the small intestine nor absorbed, the intake of inulin or oligofructose never results in increased glycemic levels. It even was reported that their consumption can result in reduced glycaemic peaks upon simultaneous consumption of easily digestible carbohydrates (sugar, starch) (Yamashita, 1984). These ß(2-1) fructans are completely metabolised by the intestinal population, which results in the formation of metabolites (short chain fatty acids : acetic, propionic, butyric and lactic acid), which are absorbed, and function in the body as substrates for energy metabolism.

The amount of energy which is put available is significantly less than the energy content of the original oligosaccharides, the more since energy is lost via production of gas (expiration) and of bacterial biomass (excretion) (Roberfroid et al. 1993)

Potential Health effects: Reduced caloric value is a major claim. Since blood sugar levels are not affected by inulin and oligofructose, they also can be consumed by diabetics.

2.1.2 Improvement of the composition of the intestinal flora

Inulin and oligofructose arrive almost quantitatively in the colon (Ellegård et al. 1996, Knudsen et al. 1995). Whereas the upper part of the intestine normally has only very low levels (<10^6 cfu/g) of bacteria, the colon contains an important bacterial ecosystem. More than 75% of the solid matter present in the colon is bacterial biomass. This bacterial population moreover is very complex, as it is composed of over 400 different species, which all to a certain extent interact. As inulin or oligofructose arrive in the colon they are completely fermented. The long chain inulin being fermented slower than the short chain oligofructose may arrive in a more distal part of the colon.

The fermentation of these food ingredients is particular in that they appear to stimulate the growth of different groups of bacteria to a different extent.

Both inulin and oligofructose selectively promote the growth of a.o. intestinal bifidobacteria in vivo and yet are called *bifidogenic* substrates (Table 1). It is also for this property that NDO are considered prebiotics. It is observed that when bifidobacteria are increased, the presence of other (potentially pathogenic) groups significantly is reduced (in vivo human volunteer studies).

Potential health effects: The direct consequence of modifying the intestinal flora towards a population with more bifidobacteria and lactic acid bacteria and therefore with less potentially harmful bacteria is the prevention or treatment of intestinal infections. The altered bacterial population results in a less toxigenic (less carcinogenic) degradation of bile acids (Bouhnik et al. 1996; Kashimura et al. 1993) . There are indications that cell walls of lactic acid bacteria would stimulate the immune system (Gaskins et al. 1996; Kanno, 1989)

Another direct consequence of these functional effects is the prevention or treatment of diarrhoea (of bacterial origin, or viral origin e.g. rotavirus) (Saavedra et al. 1994; Chandra et al. 1995).

Indirectly, the production of mostly acidic metabolites (SCFA and lactate) locally stimulates the mobility, and yet the transit time in the intestine (Cherbut 1995) . The overall stimulation of bacterial growth increases the mass and yet the volume of the intestinal content (Gibson et al. 1995; Den Hond et al. 1997). Some SCFA (esp. propionate) which are absorbed would alter the hepatic lipid metabolism.

Several beneficial properties are ascribed to the bifidobacteria. They would stimulate the immune system, synthesise vitamins (of the B-group) (Gibson and Roberfroid 1995). They produce a protein which has antibacterial activity against a wide range of pathogens (*Listeria, Salmonella, Shigella*, etc.) (Wang 1993).

2.1.3 Normalising dysbalanced lipid metabolism

A lot of research has been done in this field. In rats it was observed that the intake of inulin as well as oligofructose significantly reduces the serum triglyceride levels. This phenomenon finds its origin in altered lipid metabolism, of which the hepatic lipogenic enzyme system is down-regulated. This modification is at the genetic level, since it is the expression of all enzymes which are involved in lipogenesis which is decreased. It is presumed that the consumption of these NDO by rats influences the production of hormones such as insulin and GIP (Kok et al. 1996). Other publications report on the reduction of the serum cholesterol levels in animal models. Published data of human feeding studies give less consistent results (Table 2). The involvement of distinctly different populations however does not allow direct comparison. Notwithstanding this constatation, it is observed that in different studies serum triglycerides were decreased.

Potential health effects: Reduced levels of serum triglycerides and cholesterol (LDL) mean decreased risk of cardiovascular diseases. Extrapolation of the involvement of hormones has led scientists to presume that this might have impact on obesity.

2.1.4 Interaction with the mechanisms of carcinogenesis

This is a topic which is gaining importance. In experiments with long-chain inulin-fed rats which have been injected with carcinogenic compounds (nitroso-methyl ureum or azoxymethane), a significant reduction of numbers of (multifocal) aberrant crypt foci (ACF) as a marker of carcinogenesis has been observed (Reddy et al. 1997).

In an analogous experiment (post-initiation this time) Rowland et al. confirmed the anticarcinogenic effect of long chain inulin and additionally demonstrated a synbiotic activity (synergistic activity between a prebiotic and a probiotic compound) between long chain inulin and bifidobacteria.

Recent publications mention a significant slowing down of the development of tumour cells in the thighs or peritoneum of mice (Taper et al. 1997).

The mechanisms involved are up to now not established. Possible mediators are the immune system (e.g. increased phagocytic activity of the natural killer cells) or the stimu-lation of the hepatic detoxification mechanisms (cytochrome P450). Another thinking route is the stimulation of apoptosis by the butyrate which is produced by fermentation of some NDO.

Potential Health effects: Present results are indicative of a chemopreventive action of the fructose based NDO. Cancer, and more particularly colon cancer is a major cause of death in the western population. There is a strong cause-effect relationship between diet and colon cancer. The long chain inulin, as it is slower fermented, has the additional advantage to arrive in a more distal part of the colon where colonic cancers most frequently occurs.

2.1.5 Improved gut function

Fructose based NDO were shown to improve the gut function by increased faecal bulking and decreased intestinal pH by stimulating the bacterial fermentation as mentioned previously. Recent experiments with long chain inulin have demonstrated an increased stool frequency of healthy but chronically slightly constipated persons (Den Hond et al. Wageningen ref.)

Potential health aspects: Improved gut function increases the quality of life. It is mainly during young age (0-10 years) and during old age (>65) that people have to cope with intestinal discomfort (cramps, diarrhoea, etc.).

2.1.6 Improved mineral absorption (Ca, Mg)

Rat experiments have given strong indication that minerals such as calcium and magnesium are significantly more absorbed when the animals are administered (fructose-based) NDO (Delzenne et al. 1995; Ohta et al 1993).

In analogy with the rat experiments which always were carried out with growing animals, experiments with pubertal humans being administered oligofructose (15g/day) have confirmed these observations . Another study with adults who were administered 40g/day of inulin also resulted in significantly increased mineral absorption (Table 3).

Results from Scholz-Ahrens (1998) with ovari-ectomised rats as a model for postmenopausal women indicate that the intake of oligofructose reduces bone demineralisation. In combination with a higher Ca intake, the internal (trabecular) bone structure even was significantly improved.

Potential health effects: It is during young age that humans build up the calcium reserve for the rest of their lives. The intake of inulin or oligofructose increases the efficiency of the Ca uptake. The extra absorbed Ca is used to build bone structure.

Post menopausal women inherently loose bone calcium. Rat experiments gave preliminary indications that the demineralisation of the bone in postmenopausal women is reduced by consuming oligofructose.

Both the improved storage of minerals and the reduced demineralisation in post-menopausal women, suggest a potential of inulin and oligofructose to prevent osteoporosis.

Table 1: Overview of human nutrition studies investigating the prebiotic (bifidogenic) properties of different (NDO).

Reference	Dose (g/d) Type of NDO	No. volunteers (M/F) * Age (yr) Supplement period	Log increase in Bifido-bacteria	Statistical signifiance
Bouhnik *et al*. (1996*b*)	12.5 Oligofructose	20 (10M/10F) 22-39 12d	1.2	*P* <0.01
Gibson *et al*. (1995)	15 Oligofructose	8 (M) 20-25 2wk	0.7	*P* <0.01 §
Gibson *et al*. (1995)	15 Inulin	4 (M) 20 2wk	0.9	*P* <0.001
Kleessen *et al*. (1997)	20 and 40 Inulin	10 (F) 68-89 19d	0.90 and 1.30	*P* <0.05 §
Menne *et al*. (1997)	8 F_n-type oligofructose	8 (5M/3F) 20-50 2wk	0.9	*P* <0.05 §
Mitsuoka *et al*. (1987)	8 Oligofructose	23 (M) 50-90 2wk	0.9	*P* <0.005
Rochat *et al*. (1994)	8 Oligofructose	38 (M) Adult 2wk	1.35	*P* <0.01 (increase in lactobacillus *P* <0.05)
Buddington *et al*. (1996)	4 Oligofructose	12 (6M/6F) 20-34 25d		*P* <0.03

* M, Male; F, Female.

§ Higher counts of LAB observed upon adminstration of NDO.

Table 2: Summary of published human nutrition studies (including ENDO project results) of the effect of NDO on lipid metabolism.

Reference	NDO Dose (g/d) Intake period (d)	Number of volunteers (M/F) Type Age (yr)	Fasting serum levels
Yamashita *et al*. (1984)	Oligofructose 8 14	28 M (13M/15F) NIDD 47(±7)	↓Total-C ↓ LDL-C =HDL-C =TG ↓Glucose
Alles *et al*. (1997)	Oligofructose 15 20	20 (9M/11F postmenopausal) NIDD ?	No effect
Canzi *et al*. (1995)	Inulin 9 28	12 M Normolipidemic 23(±0.5)	↓Total-C ↓LDL-C =HDL-C ↓TG N.M. Glucose
Pedersen *et al*. (1996)	Inulin 14 28	64 F Normolipidemic “Young”	Reduced LDL/HDL ratio; No effect on Total-C, TG or glucose
van Dokkum *et al*. (1997)	Inulin, oligofructose, TOS 15 21	12 M Normolipidemic 23(±3)	No effect
Davidson *et al*. (1998)	Inulin 18 42	24 (12M/13F-Caucasian) Hyperlipidemic 60(±5)	↓Total-C ↓LDL-C =HDL-C =TG N.M. Glucose
van Vliet *et al*. (1997)	Oligofructose 15 21	50 M Hyperlipidemic (TG,2.5±0.9mmol/l) 50(±7)	No effect
Williams C. et al. (unpublished)	Long chain inulin 10	54 Hyperlipidemic	=Total-C =LDL-C =HDL-C ↓TG (-19% after 8weeks) =Glucose

C, cholesterol; Total-C, LDL-C+HDL-C; TG, triglycerides; N.M., not monitored; M, Male; F, Female.

Table 3: Overview of human feeding studies investigating the effect on mineral absorption by non-digestible oligosaccharides (NDO) consumption.

Reference	NDO Dose (g/d) Intake period (wk)	Number of male volunteers Age (yr)	Result
Coudray *et al*. (1997)	Inulin 40 4	9(M) 19-24yr	Ca, 58% increase (from 21.3 to 33.7%) Mg, Fe, Zn, no effect
Van den Heuvel *et al*. (unpublished)	Oligofructose, inulin, TOS 15 3	12 (M) 20-26yr	No altered Ca or Fe absorption with any NDO tested.
Van den Heuvel *et al*. (1997 and unpublished)	Oligofructose 15 1	12 (M) 15-16yr	Ca, 26% increase (from 47.8 to 60.1%)

3 Conclusion

Nutritional research which has been carried out up to now with inulin and oligofructose has demonstrated that many physiological parameters are affected. They selectively promote the growth of intestinal bacteria, favouring the growth of bifidobacteria and lactic acid bacteria (lactobacilli). Dysbalanced lipid parameters or gut function tend to be normalised. There are indications that initiation of cancer by carcinogens can be reduced. The improved mineral balance potentially can improve the status of the bones, which may have major health consequences (prevention of osteoporosis) in an ageing (Western) population.

At present promising indications are available. They justify further human nutrition studies. To demonstrate distinct health benefits of these food ingredients is the challenge of the next decennium.

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Effect of Copper in the Food Chain on Human Health (FOODCUE)

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Aims

- To investigate the effect of different levels of intake of Cu on oxidative and other mechanisms associated the chronic disorders of coronary heart disease, cancer and osteoporosis
- To evaluate Cu absorption and bioavailability from different sources using *in vivo* and *in vitro* methods

Task 1: study outline

Copper supplementation trial with-free living volunteers aged 20-40

To assess the effect of Cu supplementation on the composition and physical characteristics of circulating levels of lipoproteins, the susceptibility of LDL to *in vitro* oxidation, inflammatory status, bone turnover measures and lymphocyte DNA oxidant damage in free-living healthy men and women aged 22-45 years.

Task 1: progress and major findings to date

- Diamine oxidase activity may prove to be a more sensitive index of Cu status than the existing traditional methods commonly used
- Dietary Cu, even at these high doses, does not promote susceptibility to Cu or peroxynitrite induced *in vitro* oxidation nor cause any increase in lymphocyte DNA damage
- Little physiologically relevant effect of Cu supplementation on haemostatic variables

No significant effect of Cu supplementation on bone turnover measures

Task 2: study outline

Copper supplementation trial with free-living volunteers aged 50-70 years

To assess the effect of Cu supplementation on the composition and physical characteristics of circulating levels of lipoproteins, the susceptibility LDL and erythrocyte membranes to *in vitro* oxidation and inflammatory status in free-living healthy men and women aged 50-70 years.

Task 2: progress and major findings to date

- No significant alteration in inflammatory status
- Supplementation with 3mg $CuSO_4$ and with 6mg Cu-amino acid chelates resulted in significantly decreased vulnerability of erythrocytes to *in vitro* induced peroxidation
- Diamine oxidase activity may prove to be a more sensitive index of Cu status than the existing traditional methods commonly used

Task 3: study outline

Metabolic bioavailability study with healthy female volunteers aged 18-30 years

^{65}Cu administered as an extrinsically labelled meal or intravenously to facilate measurement of absorption and endogenous excretion of Cu. The effects of Cu supplementation on Cu status, oxidant/antioxidant status and on cardiovascular disease and bone health indices are also being investigated

Task 3: progress to date

- Intervention period completed
- Analyses of indicators of coagulation activity, plasma fibrinogen activity, bone health, putative indices of body Cu status, TGF-• and assessment of LDL to *in vitro* oxidation have been completed
- Determination of oxysterols in plasma lipoprotein fractions is ongoing

The double blind nature of this study means that no conclusions can be made at this stage

Task 4: study outline

Development of alternative techniques of assessing Cu bioavailability

- A pilot study to test the feasibility of plasma albumin-Cu enrichment following an oral dose of ^{65}Cu –labelled food as a semi-quantitative assessment of bioavailability
- An investigation of the validity of using rare earth elements as non-absorbable faecal markers

Task 4: progress and major findings to date

- Holmium has been demonstrated to be a suitable rare earth marker
- An elegant dialysis chelex method of selectively removing albumin-bound Cu has been developed
- Cu isotope analysis by ICP-MS is ongoing at present

Task 5: study outline

Metabolic dietary manipulation study with healthy male volunteers aged 18-55 years

Assessment of the effects of a low Cu diet on adaptive responses of Cu absorption on interactions between Cu and fructose and on various biochemical indices relating to oxidant/antioxidant status and to cardiovascular and bone health

Task 5: progress and major findings to date

- Intervention period has been completed. Blood, urine, faecal and breath samples have been collected and processed
- Analysis of putative indices of body Cu status, fructose tolerance, fibrinolytic activity and susceptibility of LDL to *in vitro* oxidation have been completed. Data evaluation is underway.
- Determination of oxysterols in plasma lipoprotein fractions and measurement of TGF-• are ongoing
- Analysis of bone turnover measures indicate that low Cu diets increased the rate of bone resorption

Task 6: study outline

Metabolic Cu studies with healthy adult volunteers to provide data on the dietary factors which affect Cu absorption from individual foods

Cu absorption from high Cu foods and intrinsically and extrinsically labelled foods is being assessed. The non-absorbable rare earth element, Holmium, is being used as a faecal marker

Task 6: progress

- Volunteers have been recruited and screened
- Hydroponically intrinsically labelled chick peas, soya beans, melon seeds and sunflower seeds have been successfully cultivated, harvested and their isotopic enrichments measured using ICP-MS
- Subjects are being fed test meals. Blood and faecal samples have been collected and processed

Task 7: study outline

In vitro absorption and metabolic studies with the human intestinal cell line Caco-2

To provide *in vitro* data on the molecular mechanisms of Cu absorption and metabolism in the intestinal cell and on the effects of the interaction of Cu with other components of the diet

Task 7: progress and major findings to date:

Detailed preliminary experiments utilizing permeable filters to mimic the *in vivo* physiological situation have established the culture conditions necessary for optimum Cu uptake.

Cu modified paracellular permeability in Caco-2 cells has been observed.

Cu histidine and Cu glutathione complexes were able to inhibit Cu damage to tight junctions.

The presence of a Cu transporter in the apical membrane of Caco-2 cells has been indicated.

Experiments utilizing ^{64}Cu show histidine to be inhibiting Cu uptake as compared to $CuCl_2$.

Urinary excretion of anthocyanins by one male volunteer after ingesting blackcurrant juice

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Introduction

Recent interest in flavonoids is based on epidemiological surveys as well as animal studies showing a close relationship between the consumption of flavonoids and a reduced incidence of cancer and heart diseases [Hertog 1993, Hertog 1995, Salah 1995, Keli et al. 1996, Hollman/Hertog 1997]. Progress in understanding the mechanisms by which flavonoids exert these health effects is limited because very little information is available regarding the existence of these compounds in human or animal fluids. Anthocyanins (fig. 1) and their aglycones, the anthocyanidins, are a major group of flavonoid compounds being particularly present in various berries (e.g. blackcurrants) and grapes in relatively high concentrations. Up to now to our knowledge, there exist no data on the intestinal absorption as well as the urinary excretion of delphinidine-3-glucoside (del-3-gluc), delphinidine-3-rutinoside (del-3-rut), cyanidine-3-glucoside (cya-3-gluc) and cyanidine-3-rutinoside (cya-3-rut), being the most important anthocyanins in blackcurrants.

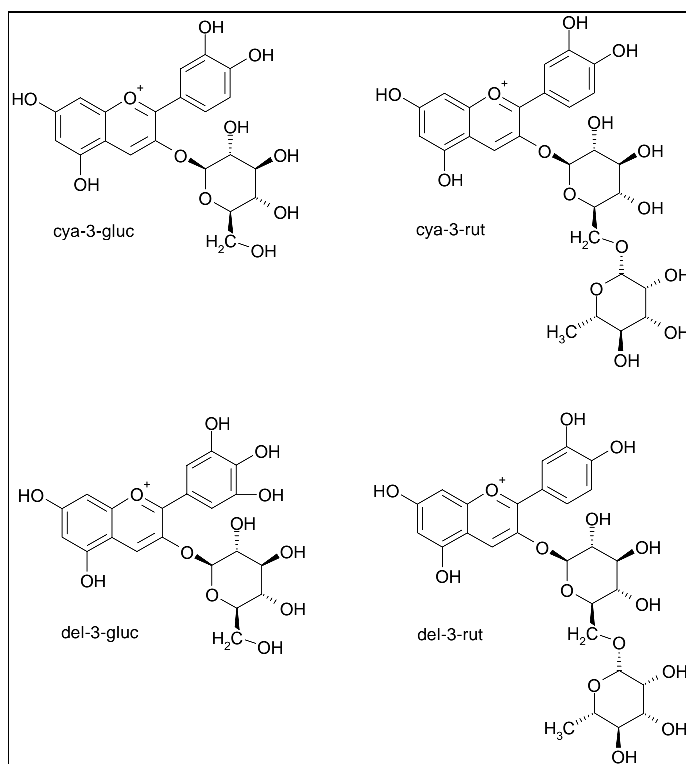

Fig. 1: Structures of blackcurrant anthocyanins

Method

Urinary samples of a healthy male volunteer were collected before (0 h) and 1, 2, 3, 4, 5, 6 and 7 hours after an oral administration of 300 ml blackcurrant juice (containing 121 mg del-3-gluc, 159 mg del-3-rut, 8 mg cya-3-gluc and 211 mg cya-3-rut). All samples (7 ml) were mixed with formic acid (2 ml) and evaporated to a volume of 0,1 ml under vacuum with a rotary evaporator, always keeping the water-bath temperature by 50°C. The evaporated samples were diluted with HPLC mobile phase (0,5 ml) before HPLC analysis. Del-3-gluc, del-3-rut, cya-3-gluc and cya-3-rut were separated with an isocratically reversed-phase (RP) HPLC method, described in the following table.

Tab. 1: HPLC parameters for the determination of blackcurrant anthocyanins in urine

column:	100 RP-18; 250 mm x 4 mm I.D.; 5 µm
guard column:	100 RP-18; 4 mm x 4 mm I.D.; 5 µm
mobile phase:	81% H_20 : 9% acetonitrile : 10% formic acid (v/v/v); pH 1,9
flow:	1,2 ml / min.
detection (UV/VIS):	520 nm
sample-loop:	20 µl

Results and discussion

The following tables and figures are showing the obtained data.

Tab. 2: Concentrations of del-3-gluc in human urine after ingesting blackcurrant juice

urine sample	concentration (µg/ml)
U 0 (*before ingesting*)	-
U ¼ (15 min. after ingesting)	-
U 1 (1 hr. after ingesting)	0.17
U 2 (2 hr. after ingesting)	0.069
U 3 (3 hr. after ingesting)	0.041
U 4 (4 hr. after ingesting)	-

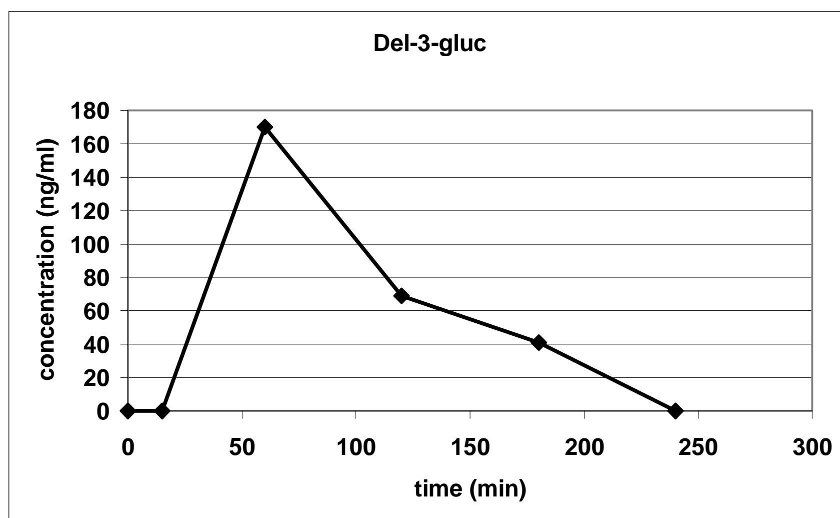

Fig. 2: Concentration-time-profile of del-3-gluc in human urine

Tab. 3: Concentrations of del-3-rut in human urine after ingesting blackcurrant juice

urine sample	concentration (µg/ml)
U 0 (*before ingesting*)	-
U ¼ (15 min. after ingesting)	-
U 1 (1 hr. after ingesting)	0.38
U 2 (2 hr. after ingesting)	0.20
U 3 (3 hr. after ingesting)	0.14
U 4 (4 hr. after ingesting)	0.063
U 5 (5 hr. after ingesting)	0.053
U 6 (6 hr. after ingesting)	0.01
U 7 (7 hr. after ingesting)	-

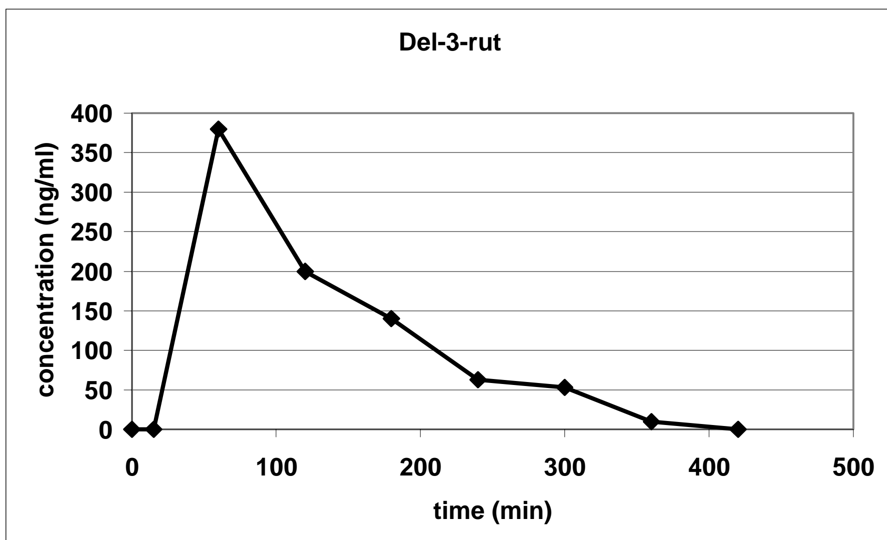

Fig. 3: Concentration-time-profile of del-3-rut in human urine

Tab. 4: Concentrations of cya-3-gluc in human urine after ingesting blackcurrant juice

urine sample	concentration (µg/ml)
U 0 (*before ingesting*)	-
U ¼ (15 min. after ingesting)	-
U 1 (1 hr. after ingesting)	0.02
U 2 (2 hr. after ingesting)	0.0085
U 3 (3 hr. after ingesting)	0.0053
U 4 (4 hr. after ingesting)	-

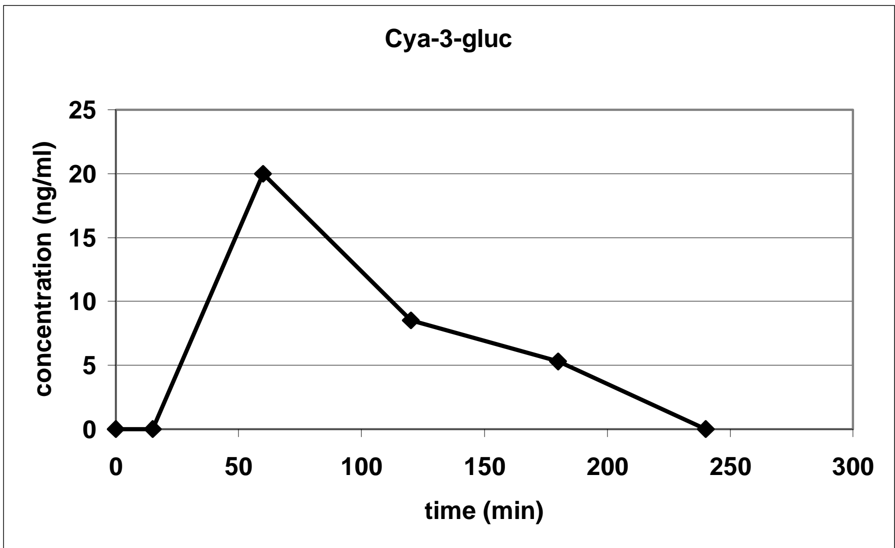

Fig. 4: Concentration-time-profile of cya-3-gluc in human urine

Tab. 5: Concentrations of cya-3-rut in human urine after ingesting blackcurrant juice

urine sample	concentration (µg/ml)
U 0 (*before ingesting*)	-
U ¼ (15 min. after ingesting)	-
U 1 (1 hr. after ingesting)	0.46
U 2 (2 hr. after ingesting)	0.20
U 3 (3 hr. after ingesting)	0.13
U 4 (4 hr. after ingesting)	0.052
U 5 (5 hr. after ingesting)	0.046
U 6 (6 hr. after ingesting)	-

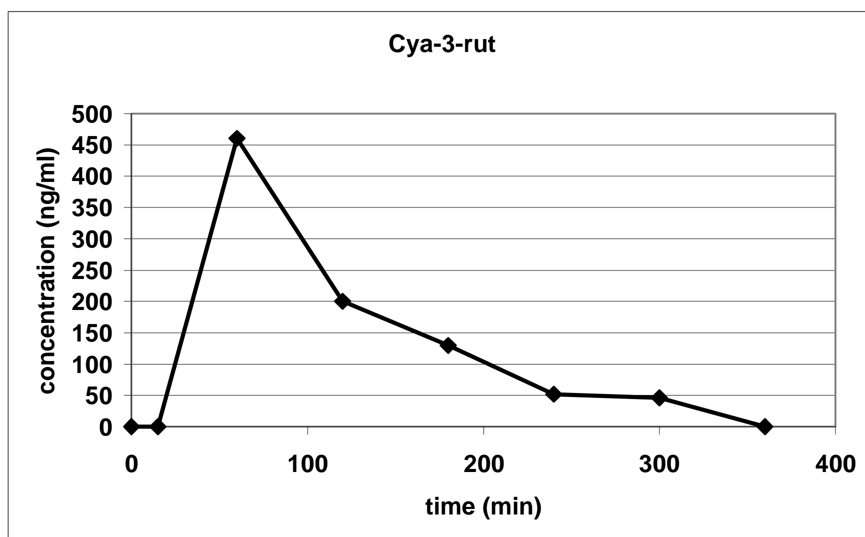

Fig. 5: Concentration-time-profile of cya-3-rut in human urine

Maximum concentrations (c_{max}) of del-3-gluc (0,17 µg/ml), del-3-rut (0,38 µg/ml), cya-3-gluc (0,02 µg/ml) and cya-3-rut (0,46 µg/ml) could be detected after 1 hour (t_{max}).

These results demonstrate for the first time that humans can absorb (and excrete) remarkable amounts of blackcurrant anthocyanins as glycosides being of relevance for biological interferences. Contrary to the widely held view, absorption of certain glycosides in the small intestine seems possible. In conclusion, research on human absorption, distribution, metabolism and excretion of flavonoids respectively anthocyanins is needed to fully evaluate their potential role in human health and disease.

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Absorption of gallic acid in humans after tea consumption

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Introduction

Gallic acid (GA) in free or bound forms is found in significant amounts in tea leaves, from which it is extracted in hot water infusions. GA (see fig. 1 for structure) is a strong antioxidant and possesses antiallergic, anti-inflammatory, antimutagenic and anticarcinogenic activities [Kondo et al. 1994, Li et al. 1994, Fuhrmann et al. 1995, Whitehead et al. 1995]. In spite of these protective effects, there are no data available about the extend of its absorption and metabolism in humans. Therefore our study was performed with adult humans to estimate the absorption and metabolism of GA after oral administration.

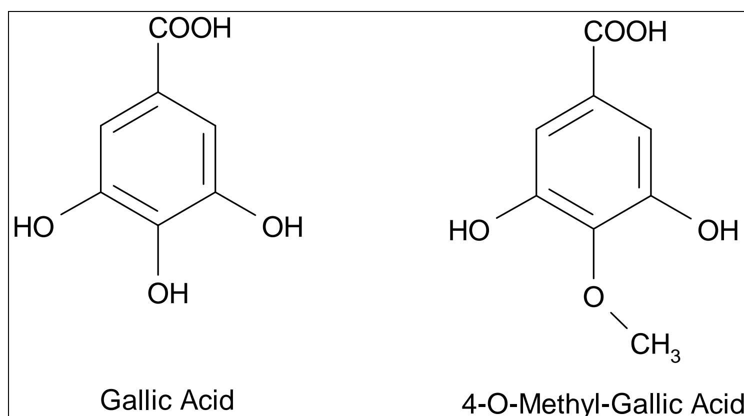

Fig. 1: Structures of GA and 4OMGA

Method

After following a GA free diet for 24h, plasma samples of 4 healthy volunteers (2 males and 2 females) were collected before (0 h) and 0.75, 1.5, 2.25, 4, 8 and 10 hours after an oral application of black tea (containing 50mg GA). 2 ml of plasma were mixed with an equal volume of 1 M hydrochloric acid and hydrolyzed in a boiling water bath under argon for 30 min. After hydrolysis the samples were cooled to room temperature and extracted twice with a fourfold volume of ethyl acetate, centrifuged (1800 g for 10 min.) to separate the organic fractions and evaporated to dryness under vacuum with a rotary evaporator. Each extract was redissolved in HPLC mobile phase (0.5 ml for 2 ml plasma) before HPLC analysis. GA and its metabolite 4-O-methyl-gallic acid (4OMGA) were determined with an isocratic reversed-phase (RP) HPLC method, described in the following table.

Results and discussion

The following tables and figures are showing the obtained data.

Tab. 1: HPLC parameters for the determination of GA and 4OMGA in human plasma

column:	100 RP-18; 120 mm x 4 mm I.D.; 5 µm
guard column:	100 RP-18; 4 mm x 4 mm I.D.; 5 µm
mobile phase:	4.4 mM phosphoric acid in water
flow:	1.0 ml / min.
detection (UV):	220 and 270 nm
sample-loop:	100 µl

Tab. 2: Concentrations of GA in human plasma after drinking black tea

plasma sample (time)	concentration (mg/l)
P 0 (*before application*)	-
P ¾ (45 min. after application)	0.099
P 1½ (90min. after application)	0.282
P 2¼ (135 min. after application)	0.201
P 4 (4 hr. after application)	0.106
P 8 (8 hr. after application)	0.054
P 10 (10 hr. after application)	-

Tab. 3: Concentrations of 4OMGA in human plasma after drinking black tea

plasma sample (time)	concentration (mg/l)
P 0 (*before application*)	-
P ¾ (45 min. after application)	0.083
P 1½ (90min. after application)	0.172
P 2¼ (135 min. after application)	0.495
P 4 (4 hr. after application)	0.366
P 8 (8 hr. after application)	0.051
P 10 (10 hr. after application)	-

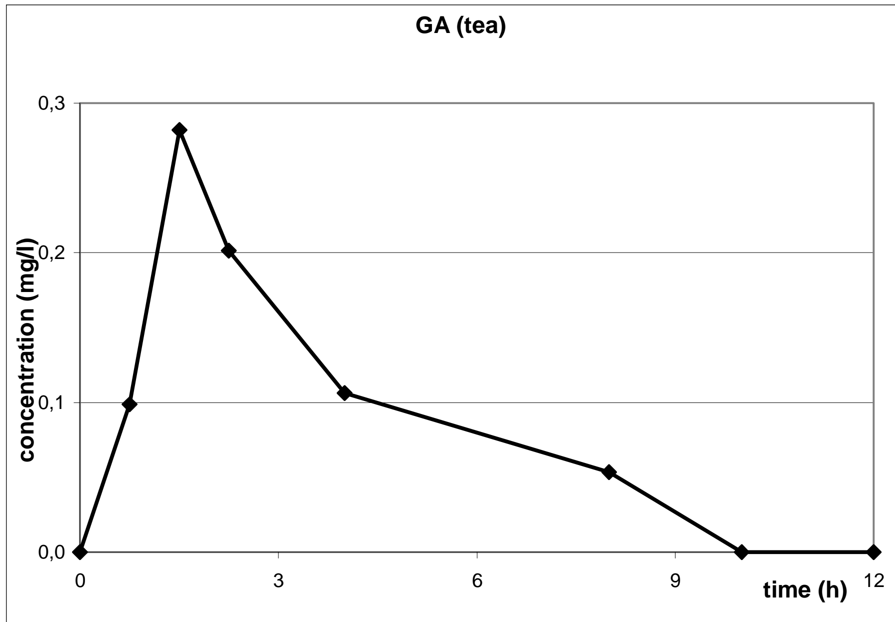

Fig. 2: Concentration-time-profile of GA in human plasma

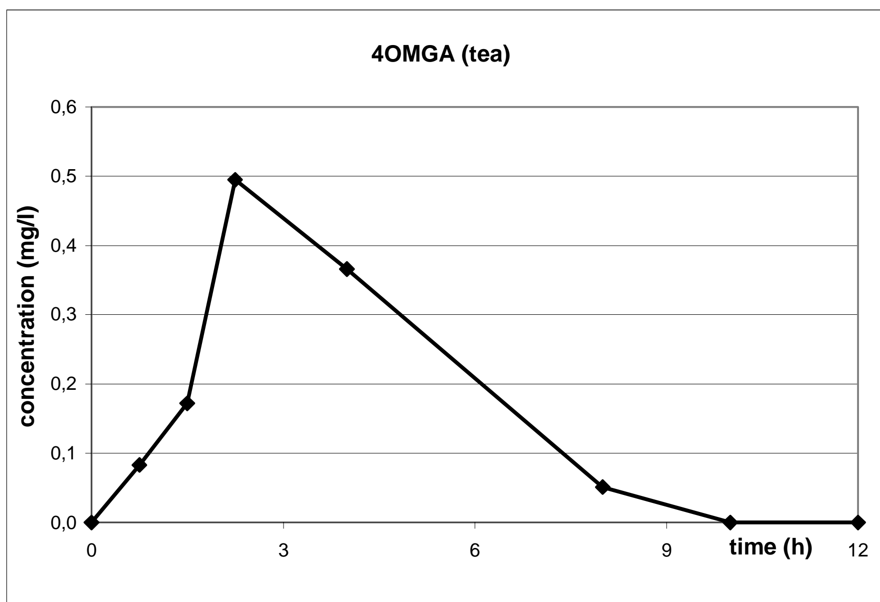

Fig. 3: Concentration-time-profile of 4OMGA in human plasma

Maximum concentrations (c_{max}) of GA (0.288 ± 0.089 mg/l) and 4OMGA (0.595 ± 0.156 mg/l) could be detected after 90 min. (GA) and 150 min. (4OMGA).

In this paper we have first established that GA from black tea is absorbed in humans in remarkable amounts. We can therefore reasonably assume, that the in vivo antioxidant effect of black tea as it was demonstrated by Serafini et al. (1996) might be caused by GA and its metabolite 4OMGA.

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New methodologies for studying diet and gut maturation in early life

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All nutritionists and paediatricians would agree that breast milk is the preferred source of nutrition for babies. For a variety of reasons, however, some babies are fed on formula milks and this could mean that they are disadvantaged from the point of view of the development of a gut microflora capable of reducing predisposition to disease in adulthood. It is therefore essential that formula milks be improved to contain certain ingredients, which could increase the retention of a breast-fed style microflora. A second critical period of gut colonisation is during the weaning period. Very little is known about the changes that occur in the colonic microflora during weaning nor of the impact of these changes on susceptibility to disease in later life.

It is well established that breast fed babies have a very different colonic bacterial flora to that of formula fed babies. The breast fed baby has more bifidobacteria and lactobacilli than formula fed babies who have more *enterobacteriaceae* and bacteroides spp. (Balmer *et al.*, 1989a). This difference in bacterial profile is thought to be a factor in the increased incidence of gastrointestinal infections seen in formula fed babies (Howie *et al.,* 1990). Some components of breast milk may thus promote retention of a colonic microflora with increased resistance to pathogens. To date, formula milks have not been able to achieve the same effects.

The bacterial metabolic activity and fermentation capacity of breast fed and formula fed infants also differ (Edwards *et al.,* 1994, Parrett *et al.,* 1994). Breast fed babies produce more lactic acid and less propionic acid and are less able to ferment complex carbohydrates than formula fed babies. This difference appears to persist well into weaning and pilot studies of the faecal bacterial activity of weaning children have suggested a slow maturation of the flora from that of the neonate to that of the adult. Degradation of mucin, conversion of bilirubin to urobilirubin, metabolism of cholesterol to coprostanol and inactivation of faecal tryptic activity are still increasing by the age of 2 years (Midtvedt *et al* 1988) Development of the ability to ferment complex carbohydrates is also a slow process with only 42% of adult values in infants during late weaning (7-9 months; Parrett *et al* 1997), further supported by the significant amounts of starch found in faeces of children of up to 3 years (Verity 1995). The exact mechanisms controlling this difference in colonisation and maturation are unclear. Despite many modifications to formula milks to mimic human milk more closely, the faecal flora of the breast fed infant can not be reproduced by artificial feeding (Balmer and Wharton 1989). The role of human milk in the establishment of the colonic microflora may be a combination of nutritional and non-nutritional factors. Nutritional factors include lactose as a major fermentation substrate, specific nutrients for bifidobacteria and iron, which is essential for the growth of most species except bifidobacteria. Despite some evidence for effects on the faecal flora in early life,

these compounds do not account for the substantial differences in bacterial profile seen in formula fed and breast fed babies (Balmer *et al.,* 1989a,b).

The colonisation of the gut by bacteria also influences the development of the immune system. The interaction between the intestinal microflora and the host mucosal immune system is complex (Moreau 1991, Char *et al*., 1994). Intestinal bacteria are considered vital for the maturation and maintenance of intestinal immunity. Food and age of the host are important influences on the gut associated immune system.

The development of the intestinal flora during weaning has been very little studied and the effect of different weaning foods and the order in which they are given are unknown. Exposure of the colonic bacteria to new substrates induces shifts in the component populations and thus it is not surprising that weaning is the most likely time for the more resilient breast fed babies to suffer infectious diarrhoea. Since the microflora established during and post weaning will remain into adulthood and may influence adult health, it is crucial that more information is gained concerning the weaning foods most likely to promote development of a microflora conferring health benefits.

Investigations of the factors that determine the intestinal microflora are hampered by ethical and practical problems of studies in babies. The variability in bacterial populations demands a large sample size for *in vivo* studies and diet can not be altered without good ethical reasons. The alternative is to use *in vitro* or animal models. Many of the in vitro studies of formula ingredients have been carried out in pure cultures of bacteria. Since the complexity of the human gut ecosystem makes interaction, competition for substrates and nutrients and synergism between bacterial species inevitable, the information gained from such studies is limited in its relevance. To date there are no realistic *in vitro* models of the infant gut microflora.

As part of a research project funded by the European Commission, *FAIR CT97 3181* models of the infant gut are being developed which will allow detailed evaluation of infant formula and weaning food ingredients. These models will allow a systematic approach to help unravel the mechanisms behind gut maturation and will help the identification and development of foods that promote health, both in the young and later in the adult.

Two major models are being developed; an *in vitro* continuous culture model and a human flora associated (HFA) rat model. These will be based on the flora of breast fed infants and weaning infants. A further *in vitro* model will investigate bacterial adhesion and translocation. The maturation of the gut and the effect of diet will be observed in these models by monitoring changes in bacterial populations and activities. This will be achieved by use of novel mRNA probes and measurement of a range of biologically important bacterial products (short chain fatty acids, lactate, ammonia, phenols, cresols and metabolites of bile acids, bilirubin, cholesterol and mucin) and bacterial enzyme activities (β-glucuronidase and β-glycosidase). Gut mucosal turnover and the gut associated immune system will be characterised in the HFA rat model. The continuous culture and rat models will be validated against the faecal characteristics of infants at different ages and stages of gut maturation and dietary experience. 150 infants (75 breast fed and 75 fed the same infant formula) are being recruited into the study to allow for drop out and change in feeding practice.

This project is in its first year. So far the main activities have been to design the models, to test the feasibility of the tasks and to optimise sample exchange and assay procedures.

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Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria

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The human colonic microflora is a complex bacterial ecosystem comprising of more than 400 bacterial species [1]. The composition and functioning of this microflora plays a key role in the protection of the host against several pathogenic conditions such as: colonic cancer [2], gastro-enteritis [3] and immunological disorders [4]. Furthermore, the colonic microflora plays a role in digestion and the production of vitamin K-12 [5]. Because of the large influence of the gut microflora on the health of the host, accurate assessment of the composition of this bacterial ecosystem has been a point of interest in both clinical microbiology and food microbiology. Historically, quantifying bacterial genera and species in the gut is performed by culturing faecal material on selective media, which a cumbersome and inaccurate. In order to bypass these biases, an automated microscopy-based method using fluorescent 16S rRNA-targeted oligonucleotide probes directed against the predominant groups of intestinal bacteria was developed and validated. The probes used in this study comprise: a probe specific for almost all bacteria present in the gut flora (EUB338), a genus specific probe for Bifidobacterium spp. (BIF164), a digenus-probe specific for Bacteroides spp. and Prevotella spp. (Bac303) and a trigenus-probe specific for Ruminococcus spp., Clostridium spp. and Eubacterium spp. (REC482). The sequences of these probes are listed in Table 1. To enumerate all bacteria, including those which do not hybridize, 4',6-diamidino-2-phenylindole (DAPI), which stains all objects containing DNA, was also included. The probes have all been designed and validated for *in situ* application [6] and standard protocols for *in situ* hybridization of faecal flora were used [7]. Furthermore, the method makes use of the Leica 600 image analysis system, a Kodak MegaPlus camera model 1.4 and a Leica DM/RXA ultra-violet microscope mounted with a servo-controlled, eight-slides stage. Software for fully automated image acquisition and analysis was developed and tested. The software was developed using the QUIPPS-environment, and mainly consists of two parts:

(i) A user interface in which all parameters can be entered. This interface consists of a single form designed in Excel 7.0 (Microsoft Corporation, Seattle, USA). The user focuses manually on each separate well of all slides. Every time an image is in focus, the vertical position of the stage (expressed as 'the Z-coordinate') is entered and stored in a database. This newly entered Z-coordinate is averaged with the already stored Z-values, together with the corresponding coordinates which determine the location of the microscopic field on the slide (i.e. the X- and the Y-coordinate). The X- and Y-coordinate determine the planar location of the image and can only be adjusted during the initial parameter-input. From the array of Z-values a mean Z for each well is calculated, yielding an advancing mean Z-value which is used during the servo-controlled slide switching. The autofocus procedure which follows after the X, Y and Z-coordinates of the starting point have been determined, consists of the acquisition of a consecutive series of images with 1 μm interspace in the Z-dimension. From each separate image a 'focus-parameter' is calculated. Because this parameter describes the integral contrast in the image, the image with the highest parameter-score is assumed to be in focus. Thereafter, a threshold-value for segmentation of the gray-level images into binary images is determined and applied throughout the entire analysis-sequence.

(ii) The actual analysis-sequence. The analysis-sequence consists of the following steps which are repeated 25 times per well, over 6 wells per slide. This, again, is then repeated for each separate slide (up to 8) leading to a total number of 1200 images which are digitized and processed during one analysis run. The processing of one single image comprises of the following steps:

(a) Autofocus under phasecontrast illumination using the mean Z-value which was calculated from the previously stored array of Z-values.

(b) Acquisition using a BGR filter (Leica) and an exposure time of 0.5 sec under ultra-violet illumination. During exposure, two images are acquired and averaged to account for fluctuations in the light-signal emitted by *in situ* hybridized gut microflora.

(c) TopHat filtering, which enhances local contrast and equalizes global variations in background gray-levels, enabling subsequent use of a global threshold value for binarization.

(d) Binarization using a user-defined threshold value for segmentation of the image into (bacterial) objects and background.

(e) Automatic counting of the number of objects in the binary image.

(f) Counting data are exported to the Excel-spreadsheet program for further analysis. The total number of bacteria per gram of faeces was calculated by multiplying the total number of bacteria counted by the ratio between the area of a well and the area of a microscopic field. This yields the total number of bacteria in the well. That number is subsequently multiplied by 100 to convert the volume applied to millilitres and, after that, multiplied with the appropriate dilution-factor. The performance of the automated counting procedure was evaluated by measuring the following parameters 25 times: mean time needed to obtain a correctly focused image (MFT), mean image analysis and storage time (MIAST), mean time needed for change of wells (MWCT), mean time needed for change of slides (MSCT) and the percentage of fatal out-of-focus incidents (POOF). The values of the performance parameters: MFT, MIAST, MWCT, MSCT and POOF were found to be: 40 sec, 10 sec, 5 sec, 5 sec and 0.01% respectively. Repeated measurement revealed that these values did not fluctuate more than 0.5%. The value of the POOF-parameter heavily depends on the quality of the microscopic preparation (e.g. clumping of the bacteria), but never exceeded 0.01%. The bulk of the analysis time is consumed by the focusing-procedure. Speeding up the automatic focusing routine by reducing the number of images read and calculated will probably enhance the number of fatal out-of-focus incidents while there is no true time-gain. This becomes clear if it is realized that a complete analysis (consisting of 1200 images) takes about 20 h to complete. Because such an analysis is usually performed overnight, the results will always be accessed the following day. Furthermore, the percentage of fatal-out-of-focus (POOF) accidents is reasonably low. It is therefore concluded on the basis of the POOF-value that using a knowledge-base which defines the starting point for the autofocusing routine enables the use of prolonged automated analyses while reliability is maintained throughout the entire run. In Table 2 the values of the coefficients of variance due to errors in the process of slide manufacturing (CV_{assay}) and due to difference in gutflora-composition (CV_{inter}) are listed. As can be seen from Table 2, CV_{assay} values obtained using the DAPI-, EUB338- and BIF164-probes range from 0.10 to 0.14. The accompanying CV_{inter} values are about three times as large (ranging from 0.32 to 0.48) as the CV_{assay}-values. This implies that this method is accurate enough to significantly detect differences in gut-flora composition between volunteers. In other words, when comparing the values obtained by means of this method using EUB338, DAPI and BIF164 on faecal samples from two different volunteers,

about one quarter of the difference between those values is determined by variations in the process of slide manufacturing, while about three quarters of the difference is determined by real differences in flora-composition between volunteers. Remarkable also is the fact that the results obtained for the DAPI stain, which is a low molecular weight intercallant, are comparable with those for EUB338, which comprises of a medium molecular weight oligonucleotide. This implies that permeabilization of the cell wall of the different bacterial species which make up the gut microflora is probably no limiting factor. Also remarkable is the fact that the CV_{inter} values of the EUB338 and the DAPI are comparable with the CV_{inter} value obtained using the BIF164-probe. Although the CV_{assay} value obtained with the REC482-probe (0.17) is still comparable with the corresponding values obtained with the previously mentioned probes, the CV_{inter}-value (0.26) is considerably lower. This probably results from the broad specificity of the REC482-probe (it covers three different genera) which makes it hard to distinguish small differences in gutflora-composition. The CV_{assay} value obtained using the BAC303 probe is considerably higher than the other values obtained. An extra assay-related source of error which may be responsible for this observation is the fast diminishing of the signal emitted by the BAC303-positive bacteria, which was observed during the process of image analysis. However, the larger assay error obtained using the BAC303 probe does not appear to prevent its applicability in distinguishing between Bacteroides and Prevotella concentrations between gut microfloras from different volunteers because the accompanying CV_{inter}-value amounts up to 0.50. In conclusion, the combined use of automated microscopy in combination with fluorescent *in situ* hybridization yields a powerful strategy to quantify groups of intestinal bacteria. With this method differences in gutflora-composition between volunteers can be detected and it is faster than traditional means of microbiological quantification procedures like culturing. This method could be used in studies where large numbers of faecal samples need to be processed e.g. studies concerned with the influence of feed-additives on the gut microflora. Finally this methodology may be of aid in studies concerned with tracking of a specific micro-organism (e.g. a probiotic). It is concluded that the combination of automated microscopy and fluorescent *in situ* hybridization enables distinction in gutflora-composition between volunteers at a significant level. Furthermore, in a 20 h timespan, the number of bacterial cells in 48 separate faecal samples can be measured automatically.

Table 1: Oligonucleotide probes used in this study*

Name	Target group	Sequence (5'→3')
EUB338	Bacterial Kingdom	GCTGCCTCCCGTAGGAGT
BIF164	Bifidobacterium spp	CATCCGGCATTACCACCC
BAC303	Bacteroides spp. Prevotella spp.	CCAATGTGGGGGACCTT
REC482	Clostridium coccoides-Eubacterium rectale	GCTTCTTAGTCARGTACCG

* All probes consist of single strain DNA covalently linked with fluorescein iso thiocyanate at the 5'-end.

Table 2: CV_{assay}*and CV_{inter}**values obtained with the automated FISH-method for quantification of bacterial genera in human faeces.

Probe	CV_{assay}	CV_{inter}
DAPI	0.10	0.32
EUB338	0.12	0.44
BIF164	0.14	0.48
BAC303	0.30	0.50
REC482	0.17	0.26

* CV_{assay} : Coefficient of variation (standard deviation/mean) due to fluctuations in the process of slide manufacturing.
** CV_{inter} : Coefficient of variation due to normal differences in flora composition between human volunteers.

Acknowledgments

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Biomarkers of exposure and effect for studying food, nutrition and well being

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1 Introduction

The effects of exposure to dietary constituents (in addition to the nutritional consequences) have been the focus of considerable efforts. It is possible to investigate these in a number of different ways, however, almost all can be assessed in one of two distinct categories. Direct measurement of the biological effect may be obtained by monitoring a clinical endpoint. This type of approach can be difficult to achieve in many cases since the effects of dietary constituents tend to be chronic rather than acute. Hence, long term, generally non-predictive testing is carried out. The second type of approach involves the use of predictors of biological function. These can take many forms including animal models, *in vitro* systems and measurements of indicators in blood, urine, hair etc. Such predictors of biological effect are collectively called *biomarkers*. They can be defined as short term measurements which reflect long term clinical effects and which are responsive to, and predictive of, the effects of specific dietary components. They can be divided into two distinct groupings - biomarkers of exposure and biomarkers of effect.

2 Biomarkers of exposure

A biomarker of exposure is a measure of the level of exposure of an individual to a specific component and, in addition, also encompasses those factors which influence the exposure. There are various ways of measuring exposure with varying degrees of invasiveness. The decision as to which approach is best must take into account factors such as the nature and metabolism of the chemical species, and the level and frequency of exposure.

2.1 Dietary exposure from food

One approach to measuring exposure is to measure the levels of specific components in foods. This forms the basis of much of the traditional monitoring process carried out by legislative authorities. For example, in the UK, surveillance programmes exist which focus on a diversity of analytes such as heavy metals, dioxins, polycyclic aromatic hydrocarbons, veterinary drugs, pesticides, mycotoxins and packaging materials. One problem with this type of approach is attempting to define what is meant by an acceptable or "safe" level of contamination. A simple illustration is the measurement of levels of the mycotoxin, aflatoxin B_1.

Aflatoxin B_1 is produced by the fungus *Aspergillus flavus.* It is endemic in crops, such as peanuts, pistachio nuts and figs, which are grown in parts of the world where the climate favours the producing organism (generally hot and damp conditions). Aflatoxin B_1 is considered to be a genotoxic carcinogen with the primary site of action being the liver. Definitive proof of its role as a causative agent in heptocellular carcinoma in humans has not yet been established but its classification as a suspected genotoxic carcinogen means that the acceptable level for its presence in food should be the limit of analytical detection (currently of the order of 0.1 ppb). In fact, such a limit is practically unenforceable since contamination is both widespread at a low

level and extremely heterogeneous. For practical purposes, therefore, a limit of 4ppb has been adopted in Europe [1].

The establishment of a limit for a compound in foods can help in the prediction of lifetime exposure when surveillance data are combined with information from food intake surveys. There are, however, several uncertainties associated with such measurements including variations in diet and the heterogeneous nature of most contamination. In addition, in cases where the dietary constituent is considered beneficial there is much less legislative or social impetus to carrying out extensive surveillance. An alternative approach would be to carry out a more direct measurement of individual exposure to the constituent of interest.

2.2 Direct exposure measures

The direct measurement of exposure is, in principle, a more effective reflection of the real life situation. In this case the target species is measured in a suitably accessible body product. For example, dietary exposure to a range of micronutrients is determined using blood, faecal and urinary measurements. There is however considerable uncertainty about the relevance of many biological markers, such as serum concentrations of a nutrient, as evidence of an individual's "status" for that nutrient [2]. Factors such as the individual's lifestyle and diet, the efficiency with which a nutrient is absorbed, metabolised and utilized (bioavailability), as well as the appropriateness of the analytical methodology, all influence the interpretation of the measurement made. In contrast, for some food additives, a biomarker of exposure can be found that accurately reflects dietary intake because the additive is not extensively metabolised and is excreted in a dose responsive manner. For example, the intense sweeteners saccharin and acesulfame-K, are excreted unmetabolised in urine and little variation is found between individuals. Thus a urinary biomarker of exposure can be used to calculate dietary exposure to the two additives [3].

2.2.1 Metabolism and bioavailability

For some compounds the metabolism and bioavailability pathways are complex. For example, the majority of dietary phytoestrogens are isoflavones, such as genistein and daidzein. They occur in foods as the corresponding glycosides, (daidzin and genistin), and are rapidly hydrolysed upon ingestion to produce the aglycones which are considered to be the major, bioavailable active species. The metabolism of daidzein and genistein leads to production of the corresponding glucuronides and sulphonates and eventual excretion. A certain proportion of individuals are able to convert isoflavones into equol - a reaction possibly mediated by gut microflora. The extent of metabolism of genistin and daidzin including hydrolysis of the glycoside, sulphonation/glucuronidation and conversion to equol are all factors which influence individual exposure to the compounds.

The major estrogenic contaminants in foods are probably dioxins. Both dioxins and polychlorinated biphenols (PCBs) occur widely in the environment. In contrast to the phytoestrogens, these compounds are essentially unmetabolised and are highly fat soluble. This means that they are laid down in adipose tissue and have a very long *in vivo* half life. Therefore, estimates of dietary exposure based upon measurements of levels in blood are not particularly useful.

2.3 Indirect exposure measures

A number of studies have been carried out in which exposure to specific agents is measured indirectly. This includes factors such as enzyme induction, altered gene expression, production of stress molecules and changes in cell morphology. While some of those appear to be typical of non-specific responses, they also impinge upon biomarkers of effect. It should be clear, however, that the clear difference between biomarkers of effect and indirect measures of exposure is in the clinical outcome. For an indirect measure of exposure to be valid, it must be unconnected with the clinical outcome resulting from that exposure.

3 Biomarkers of effect

A biomarker of effect is, almost by definition, less tangible than a biomarker of exposure. The crucial aspects of biomarkers of effect are that they should relate long term, clinical endpoints to specific dietary components and that the identified biomarkers should change in response to changes in dietary constituents and should be predictive of the long term health effect. In this way association is distinct from causal relationships.

3.1 Diet health issues

In order to determine the validity of biomarkers of effect it is essential to be able to determine and where possible quantify, a number of factors. These include:

(a) How strong is the link between diet and health?
(b) How relevant is the biomarker as a predictive tool to determine clinical outcome?
(c) How responsive is the biomarker to changes in diet?
(d) How do the changes in the diet correlate with long term clinical outcomes?

Based upon these types of considerations, a range of biomarkers of effect and disease/health issues have been assessed [4] and the following conclusions arrived at:

(a) The major diet/health issues are:
    (i) Cancer
    (ii) Circulatory disorders
    (iii) Osteoporosis
    (iv) Allergy
    (v) Obesity and effects arising from it

(b) In terms of biomarkers of effect, the causal relationship for most of these is incomplete. This is because a mechanistic underpinning for the effects has, in many cases, not been adequately arrived at.

(c) Specific dietary constituents which affect the health issues outlined in (a) include:
    (i) Antioxidants
    (ii) Protective factors
    (iii) Phytoestrogens
    (iv) Allergenic proteins
    (v) Dietary lipids

(d) Further work is necessary to develop a co-ordinated approach which will answer the issues raised under (b), to link the health issues at (a) with the diet constituents (c) and to develop methods for the development and implementation of dietary measures to improve the health of populations.

4 Conclusion

Biomarkers have the potential to contribute significantly to the area of food, nutrition and health as well as aiding better approaches to risk assessment. They are an integrated part of the plan for a coherent strategy to improve the diet and health of the consumer and to increase European competitiveness through the production of products with greater appeal and world-wide marketability.

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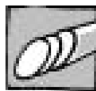

Meat

(special FLAIR-FLOW contribution)

Influence of oxidant systems on lipid and protein oxidation in homogenates and microsomes from turkeys (Pectoralis major muscle) fed different fat sources and vitamin E levels

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Abstract

This work deals with the effects of a chemical (Fe^{+3} / sodium ascorbate) or an enzymatic (Cytochrome P450 oxidase / NADPH) oxidative system on lipid and protein oxidation in pectoralis major muscle from turkeys fed different fat sources (rapeseed oil, soya oil and tallow) and vitamin E levels. The measurements were done on homogenates, without myofibrils, and on microsomal membranes. Vitamin E supplementation lowered lipid oxidation in muscle homogenates and microsomal fractions. This effect was influenced by dietary fat source and by the oxidant system. To a less extent, vitamin E supplementation can also lower protein oxidation and lowering is also influenced by the dietary fat source and the oxidative system (chemical / enzymatic).

These experiments, which measure the oxidative processes of different muscle fractions with different free radical generator systems, could be an efficient way of studying the oxidation mechanisms during storage of meat.

Introduction

Diets used for fast growing poultry are usually rich in polyunsaturated lipid to obtain high energy level. But in monogastric animals, such as turkey, the dietary fat source affects directly the triglyceride and, to a lesser extent, the membranal phospholipids composition. Membranal polyunsaturated fatty acids are well known to be good targets leading to oxidative processes in meat which affect organoleptic qualities during chill storage as flavour. Lipid oxidation can also interact with muscle pigment, which affect colour properties as in beef, and with other muscle proteins. Protein oxidation is also implicated in structural and functional changes of muscle proteins (Decker et al., 1992; Martinaud et al., 1997) and its role in texture is still not known.

In meat, oxidative processes depends on pro-oxidant compounds, such as free and haeminic iron, or enzymatic system such as membranal cytochrome P450. On the contrary, many antioxidant compounds such as vitamin E or vitamin C, glutathione and muscle antioxidant enzymes can lower lipid oxidation. Many studies in beef (Arnold et al., 1993), in pork (Monahan et al., 1992), in chicken (Sheehy et al., 1993) and turkey (Mercier et al., 1998) have reported a beneficial effect of vitamin E supplementation on lipid oxidation in meat or in membranal systems (Monahan et al., 1993). On the contrary, a few experiments have been conducted to study whether or not vitamin E supplementation could reduce protein oxidation (Mercier et al., 1998).

The aim of this work is to study lipid and protein oxidation in pectoralis major muscle fractions (on homogenates, without myofibrillar proteins, and on microsomal membranes) from turkeys fed different fat sources and vitamin E levels. Two oxidant systems (chemical and enzymatic) were used to study the mechanisms of lipid and protein oxidation.

Materials and methods

Male BUT 9 strain turkeys were fed basal diet enriched with 6% of one of the following fat sources: soya oil, rapeseed oil or tallow. For each diet, three tocopheryl acetate (Hoffman-Laroche, France) levels were used: a basal level (30 ppm) and two supplemental levels (200 or 400 ppm). Animals (n= 6)were slaughtered at 16 weeks of age and Pectoralis major muscles were taken at slaughter, put in a plastic bag, stored on ice at 6 h pm and, after 6h, frozen at -80°C before analysis.

Ten grams of muscle were homogenised with a waring-blender in 50 ml buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.4). The extract was centrifuged at 3000 g for 15 min and the supernatant was collected and used for homogenate oxidation.

Microsomal membranes were obtained by differential centrifugation method (Apgar and Hultin, 1982). Microsomes were resuspended in the homogenate buffer at about 1 mg of protein/ml.

The chemical oxidation was carried out using the $FeCl_3$ (0.1mM)/sodium ascorbate (0.5mM) at 37°C.

The enzymatic induction was carried out using the microsomal cytochrome P450 oxidase NADPH dependent system with NADPH (2mM), ADP (2.6 mM), $FeSO_4$ (0.11mM).

Lipid oxidation was measured by the TBA-RS values according to the method of Lynch and Frei (1993). Results were expressed in nmol/ml for muscle homogenates and in nmol/mg of microsomal protein. Protein oxidation was measured by the carbonyl content, according to the method of Oliver et al.(1987), and expressed in nmol/mg of protein.

Results and discussion

Previous work (Mercier et al.,1998) showed that muscle vitamin E content was greatly affected by vitamin E supplementation in the diet. The vitamin E content in Pectoralis muscles from supplemented (200 or 400 ppm) animals was almost 6-fold greater than in control ones(30 ppm) with significant differences between dietary fat sources (results not shown). The higher vitamin E content was found in supplemented animals fed tallow followed by animals fed rapeseed and soya oil. It was also shown that muscles from supplemented animals receiving 400 ppm tocopheryl acetate had about twice the vitamin E content than muscles receiving the 200 ppm supplementation (to be published). In the microsomal fraction, the vitamin E content was determined for the 200 ppm supplementation and it was observed (results not shown) about identical differences between control and supplemented animals.

Figure 1 shows the effect of Fe^{3+} / ascorbate system on lipid and protein oxidation in muscle homogenate from control and supplemented (400ppm) animals.

At T0, the TBA-RS values in control and supplemented animals were similar and no fat source effect was noted. After 2 and 6 hours induction, control muscle homogenates exhibited significantly higher TBA-RS values than supplemented ones. These results were in agreement with those of Santé et al. (1994). When dietary fat sources were compared, for control animals after a 6 hour induction, significant differences between soya and tallow fed animals were found. Animals fed rapeseed showed intermediate values. For the muscle homogenates from supplemented animals, the TBA-RS values obtained after 2 and 6 hours induction were low and similar whatever the feeding mode. Gandemer et al., (1996) have shown that animals fed soya exhibited a high content in polyunsaturated fatty in neutral lipids and, to a less extent, in polar lipids. This result is in agreement with the TBA-RS levels obtained after a 6 hour induction.

For protein oxidation, measured by the carbonyl content (Figure 1), a significant difference between control and supplemented animals for soya and rapeseed fed animals was evident at T0. After 2 and 6 hours induction, significant differences between control and supplemented muscle homogenates from animals fed rapeseed and soya was noted. In muscle homogenates from tallow fed animals, no difference between control and supplemented was observed. This results suggest that protein oxidation is dependent on lipid hydroperoxide formation. Moreover, after a 6 hour induction, the maximum carbonyl content was obtained for soya and rapeseed oil fed animals. In this experiment, the differences in oxidative status could be directly linked to the higher content in vitamin E in supplemented animals and to differences in fatty acid composition in relationship with the dietary fat source.

Figure 2 shows the effect of an enzymatic induction using the cytochrome P450 oxidase NADPH dependent in muscle homogenates of control and supplemented (200ppm) animals.

At T0, significant differences between control and supplemented animals were observed as in the first experiment using a chemical induction. After a 1hour induction, the TBA-RS values increased in all control samples, and more particularly in samples from animals fed soya oil as previously shown. Except in homogenates from soya fed animals, no evolution in TBA-RS values was observed in supplemented ones. After 5 hours induction, TBA-RS values of control samples were significantly higher than those of supplemented ones for rapeseed and tallow fed animals. For muscle homogenates from animals fed soya oil, no difference in lipid oxidation between control and supplemented was noted. In this experiment, the vitamin E supplementation was only 200 ppm and, with this enzymatic oxidant system, the TBA-RS values of control samples were about twice to those noted in the first experiment. In very polyunsaturated medium such as in the soya sample, and with a constant free radical production due to an enzymatic system, it is possible that vitamin E could be consumed and ,after 5 hours induction, TBA-RS values of supplemented samples were identical to those noted for the control ones.

Results obtained for protein oxidation with the enzymatic system (Figure 2) showed a less increase compared to the increase in TBA-RS values and to the first experiment. After 1 hour induction, no difference in carbonyl content was noted between control and supplemented animals. After 5 hours induction, significant differences were noted between control and supplemented samples essentially for animals fed tallow and, to a less extent, for animals fed rapeseed oil. As for TBA-RS values, no difference between control and supplemented samples was observed for muscle homogenates from turkey fed soya oil. In this experiment, the free radical production site is in, or near, the membranal enzymatic system. In homogenates, it is possible that only membranal proteins were affected by the free radicals generated by the enzymatic system. Moreover, with this enzymatic system, control samples exhibited twice the TBA-RS values than in first experiment. This result could indicate that the « biological » site of free radical production was more efficient in increasing the TBA-RS production compared to the chemical system.

In experiment 3, microsomal membranes were prepared from control and supplemented (200 ppm) animals. Figure 3 shows the lipid and protein oxidation after a chemical induction with the Fe^{3+} / ascorbate system. At T0, significant differences in TBA-RS values appeared between control and supplemented samples whatever the dietary fat source. This result could be explained by the time between slaughter of the animals and the preparation of the membranes. After 2 and 5 hours induction, with the chemical system, in microsomal membranes from turkeys fed rapeseed or tallow as fat source, significant differences between control and supplemented samples were observed.

This result was in agreement with those of Monahan et al. (1994) which obtained significant differences between membranes from control and vitamin E supplemented pigs. When animals were fed soya oil, no significant difference in TBA-RS values was noted between control and supplemented samples (Figure 3). Comparatively to results obtained with muscle homogenates with the same induction system (Figure 1), a greater increase in TBA-RS values was noted after 1 hour induction with microsomal membranes (Figure 3). This increase is particularly important when animals were fed rapeseed oil or tallow.

For protein oxidation, Figure 3 shows no difference between control and supplemented samples at T0. After 2 and 5 hours induction, there were no or few differences in carbonyl content between the control and the supplemented samples; after a 5 hour induction, the carbonyl content was higher than those observed in the muscle homogenates with the same chemical system (Figure 1) particularly when animals were fed rapeseed oil or tallow. This result could be explained by the lipidic environment of the microsomal proteins and the higher TBA-RS values obtained in this system.

In the last experiment (Figure 4) microsomal membranes were oxidized by the same enzymatic system used in muscle homogenates of experiment 2. Lipid and protein oxidation were measured on membranes from control and supplemented (200 ppm) animals. At T0, no significant differences between control and supplemented sample was observed. After a 2 hours induction, all control samples reached a TBA-RS value around 30 nmol/mg of protein and no more evolution of TBA-RS value was noted after a 5 hours induction. Asghar et al. (1990) showed with the same enzymatic system differences in TBA-RS values in microsomes from broilers which are fed with different fat sources. For rapeseed oil and tallow, a significant effect of the vitamin E supplementation was obtained on TBA-RS values.

For protein oxidation, carbonyl contents at T0 were the same in control and supplemented samples. After a 2 hours induction, the protein oxidation in membrane from tallow fed animals were significantly lower than in supplemented animals. In rapeseed and soya fed animals, whatever the incubation time, no differences between control and supplemented samples were shown. In this experiment, done on microsomal membranes, contrary to results obtained in muscle homogenates, the protein oxidation rate was higher than in experiment 2 (about twice).

Conclusion

In this work, we have shown the different evolution of lipid and, more originally, of protein oxidation in different “model systems” and with different oxidative systems. It appeared that dietary fat source in animal breeding can directly influence the oxidative status of meat. Previous study showed that the dietary fat sources could modulate the muscle vitamin E intake (Mercier et al., 1998). Moreover, other works on muscle lipid composition (Gandemer et al., 1995) had shown a significant effect of the dietary fat source on the triglycerides, and to a less extent, on phospholipids fatty acid composition. The similar study done on microsomal and mitochondrial membranes (work in progress) could give more information on the influence of dietary fat source on phospholipids composition. More recent studies done in our laboratory on turkeys or done on rats (Venkatraman et al., 1998), have shown that fatty acid composition in diet can also modulate the antioxidant enzymes activity, and add to understand the oxidative evolution in meat.

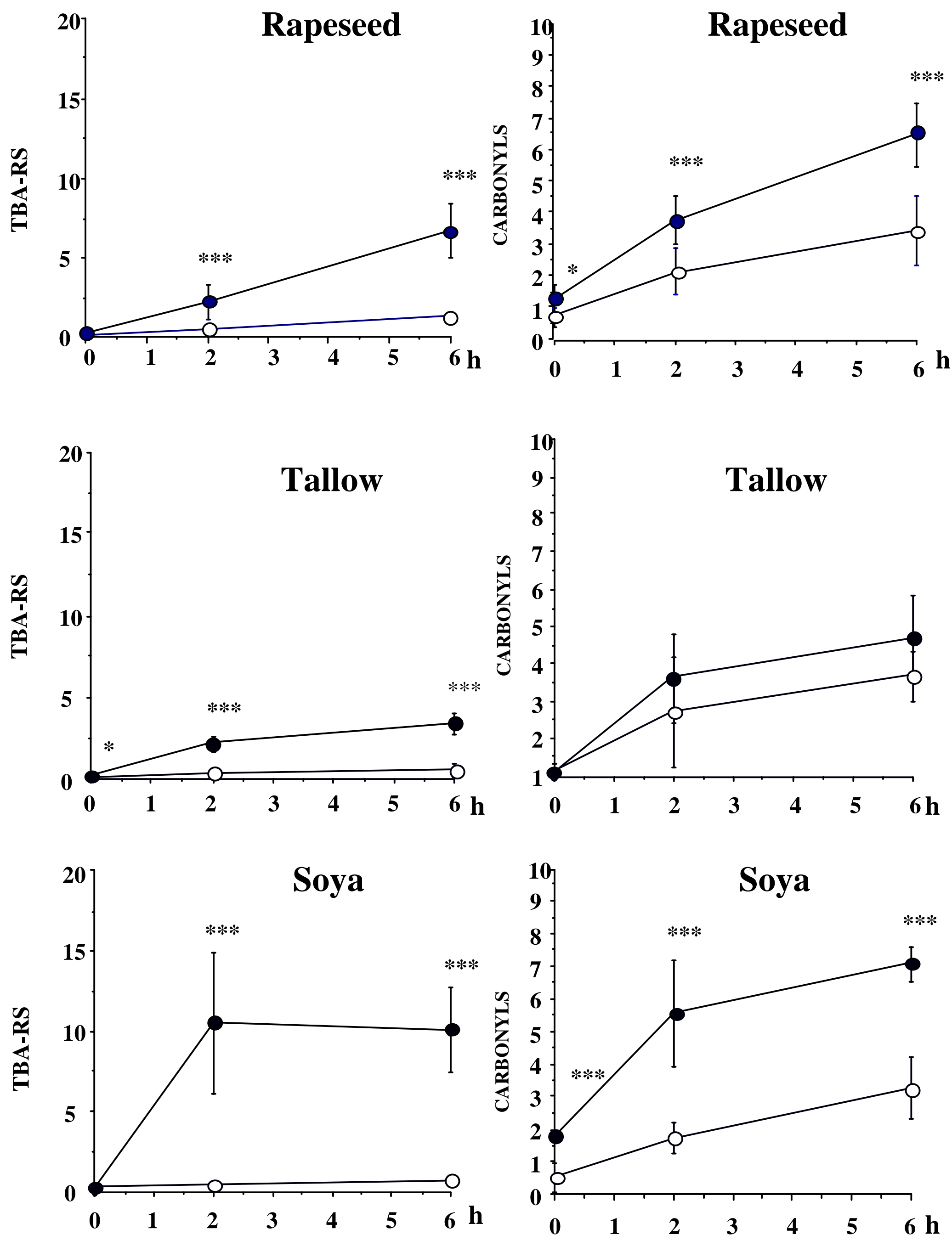

Figure 1: Means TBA-RS (nmol/ml) and carbonyl values (nmol/mg of protein) after 0 ; 2 and 6 hours incubation of muscle homogenate with Fe^{3+} / ascorbate from control and supplemented animals .

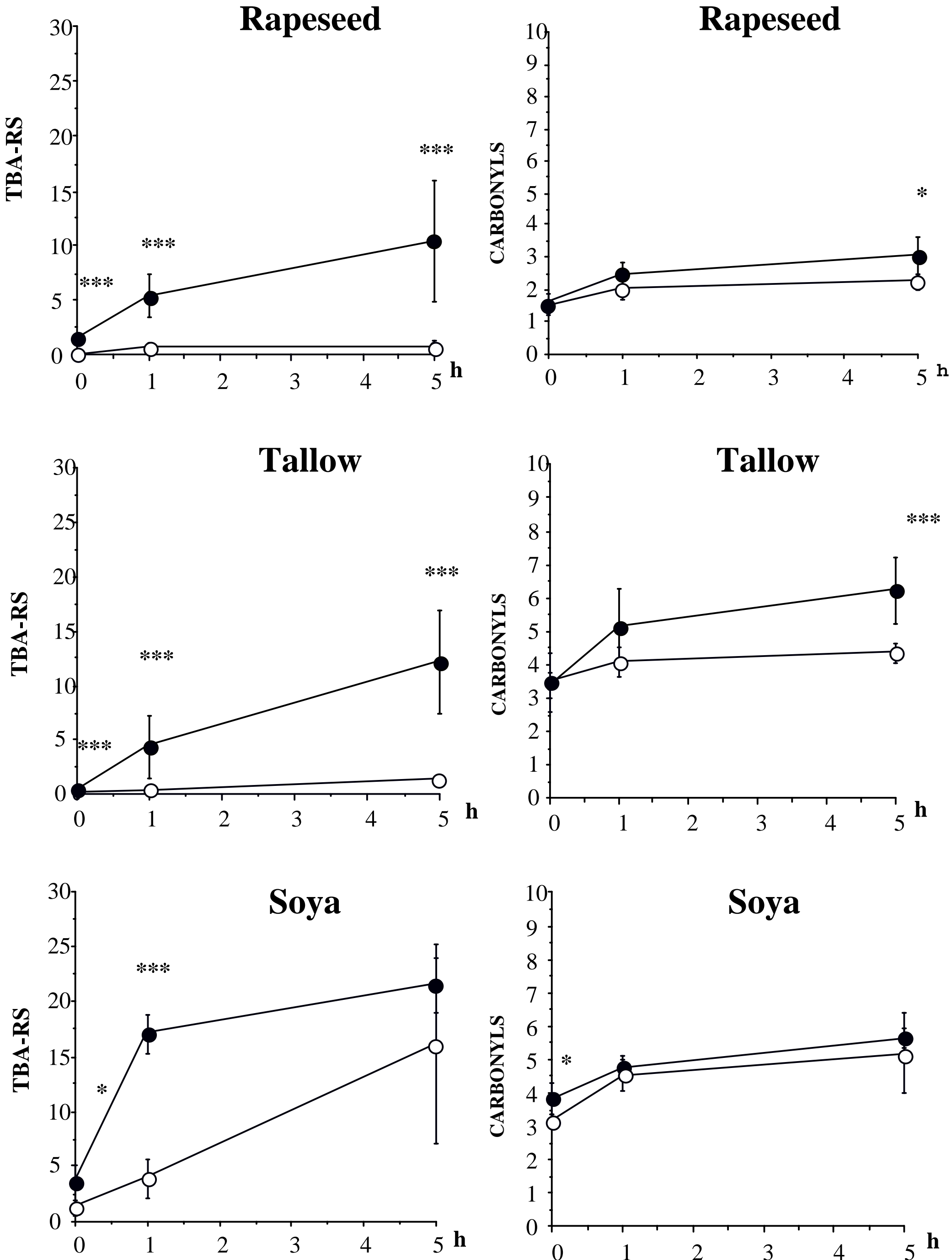

Figure 2: Means TBA-RS (nmol/ml) and carbonyl values (nmol/mg of protein) after 0 ; 1 and 5 hours incubation of muscle homogenate with an enzymatic system from control and supplemented animals .

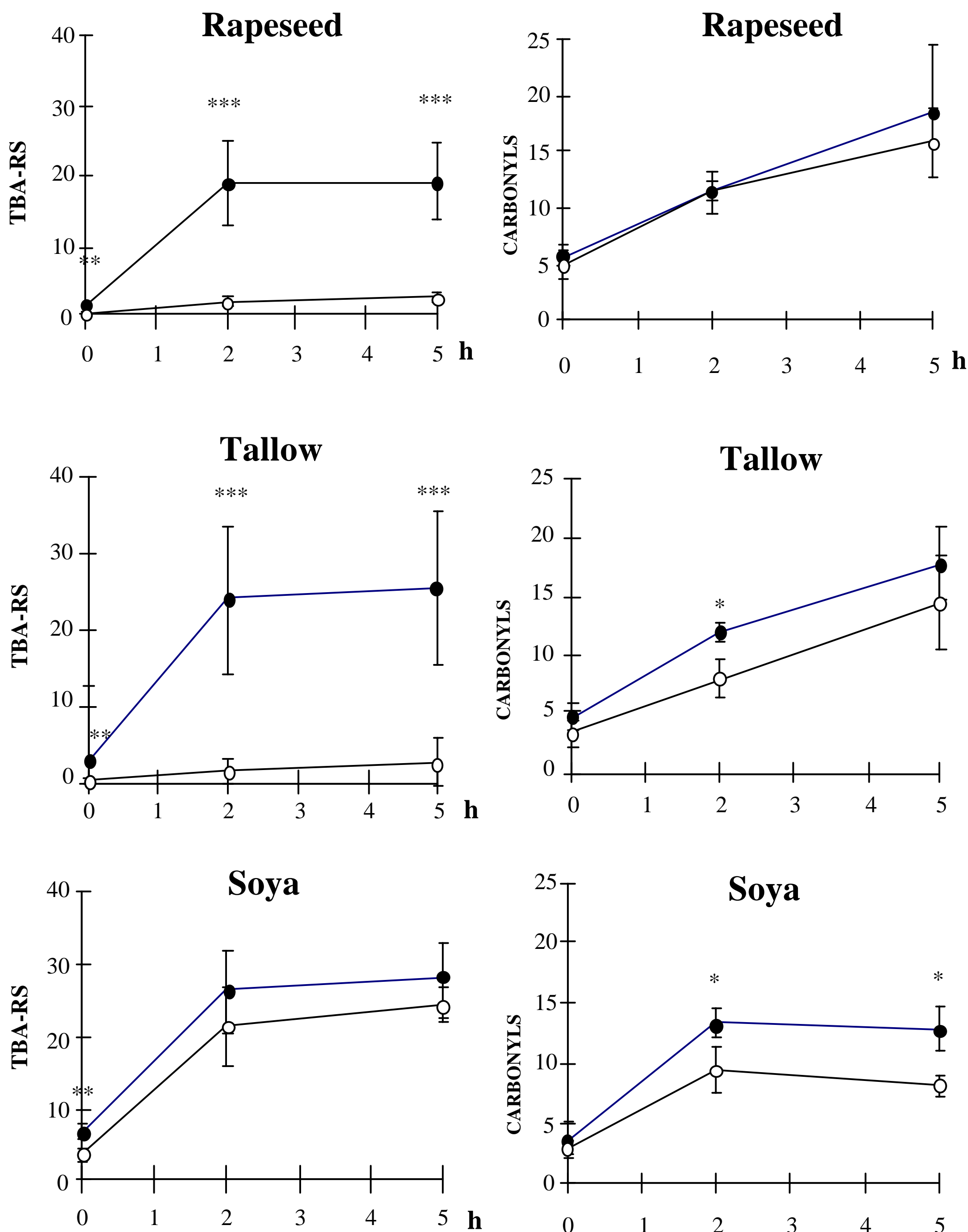

Figure 3: Means TBA-RS (nmol/ml) and carbonyl values (nmol/mg of protein) after 0 ; 2 and 5 hours incubation of microsomal membranes with a Fe^{3+} /ascorbate system from control —●— and supplemented animals —○— .

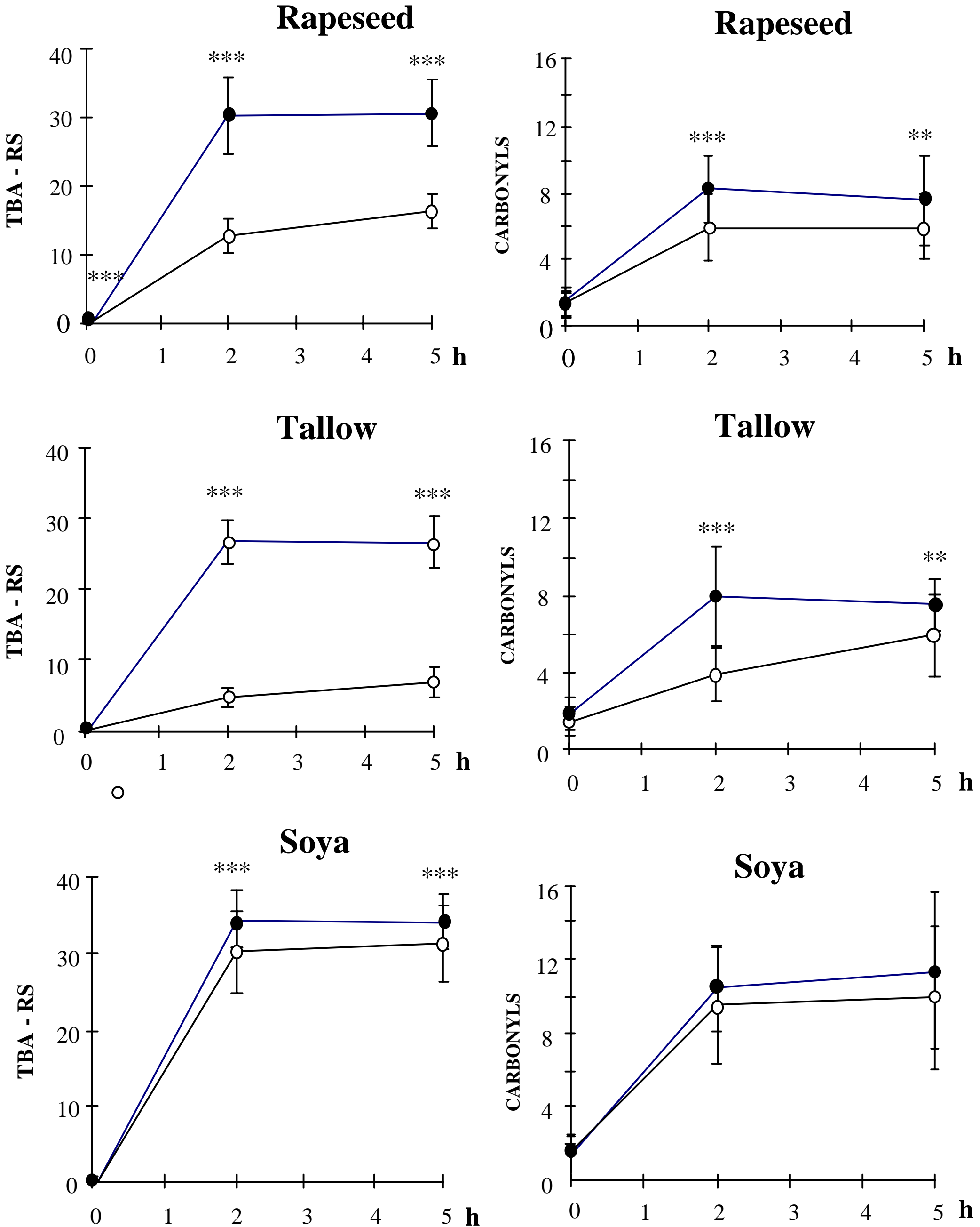

Figure 4: Means TBA-RS (nmol/ml) and carbonyl values (nmol/mg of protein) after 0 ; 2 and 5 hours incubation of microsomal membranes with an enzymatic system from control —●— and supplemented animals —○— .

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Vitamin E and rapeseed oil in feed of pigs - influence on the quality of meat products

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Abstract

Vitamin E, added to feedstuff, has proven to have an antioxidative effect towards fresh pork. Improvements are seen in muscle and adipose tissue. Development of rancidity, drip loss and discoloration are retarded. Rapeseed oil in the diet is known to have an adverse effect to meat quality. The amount of unsaturated fatty acids in tissues is enhanced by rapeseed oil. Consequently fat will become soft and more susceptible to oxidation. Positive and negative effects in fresh pork can be balanced by dietary treatment. Which requirements regarding meat and fat quality are needed for production of raw, cured and smoked meat products? Raw sausages (salami type) and raw hams were manufactured and stored.

Materials and methods

Basis for the meat products were two feeding trials, described in the preceeding paper no. I. 'Influence on Animal Performance and Fresh Pork' by Honikel et al.. Raw sausages were produced as mixed batches of 2 resp. 3 animals of each group. Usual technological procedures were applied, with the exception that only pork (66 resp. 75 % shoulder) with a portion of 33 % belly resp. 25 % backfat was used. After 3 weeks of ripening the sausages had lost about 30 % of weight. The sausages were vacuum packaged and stored at 12 °C. After 12 and 26 weeks of storage the vitamin E-content was determined by a modification of the method of Brubacher et al. (1985). Sensory evaluation was done by a panel of 6 experienced people at the same intervals.

Raw hams were produced from frozen material. After the samples have been thawed at 1-2 °C, 3.5 % of nitrite curing salt (w/w) containing 0.5 % nitrite were added to each ham. Each portion of ham was packed separately in vacuum. The samples were tumbled for 24 hours (effective time of movement: 30 min) and cured for 3 weeks in the vacuum bag at 1-2 °C. After washing the surface, the hams were allowed to dry for 3 weeks at 5 °C and 85 % r.H.. Then the samples were smoked twice for 3 h. After 24 h of drying the hams were packaged separately in vacuum and stored at 12 °C. Ripening of these samples lasted for 9 weeks. Sensory evaluation was done directly after fermentation. Vitamin E-concentration was determined according to Pfalzgraf et al. (1995) after 6 month of frozen storage (-20 °C) under vacuum.

Results and discussion

All sausages and hams manufactured from meat with 6 % rapeseed oil were judged to be faulty products.

The qualitative defects of the sausages showed in formation of wrinkles, by loosing oil through the surface as well as by retarded drying. The products constantly showed a smeared cross section and soft and crumbly consistency. They were oily and pungent in the taste. The control group lost water at a faster rate than the ones with rapeseed oil.

Concerning the sensory evaluation of hams no big differences could be recognised between the supplemented groups. The raw hams were visually and analytically (content of nitrite, nitrate and salt) attractive, but only the control group exhibited the characteristic curing flavour. In each ham made of animals, which maintained 6 % rapeseed oil in the ration, the curing flavour was covered up by the deviating fat condition.

With increasing vitamin E-supplementation in feed there was an increase in vitamin E-concentration in all products. It became obvious that the feeding regime using a higher portion of unsaturated fatty acids (6 % rapeseed oil), despite an increased vitamin E-concentration, had a strong negative impact on raw and cured meat products.

After the feeding regime was changed to 2 % rapeseed oil, improvements were noticed in fat quality. Due to the lower level of unsaturates in the fat, its consistency was obviously improved. Fat did not smear during the production of sausage. Weight loss was accelerated and improved the fermentation of raw sausages. Neither vitamin E nor rapeseed oil did influence the weight loss of raw sausages during fermentation. These findings also corresponded to the a_w-level and the pH-level.

Vitamin E-levels in raw sausages during fermentation and storage corresponded very well to the levels supplemented in feed (fig. 1). Vitamin E-levels were found to increase when rapeseed oil and vitamin E was added to feed. The decrease of vitamin E-levels during storage was lower in the groups with additional rapeseed oil. Sensory evaluation of the sausages clearly indicated quality losses by addition of rapeseed oil to feed (fig. 2). Deductions were made for lacks in appearance, colour, consistency and taste. Faults were weighed differently: appearance (x1), colour (x1), consistency (x3), taste (x5) and other faults (x1). Enhanced vitamin E-concentrations (feed with 200 ppm) in the product could lower the quality defects to the level of the control group.

Vitamin E-levels in raw hams, both in muscle and adipose tissue, rose with increasing addition of vitamin E to feed (fig. 3). Supplementation with rapeseed oil enhanced the deposition of vitamin E in both types of tissue. Sensory evaluation was performed the same way as in raw sausages with equal weighing of faults (fig. 4). The addition of rapeseed oil to feed caused losses in sensory quality of raw hams. Increased vitamin E-concentrations in the product could lessen the faults to some extent, but could not make them up.

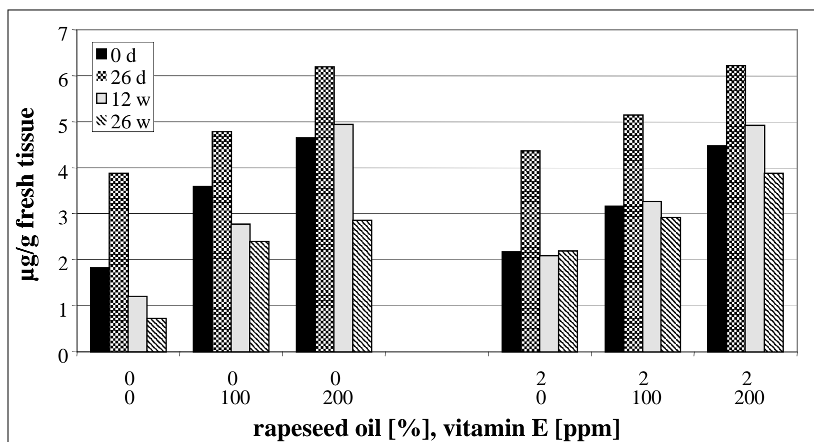

fig. 1: Vitamin E-level in raw sausages during fermentation and storage (mixed samples of 2 pigs)

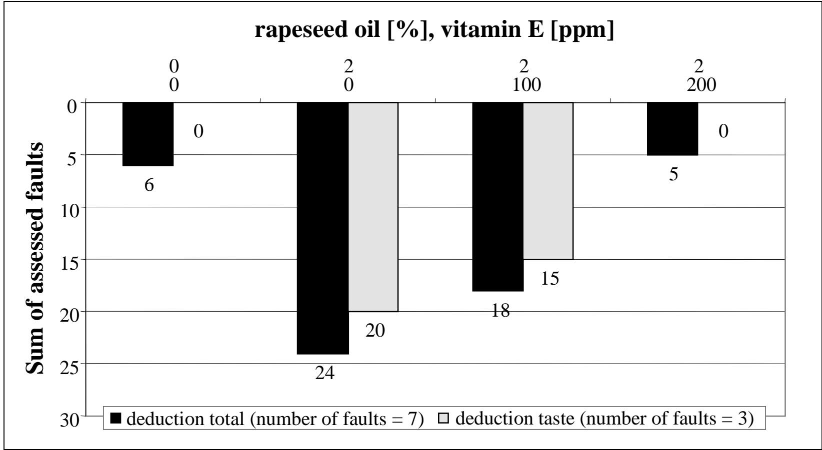

fig. 2: Sensory evaluation of raw sausages after 3 weeks of fermentation and 16 weeks of storage

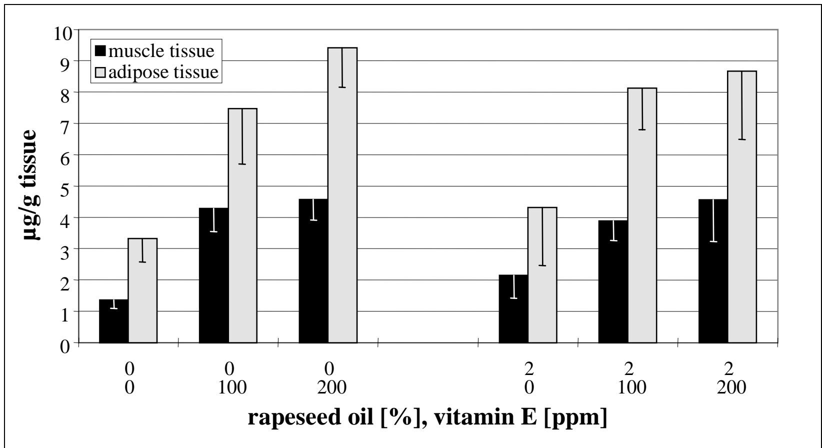

fig. 3: Vitamin E-level in raw hams after 9 weeks of fermentation (6 month frozen prior to analysis, n=5, mean - SD)

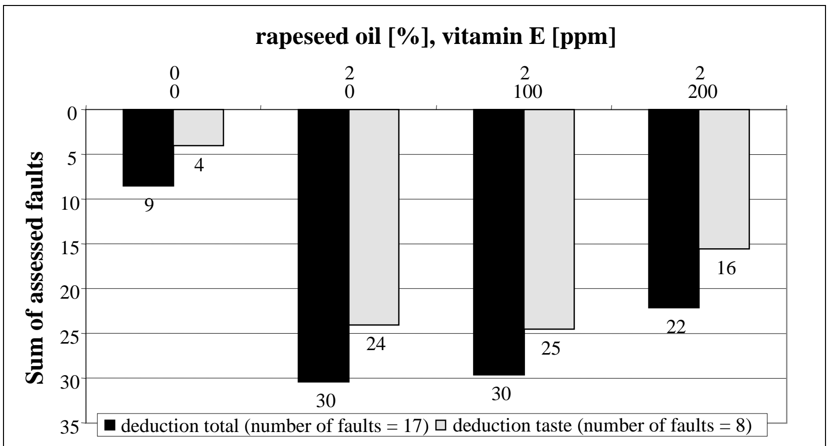

fig. 4: Sensory evaluation of raw hams after 9 weeks of fermentation (n=5)

Conclusions

The addition of 6 % rapeseed oil to feed caused severe quality deteriorations both in raw fat and muscle and in processed meat. This negative treatment effect could be lessened to some extent by increased levels of vitamin E in feed. Even high vitamin E-doses could not overcome the problems accompanied by the addition of 6 % rapeseed oil to feed. All of the sausages and hams manufactured from meat with 6 % rapeseed oil were judged to be faulty products.

Supplementation of feed with 2 % rapeseed oil resulted in improved fat quality. Neither vitamin E nor rapeseed oil showed treatment effects regarding the fermentation of raw sausages and hams. Enhancement of vitamin E in the diet caused increased vitamin E-concentrations in raw sausages and hams. Additional rapeseed oil increased vitamin E-levels in products, too.

Even 2 % rapeseed oil in the diet caused quality losses in the products evaluated by a sensory panel. Increased vitamin E-concentrations in the products could balance the negative treatment effects by rapeseed oil to some extent.

Feed supplemented with rapeseed oil (2 resp. 6 %) caused quality lacks in raw and cured meat products. Dietary vitamin E improved the quality, but could not completely make up the losses caused by rapeseed oil.

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Formation of cholesterol oxides in meat – in dependence of preparation, treatment and storage

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Summary

Studies on hazardous cholesterol oxides with a newly developed method showed, that raw, lean pork and beef samples yielded 7-Ketocholesterol up to a concentration of 150 µg/kg, while the content of other cholesterol oxides (7α-Diol, 7β-Diol, α-Epoxid, β-Epoxid, Triol, 25-Diol) was well below 100 µg/kg. 20α-Diol was not detectable. Fresh cooked or fried lean pork or beef contained in most cases rarely increased levels. After one week of storage (8°C) significant changes were observed. Whereas the CO-contents in raw pork changed only slightly, they increased in the fried samples with 7β-Diol by about a factor of 30. Also 7α-Diol, α-Epoxid, β-Epoxid and 7-Keto increased noticeably. The largest changes were observed after refrigerated storage of cooked meat. After seven days the concentration of 7β-Diol in pork raised by about a factor of 100. Considerable increases were also noticed with all other cholesterol oxides. After a normal household refrigerator storage of one or two days a considerable increase was found.

Introduction

Several cholesterol oxides (CO) are attributed to hazardous biological effects. They could act cytotoxic [1], angiotoxic [2], cancerogenic [3] and mutagenic [1]. A connection is supposed between the absorption of CO with the food and cardio vascular disorders [4]. It was proofed in animal tests that CO could initiate atherosclerotic lessions of blood-vessels [5]. There is evidence, that CO act much more effective in this manner than cholesterol itself[6].

Materials and methods

Pork and beef samples were trimmed of all visible fat. Raw and freshly cooked and fried samples were respectively analysed. Moreover, the heated samples were stored at 8°C in the refrigerator for 1, 2 and 7 days with access of oxygen. The developed method includes cold extraction, transesterfication, enrichment, derivatisation, gaschromatographic separation and mass-spectrometric detection of the CO.

Results and discussion

Influence of cooking and frying

The CO-concentrations in raw meat were surprisingly not distinguishable from that of the freshly cooked and fried samples – in spite of the loss in weight due to heating.

Influence of refrigerated storage

There was no essential influence on the CO-concentrations during refrigerator storage of the **raw** pork chops.

After one week of refrigerator storage of the **fried** meat the CO-content had increased strongly. The concentration of 7β-Diol reached 1100 µg/kg and was enhanced by about a factor of 30 after seven days of storage compared to the values immediately after frying. 7α-Diol, 7-Keto, β-Epoxid and α-Epoxid also showed considerable increased concentrations. Those oxidised at position seven exhibited the greatest increase. Only small increases were found at Triol and the two side-chain oxides 20α-Diol and 25-Diol, 20α-Diol was only found at the seventh day of storage.

After **cooking** the strongest influence of refrigerated storage was noticed – in comparison with the fried or raw meat. This was found with all CO´s. 7β-Diol reached a concentration of 3700 µg/kg after seven days which is an increase of about a factor of 100 against nonstored cooked meat. 20α-Diol was already found at the second day of storage and also 25-Diol clearly increased. Interesting is also the fact, that not only after seven days, but already after a normal household refrigerator storage of one or two days considerably CO-increases were noticed and the 1 mg/kg level was passed already after one day of storage.

Conclusions

The results raises a number of questions: Which CO-concentrations exist in prepared and chilled stored meat? What does happen in ready to eat chilled meals which rejoice an increasing popularity? Which influence could ingredients have in convenience food have, especially spices with antioxidative behaviour? Meat dishes were not analysed yet under these conditions. Also the CO-contents in preserves of meat and meat products are interesting, which could be stored at ambient temperatures for long times. The health critical CO require further research.

Acknowledgment

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Investigations about the suitability of different plant and milk proteins for manufacturing cooked sausage

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Meat products in Germany contains up to the changing of the meat law on 15th December 1995 as proteincontent nearly exclusive muscleproteins. There is the question for the domestic industry, how shall they react to the legal changings in the competition. Besides meat protein it is allowed to use other proteins like milk, bloodplasma, egg proteins and plant proteins for manufacturing meat products. "Plant protein" stands often lumpsumly for "soya protein" or "protein containing soya products".

Out of regional and agricultural policy reasons it is to be favourite to use protein products from local culture plants. A plant with a high proteine content that was tested in Bavaria during cultivation tests was the sweet lupine. In the up to now carried out research and development in the "Fraunhofer Institut for Food-Technology and Packaging" (FhLV) it was possible to manufacture protein concentrates and isolates from sweet lupine. But there was no optimising for using as additive for meat products. For the common project between FhLV and BAFF in FhLV manufactured lupine protein preparations was tested in comparisation with other milk and plant proteins of their suitability for production of cooked sausage.

These investigations was made with a basic formulation for cooked sausage, by those 7,5 % lean meat was exchanged for 2 % protein powder and 5,5 % water. Also was included other milk and plant protein they was available in this time, to enhance the strength of the experiments, there the lupine isolates in developing complete with this preparations in the market.

Nearly all of the investigated proteins cause a reduction of gelly separation in comparison with the control batch in fully preserved canned sausages. Therefore the soya isolates leads to the best results in referring to the gelly separation. But there are differences between several preparations - also by one producer. By using this proteins the firmness values of the control batches was partly lower. A part of the batches shows - in dependence of the protein preparation - similar or slight higher firmness values as the control batches. Depend on the protein preparations the results of the colour brightness (L*) was -1,4 to +1,6 in relationship to the control batches, but mostly in the positive area. The red part (a*) of the batches with protein isolates was lower for 0,8 - 2,1. The yellow part (b*) of the batches with protein isolates varied between -0,3 to +0,6 in comparison to the control batches. Altogether the control batches were best judged in sensory evaluations. In odour and flavour the milk protein had the highest acceptance of the used foreign proteins. The evaluated soya, pea and one wheat protein as well as the isolated and roller dried lupine protein were evaluated as relative neutral in flavour. These samples distinguish from the control batch on the whole in a reduced meat flavour. One wheat protein compare badly with the control batch in taste (bitter, burning). Optical all samples including protein isolates - depend on the protein isolat preparation - look more or less light-coloured.

From the evaluated sweet lupine preparations the isolated and the roller dried lupine protein come into question, because they are quite comparable with the other protein preparations used in these evaluation in waterbinding (gelly separation), consistency and tastelessness. Something less was

judged the yellow colour of the lupine protein, they influenced the colour of the cooked sausage measurable and visible. Furthermore in comparisation with for decades tested and optimized soya isolates there is any possibility to improve the waterbinding, structure and consistency forming properties. The exchange up to 7,5 % lean meat against 2 % lupine protein isolate and 5,5 % water appears possible in cooked sausage recipes and a specific spicemix, because the consumer normally has not the direct comparisation with the sausage manufactured only of meat.

Suitability of spray-dried soy sauce for the production of cooked sausages

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Abstract

Within the scope of a project with industrial contribution, the Institute of Technology at the BAFF in Kulmbach studied the effects of the addition of spray dried soy sauce in cooked sausages. New ways of versatility of such products and the potential of substituting the number one flavour enhancer glutamate had been analyzed in numerous trials. Beside extensive physical and chemical analyses according to §35 LMBG special attention was directed to examine the products colour composition and taste. The interpretation of these trials showed, that the best results concerning sensory evaluation and long-time storage where achieved with cooked sausages manufactured with 0.2 to 0.4% spray dried soy sauce.

Introduction

Many sausages are produced using flavour enhancer (glutamate, relish) to give the products a more finished taste. Also soy sauce is used as flavour enhancer. It is made by a fermentation process from soy beans and wheat and aquires a strong pleasant flavour and a dark red-brown colour. Because of the complex situation concerning foodstuffs and its artificial or chemical additives many consumers get more critical of those products containing preservatives and/or flavour enhancer. In contrast to the label "including flavour enhancer glutamate“ the note "including soy sauce“ would probably have a more positive effect on peoples attitude. In this connection only the soy sauce and not its components must be mentioned on the label, even if the same active substance (glutamic acid) is included. With the employment of spray-dried soy sauce new foodstuffs could be developed that stand out of the uniformity of the glutamate taste.

Materials and methods

Two different types of spray-dried soy sauce were tested in this trial. Product A made from soy beans and wheat, fixed to salt and maltodextrin as a carrier, is an extremely hygroscopic light brownish powder with a mild carneous flavour (NaCl: 36,5 ± 1,5% w/w, H_2O: <2,0% w/w; N_2: >3,1% w/w; $pH_{2,5\%}$: 5,15 ± 0,25). Product B is hydrolyzed only from wheat protein and is almost flavourless (NaCl: 32,0 ± 2,0% w/w, H_2O: <3,0% w/w; N_2: >4,75% w/w; $pH_{2,5\%}$: 5,2 ± 0,25). Concerning the microbiological status of the products, both powders are unobjectionable. With a total count of less then 10,000 germs per gram no coliform organism or salmonella could be found.

To consider as many different products as possible being found on the world market in this study, differing types of bologna-type sausages with variable adding of spray-dried soy sauce from 0.0% (control) to 0.6% sauce powder were produced and analyzed. Also sausages with commercial amounts of glutamate had been manufactured as a standard of comparison. Cured („Lyoner“: 25% beef; 25% porc; 25% fat; 25% ice) and non cured meat products („Gelbwurst“: 50% beef; 25% fat; 25% ice) were produced using conventional technologies and usual addings of phosphate. For the spicing only natural condiments were used.

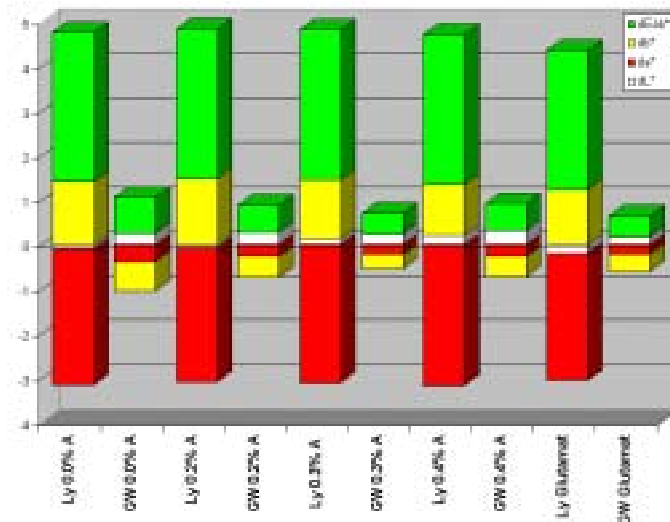

Figure 1: Measurement of the colour stability of Bologna-type sausages with product A (dL*=degree of brightness; da*=red portion; db*=yellow portion; dEab*=colour distance; Ly="Lyoner"; GW="Gelbwurst").

Results

None of the spray-dried soy sauces showed significant affects on the course of temperature during the cuttering process, the pH-value of the stuffing and the finished product, the separation of fat or jelly, the chemical composition or the firmness of the sausages. Also the colour and its stability was not influenced by either of the products (see figure 1).

The sensory evaluation of the cooked sausages with product A showed that most of the test persons accepted an addition of 0.2 to 0.4% spray-dried soy sauce type A (figure 2).

Despite a still perceptible flavour of the soy sauce, the examiners often particularly preferred the non cured meat products when soy sauce was added.

Sausages manufactured with product B as a flavour enhancer were compared to commercial glutamate bologna in three point pairing tests. As shown in figure 3, most test persons could not find a distinction between the use of soy sauce and glutamate. Those who found out the difference ranked both products equally. After 6 weeks of storage all products were tested again. As a result it was found that those sausages made with soy sauce still carried a typical finished taste, while glutamate products showed a marked lack of flavour.

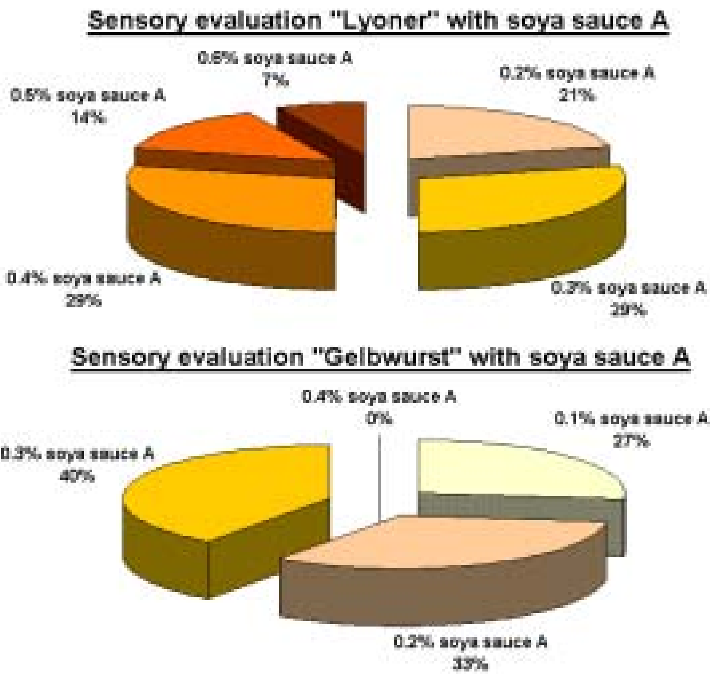

Figure 2: Sensory evaluation of bologna-type sausages („Lyoner“ and „Gelbwurst“) with Product A

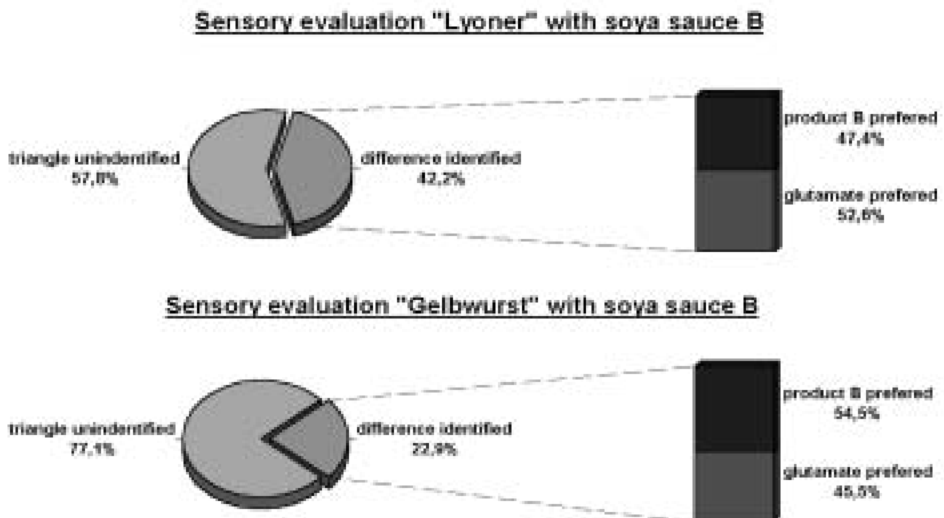

Figure 3: Sensory evaluation of bologna-type sausages („Lyoner“ and „Gelbwurst“) with Product B

Conclusion

- None of the soy sauces used in this research showed any negative chemical or physical effects on the finished products.
- Even having a typical finished flavour, cooked sausages made with soy sauce A (soy beans and wheat) could easily be distinguished from glutamate sausages.
- Most test persons could not make a distinction between soy sauce and glutamate rank when sauce B was used (only wheat protein).
- The adding of spray dried soy sauce helps cooked sausages to hold their finished flavour over a longer period of time.

Because of the soy product´s comparatively high pH-value it is assumed that an increase of soy sauce added to meat products far above the limit used in the documented trails will cause negative chemical and physical alterations in the finished cooked sausages.

Biopreservation of vacuum-packaged sliced Bologna-type sausage by Lactobacilli

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Summary

Sliced Bologna-type sausage was inoculated with a pool of different serovars of *Listeria monocytogenes* at a level of 10^3 /g and, in addition, with 10^7 /g of various lactic acid bacteria (LAB) as protective cultures. Three bacteriocinogenic strains of *Lactobacillus sakei*, strains Lb706 (sakacin A +) and strain Lb674 (sakacin P +), and *Lactobacillus curvatus*, strain Lb1071 (curvacin 1071 +), respectively, and a commercial, non-bacteriocinogenic strain, available as 'FloraCarn L-2', were investigated. The bacteriocins produced by the first three strains belong to the "pediocin family" and share an N-terminal -YGNGV- motif. The inoculated sausage slices were vacuum-packaged and stored at 7°C up to 28 days. In the absence of LAB, listerial numbers of 10^7 - 10^8 /g were reached after 14 - 21 days of storage, while listerial growth was inhibited in the presence of each of the LAB strains. Best inhibition was observed with the bacteriocinogenic LAB strains, all of which were able to reduce the initial inoculum of *L. monocytogenes*. Strongest anti-listerial activity was seen with *L. curvatus* Lb1071. In contrast, the non-bacteriocinogenic commercial strain allowed listerial growth by two decades within 14 - 21 days.

Introduction

Vacuum-packaged, sliced ready-to-eat meat products with relatively high pH and water activity (pH 6.2, a_w 0.98), where the natural microflora has been inactivated during pasteurization, may represent the main potential field for the application of protective lactic acid bacteria (LAB) in meats. Recontamination during further processing of Bologna-type sausage, cooked ham and pâté may threaten consumer health and product quality. At the same time LAB are most difficult to apply to this products in high numbers without accepting a certain drop in pH. This may however not necessarily constitute a problem, and product quality may actually benefit from the application of an appropriate LAB culture during prolonged storage if compared to uninoculated sausages (*1, 2*).

When using lactic acid bacteria (LAB) as protective cultures in the biopreservation of cooked meat products, the inoculation level is very important. We found that in vacuum-packaged, sliced Bologna-type sausage stored at 7°C, low initial numbers of *L. sakei* were not sufficiently active to suppress the growth of *Listeria monocytogenes* (L.m.), even if a potent anti-listerial bacteriocin was included in the recipe. Good inhibition of L.m. - but no reduction - was found when 10^5-10^6 cfu/g of bacteriocinogenic *Lb. sakei* were applied, while a pool of 20 random LAB isolates from this type of meat product gave no protection under these conditions (*1, 5*). In comparison, FloraCarn L-2, a bacteriocin-negative commercial culture, was reported to show a satisfying effect at 5°C when inoculated at a level of at least 10^7 cfu/g (*2*).

The aim of this study was to compare the performance against listeria of some bacteriocinogenic LAB strains and of FloraCarn L-2, a commercial protective culture for meats, on vacuum-packaged, sliced Bologna-type sausage at a storage temperature of 7°C, which is the legal maximum temperature for chilled meats.

Methods

Bologna-type sausage was purchased from a local producer and sliced under semi-sterile conditions. The procedure of *Anderson (1995)* was followed for inoculation and vacuum-packaging of the slices. Briefly, sliced Bologna-type sausage was inoculated with a pool of four different serovars of *Listeria monocytogenes* (serovars 1/2c, 4b, 1/2a, 1/2b) at a level of 10^3 /g and, in addition, with 10^7 /g of various LAB as protective cultures. Three bacteriocinogenic strains of *Lactobacillus sakei*, strains Lb706 (sakacin A +) and Lb674 (sakacin P +), and *Lactobacillus curvatus* strain Lb1071 (curvacin 1071 +), respectively, and a commercial, non-bacteriocinogenic culture, FloraCarn L-2, were investigated. The bacteriocins produced by the first three strains belong to the "pediocin family" and share an N-terminal -YGNGV- motif (*3, 4*). The inoculated sausage slices were vacuum-packaged and stored at 7°C up to 42 days.

Results

In the absence of LAB, listerial numbers of 10^7 - 10^8 /g were reached after 14 - 21 days of storage, while listerial growth was inhibited in the presence of each of the LAB strains (Fig. 1). Best inhibition was observed with the bacteriocinogenic LAB strains, all of which were able to reduce the initial inoculum of *L. monocytogenes*. Strongest anti-listerial activity was seen with *L. curvatus* Lb1071. In contrast, the non-bacteriocinogenic commercial strain allowed listerial growth by two decades within 14 - 21 days.

This experiment confirms that 'FloraCarn L-2' at high inoculation levels can prevent excessive growth of L.m. in vacuum-packaged, sliced Bologna-type sausage. However, it also shows that bacteriocinogenic strains of *L. sakei* and *L. curvatus* perform much better under such conditions.

Where LAB had been added, the pH decreased from pH 6.5 to values between pH 5.5 - 6.0. For strains Lb674 and Lb1071 pH 5.5 was reached already after 14 days, while Lb706 and FloraCarn L-2 gave a pH drop to pH 5.6 and pH 5.7 at this time, respectively.

For sensoric purpose all trials were run also in the absence of listeria. Inspite of the high initial LAB inoculum there were no deviations in smell, taste and appearance after 2 weeks of storage with any of the cultures when compared to the uninoculated control. After 4 weeks, for Lb674, Lb706 and Lb1071 smell was still fresh, appearance good, taste was only slightly more acidic, and the chewing resistance was somewhat less than with the uninoculated control. FloraCarn L-2 and the uninoculated control gave an acidic smell, and an unpleasant taste, which was "rough/pungent, cheesy and old" for the uninoculated control, and "rough/acidic" with an unpleasant aftertaste for FloraCarn L-2. After 6 weeks only samples containing Lb674 and Lb1071 were acceptable, but had a rough/acidic note by then.

Brochothrix thermosphacta was less than 10^6 - 10^7 /g or absent in the presence of LAB, but 10^7 - 10^8 /g in the absence of LAB after 3 - 4 weeks of storage.

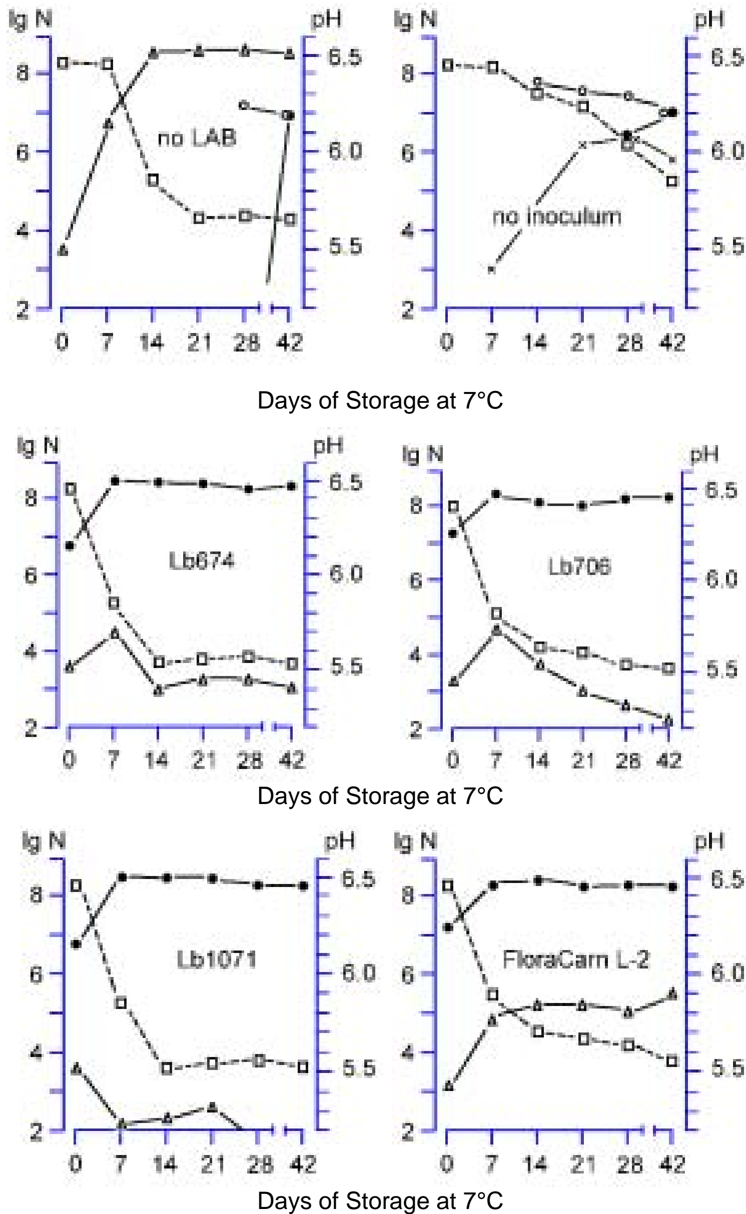

Fig. 1: Control of *Listeria monocytogenes* in vacuum-packaged sliced Bologna-type sausage by bacteriocinogenic and non-bacteriocinogenic LAB.

Legend to Fig. 1: lg N, $\log_{10}$ of colony forming units per gram sausage; triangles, *Listeria monocytogenes*; full circles, protective culture (LAB); squares, pH; open circles, *Brochothrix thermosphacta*; crosses, pseudomonads.

Bacteriocinogenic strains: *Lactobacillus sakei* strains Lb674 and Lb706; Lactobacillus curvatus strain Lb1071.

Non-bacteriocinogenic strain: FloraCarn L-2.

no LAB: no protective culture added.

no inoculum: no LAB or listeria added.

Conclusions

At inoculation levels of 10^7 LAB/g, the growth of *Listeria monocytogenes* on vacuum-packaged sliced Bologna-type sausage stored at 7°C is completely prevented by the class II-bacteriocin producers *Lactobacillus sakei* strains Lb674 and Lb706, and *Lactobacillus curvatus* Lb1071. The commercial protective culture FloraCarn L-2 efficiently prevents excessive listerial growth but still allows a significant increase of listerial numbers. Since listerial counts even decreased in the presence of the bacteriocinogenic strains, such cultures are clearly preferable over non-bacteriocinogenic ones. All LAB strains used in this study did not deteriorate the taste of the sausages by unacceptable souring.

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Review of meat chilling

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Summary

Chilling is the most important stage in the total chill chain for meat production and distribution. To optimise conventional single stage air chilling operations design data are required on the relationship between the environmental variables air temperature, velocity and relative humidity and processing variables. The most important processing variables are chilling time, weight loss and product loads. This paper outlines the data that is available for the chilling of beef sides and pork and lamb carcasses. It also illustrates how carcass weight and fatness have a substantial influence on the relationships.

In general the rate of chilling that can be achieved in single stage air chilling systems is slower than that desired by commercial companies and weight loss higher. A number of alternative chilling systems are therefore described that have the ability to reduce chilling times or weight loss or both. Ultra-rapid and immersion chilling can achieve substantial reductions in both chilling time and weight loss. However, there are potential economic and meat quality problems in their commercial use. High humidity and spray chilling systems can produce substantial reductions in weight loss and are already in commercial use.

Introduction

The increased application of temperature legislation in many countries, coupled with economic requirements to maximise throughput, minimise weight loss and operate refrigeration systems in the most efficient manner, has created a very large demand for process design data on all aspects of carcass chilling. Concurrently, there has been a growing realisation of the importance of chilling rate on meat saleability, in terms of drip potential (1, 2, 3), appearance (4) and eating quality, particularly texture (5,6,7)

European Union (EU) temperature legislation governs the chilling of beef, pork and lamb in the majority of abattoirs within the Union. The only derogations are for very small abattoirs and for retail shops cutting meat for direct sale to the final consumer. The EU legislation does not define a chilling time, only a maximum final meat temperature of 7°C before transport or cutting. As discussed by Drumm et al. (8) there are many problems in actually locating the slowest cooling point in a meat carcass. The determination of surface temperature is equally problematic although some success has been achieved using non-contact infrared thermometry (9).

Abattoir management and refrigeration contractors require reliable design data, relating processing variables to chilling time and weight loss, so that they can specify and design carcass cooling systems to meet differing requirements. To fully optimise such systems knowledge is also required of the product heat load, and its variation with time, so that the refrigeration machinery can be sized to achieve the required throughput.

It is also important that the industry is made aware of a growing number of alternatives to conventional batch air chilling systems. Many of the alternative systems offer significant advantages in terms of increased throughput, lower costs and increased product quality.

A number of major investigations to provide data on carcass chilling have been carried out. This paper summarises and updates information contained in the reviews of beef (10), pork (11) and lamb chilling (12).

Conventional chilling systems

Despite the general absence of specific regulations for chilling time, the time required to cool a carcass or side to a specified maximum temperature is the most important commercial factor determining the cost and operation of a cooling system.

Many abattoirs would like to chill beef sides within 18 h, which is the time available in one day, making allowance for loading, unloading and cleaning. However, using conventional single stage chilling regimes it is evident that only relatively light weight (<105 kg), lean beef sides can be cooled to 7°C in the deep leg during a 24 h operating cycle. Heavier sides will probably remain in chill for a further 24 h. Consequently, chilling facilities have to be twice as large, with considerably increased capital investment and running costs. Some investigations on the continuous chilling of beef (8, 13) have been carried out but such systems are not widely used.

Conventional pork chilling systems aim to reduce the mean temperature of the side or carcass to approximately 4°C, a temperature considered suitable for cutting or curing. Most producers despatch, cut or commence further processing of the chilled carcass on the day after slaughter, allowing a period of 14 to 16 h for the chilling operation.

Lamb carcasses are much smaller than pork carcasses and beef sides, so rapid cooling rates can be achieved. However, it has been known for many years that reducing the temperature of the muscles in either beef or lamb to below 10°C within 10 h post mortem is likely to increase the toughness of the meat when cooked due to a phenomenon called ‘cold shortening’. Many experimental investigations have been carried out to determine the extent of toughening under different cooling conditions and ways of alleviating the condition, by either a delay period, or electrical stimulation. In commercial operations a chilling system needs to be designed and operated to chill efficiently while maintaining meat quality. Some abattoirs would like to dispatch lamb on the day of slaughter and to meet this requirement chilling has to be completed in 8 to10 h. For others overnight chilling in 14 to 16 h is normally desired.

There is little published data on the chilling of offal. Stiffler et al., (14) and Vanderzant et al., (15) investigated the effect of five chilling treatments on weight loss and bacterial and sensory changes after storage and transportation. The five treatments used:

- air at 2°C for 24 h;
- air at 2°C for 4 to 6 h;
- air at -20°C for 2 h;
- air at -20°C for 0.5 to 1 h;
- slush ice for 2 h.

Significant differences in weight loss between treatments were measured after chilling with the faster treatments using air at -20°C, or immersion, tending to produce the lowest losses. After storage and transportation there was usually no significant difference in weight losses between treatments or with the non pre-chilled control. Bacterial counts after transport were usually lower on samples that had been pre-chilled before packaging. However, off-odour scores of non pre-chilled vacuum packed samples of beef livers, pork tongues, lamb livers and lamb tongues were lower than comparable samples that had received an initial chilling treatment.

Increasing attention is now being paid to the reduction of energy consumption, but it has been shown that in commercial chilling operations the cost of evaporative weight loss from beef sides (16) are at least an order of magnitude higher than the energy costs.

Effect of environmental and carcass variables on cooling rate and weight loss

Air temperature, air velocity and relative humidity, are the environmental factors that affect the cooling time and weight loss from sides and carcasses. Cooling rate will also be a function of the weight and fat cover of a given body.

Air temperature is the prime factor controlling cooling rate. For example in beef sides, cooling in air at a constant 4°C, compared with 0°C, will at 3 ms^{-1} increase the time to reach 7°C in the deep leg of a 100 kg side from 20.3 to 27.7 h (36% increase) and at 0.5 ms^{-1} that of a 220 kg side, from 45.9 to 68.3 h (49% increase). In systems designed to produce fully chilled sides, with average meat temperatures of 2 to 4°C, the requirement for low air temperatures becomes even more important because of the small temperature difference between the surface of the meat and the air at the end of the process.

Increasing the air velocity during chilling produces a substantial reduction in chilling times at low air velocity but similar increases at higher velocities have a much smaller effect (Table 1).

Table 1: Chilling time in h to a deep bone temperature of 10°C in beef sides of 105 and 140 kg in air at 0°C, 95% relative humidity at air velocities from 0.5 to 3.0 ms^{-1}.

	Air velocity (ms^{-1})				
Side weight (kg)	0.5	0.75	1.0	2.0	3.0
105	19.5	18.5	18.0	16.0	14.8
140	24.1	22.8	21.8	19.7	18.5
140	27.2	-	25.0	22.1	20.0

The power required by the fans to move the air increases with the cube of the velocity. A four-fold increase in air velocity from 0.5 to 2 ms^{-1} results in a 4 to 7 h reduction in chilling time for a 140 kg side weight, but requires a sixty-four-fold increase in fan power. Further increasing air velocity to 3 m/s only achieves an extra 6 to 8% reduction in chilling time. In most practical situations it is doubtful whether an air velocity greater than 1 ms^{-1} can be justified.

With beef sides. the effect of air temperature on weight loss is small, a reduction in air temperature from 4 to 0°C produces a change of <0.1%. Relative humidity (RH) has a greater effect on weight loss than either air temperature or velocity (Figure 1). Reducing relative humidity from 95 to 80% RH increased evaporative weight loss over an 18 h chilling cycle at 0°C by nearly 0.5%. Increasing yield by 0.5% can double the profitability of an abattoir.

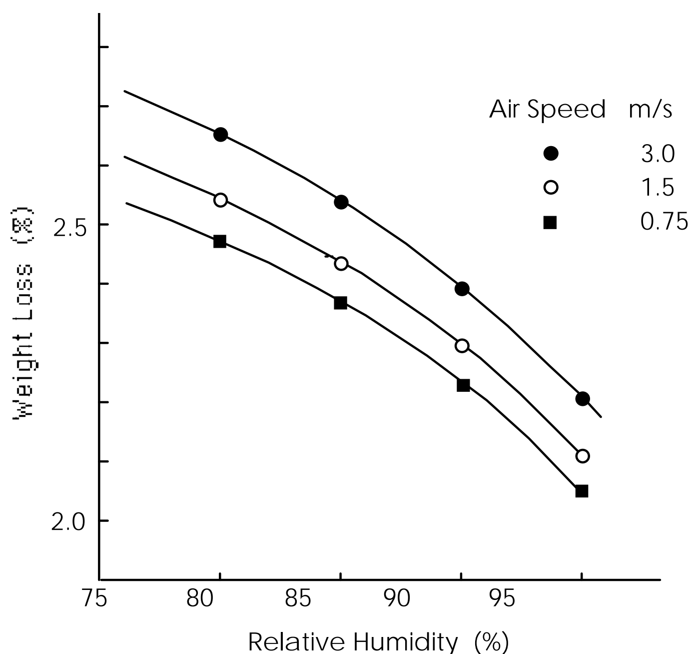

Figure 1: Weight loss from beef sides during 18 h chilling at an air temperature of 0°C at different velocities and relative humidities.

Table 2 gives the weight of the heaviest pork carcass that can be cooled to a deep leg temperature of 7°C by 16 h post mortem in a single stage chilling operation. These data were obtained in a situation where the carcasses were placed in the cooling chamber at 50 min post mortem, the temperature pull-down period was minimal (less than 30 min) and the air velocity was maintained over all the surfaces of the carcasses. Few if any of these conditions would be achieved in commercial practice and the weights should therefore be taken as a theoretical upper limit to the weights that could be cooled.

With lamb carcasses, chilling in air at 0°C, 1.0m/s will achieve a chilling time to 7°C in 10 h for carcasses up to 30 kg in weight, which will allow for dispatch on the same day as slaughter in many abattoirs. Reducing the initial temperature to –2°C, and increasing the air velocity to 3.0m/s resulted in a 6 h chilling time with lighter 15 to 17.5 kg lamb carcasses without any surface freezing.

Neglecting other constraints, imposed by eating quality considerations, severe environmental conditions are not required with lamb carcasses to produce chilling times that allow cutting or transport on the same day as slaughter. Air temperatures of, or slightly less than the desired final meat temperature in the range 0 to 4°C, and a low air velocity 0.2 to 0.5m/s will achieve overnight chilling in 14 to 16 h.

Table 2: Maximum weight of pork carcass that can be cooled to 7°C in deep leg within 16 h at different combinations of air temperature and velocity. (*Interpolated values).

Chill room air		Heaviest carcass cooled to 7°C in
Temperature	Velocity	deep leg in 16 h.
(°C)	(m/s)	(kg)
0	0.5	100
	1.0	105*
	3.0	110*
4	0.5	60
	1.0	80
	3.0	95*
6	0.5	<40
	1.0	40
	3.0	60

Refrigeration loads and operational factors

If specified cooling schedules are to be attained, refrigeration machinery must be designed to meet the required heat extraction rate at all times during the chilling cycle. Heat enters a beef chill room via open doors, from personnel, through the insulation, from lights and cooling fans, and from the cooling carcasses or sides. The product load is the major component of the total heat to be extracted from a fully loaded chill room (16).

The rate of heat release from a single side varies with time. It is at a peak immediately after loading, and then falls rapidly. The peak value is primarily a function of the environmental conditions during chilling and is not substantially affected by side weight in the region of 120 to 140 kg. In commercial systems the peak load imposed on the refrigeration plant is also a function of the rate at which hot sides are introduced into the chill room.

Investigations carried out in a commercial beef chiller, that was designed to operate in either, a slow chilling mode to avoid cold shortening, or a rapid chilling mode for a quick turnover and reduced weight loss, showed that operational factors are as important as technical specifications with respect to total weight loss (17). Over 50% of the variance in weight loss was accounted for by the difference in time that elapsed between death and hot weighing, whilst a further 11.8% was related to the time that elapsed between hot weighing and loading the beef side into the chiller.

Table 3: Cooling time to 7°C and 1°C in deep *M. longissimus dorsi* in air at 1±1°C for lamb carcasses of different average weights and fat covers (Smith and Carpenter, 1973 (17)).

Carcass weight (kg)	26.8	21.5	16.8
Fat thickness 12th rib (mm)	7.1	3.3	1.1
Cooling time to 7°C (h)	4.3	3.1	1.9
Cooling time to 1°C (h)	8.1	5.9	5.6

It is difficult to separate the effect of carcass weight from fat cover on chilling time because as carcasses get heavier they also tend to become fatter. However, Table 3 shows that the chilling time to 7°C in the *M. longissimus dorsi* of lean light weight (16.8 kg) lambs can be under half that of 26.8 kg lambs with a much thicker fat covering. It would not be unusual for a chilling system to contain lamb carcasses covering this range of weights and fat covers. The design and operation of such a system must therefore be a compromise between over long chilling periods for the smaller carcasses and under cooling of the larger carcasses.

Novel systems with future potential

The majority of carcass chilling is carried out in conventional air chilling rooms using constant environmental conditions. There are a number of obvious problems with conventional chilling operations. The duration of the process is such that chill rooms have to be large enough to hold a whole day's kill. If throughput increases, another chill room has to be built to cater for the extra carcasses. This is all too often neglected, with the result that the existing chill room is overloaded. The thermal load on the refrigeration system exceeds the design duty, and air temperature rises. This leads to increased weight loss and more rapid bacterial multiplication.

Accelerated chilling

As early as the 1950s, several progressive sausage manufacturers in the USA deboned hot (< 1 h *post-mortem*) sow carcasses (19). The resulting pre-rigor muscles were treated with salt or sometimes polyphosphates to improve the water-holding capacity for the production of frankfurters. Today some 15% of the total pork production is hot boned and nearly all the musculature transformed immediately into sausages. This is the most extreme example of accelerated processing currently in commercial operation, from pig to sausage in less than 2 h.

MIRINZ (20) have carried out investigations on the possibility of using very low temperatures (–25 or –30°C) for a short period of 30 min followed by an equalisation period at 0°C until the deep leg temperature reaches 7°C. The treatment produced a 4 h process from slaughter to deep temperature of 7°C. The products, which are then fast frozen, are claimed to be moderately tender.

Chilling pork in two stages, with the first stage consisting of a conveyorised air blast tunnel is quite common. The pre-chiller serves two requirements in that it rapidly lowers the surface temperature of the carcass, reducing the rate of evaporative weight loss, and has the capacity to absorb the initial peak heat load. Ultra-rapid chilling extends the process by extracting all the required heat from the pork carcass in a single short blast chilling operation. The carcass can then be band-sawn into primals and stored or transported on the same day as slaughter.

In experiments, sides and carcasses were cooled in air at -30°C and 1.0m/s for 4 h, and compared to controls chilled in air at 0 to 4°C and 0.5m/s for 24 h. After chilling, both the sides and carcasses were band-sawn into primal joints and vacuum-packed for equalisation. After the 4 h ultra-rapid chilling process the average temperature in the primal joints from sides ranged from -1.9 in the loin to 1.2°C in the shoulder and in whole carcasses from -2.1 in the belly to 3.0°C in the shoulder.

Evaporative loss was reduced to 1.13% for sides and 1.10% for whole carcasses, almost half that of the controls. No extra drip was measured from the primal joints but the chops from the sides which had been partially frozen during the process recorded higher drip levels. Instrumental measurements of texture carried out on loin chops from ultra-rapidly chilled pork stored for two days

showed that the meat was tougher than that of the controls. There was less increase in toughness in sides subjected to ultra-rapid chilling than whole carcasses. The lean of chops from ultra-rapidly chilled sides and whole carcasses was significantly darker ($P<0.05$) than control chops.

Immersion chilling

All frozen poultry is initially chilled by being immersed in chilled water or an ice water mixture. The procedure is very rapid and the poultry carcasses actually gain weight during the process. Whole carcasses or even sides of pork are too big to handle in this way, but it is possible to hot joint the pork into primal cuts, which are then vacuum packed and chilled by immersion in iced water or brine. The vacuum packaging prevents water pick up and overcomes any possibility of cross contamination, both of which are considered a problem in the poultry system.

In immersion chilling trials, the right sides of carcasses were cut into primals (shoulder, leg, loin and belly) and vacuum packed. They were then immersed in a tank of refrigerated agitated brine at 0°C until the slowest cooling sections in the loin and belly reached 13°C and the slowest cooling sections in the leg and shoulder fell to 10°C. These were the deep temperatures at which the primals would adiabatically equalise to 7°C. The primals were then placed in a chill room operating at 0°C. The left side was chilled for 24 h in a conventional chill room at 0°C and 0.5m/s, before cutting and packaging for storage with the immersion chilled primals.

The average temperature of the loin and belly primals was reduced to 7°C within a 2 to 3 h period and legs and shoulders in 6 h in the immersion system. Evaporative weight loss was reduced by over 2% in the immersion chilling system and this yield advantage was still maintained after 14 days further storage.

There were large differences in the texture of individual samples and between samples taken from the end and middle of the loin of the immersion cooled pork. After 14 days conditioning at 0°C there was still a considerable variation in texture between samples. However, the mean value of work done was significantly lower than that measured after 1 day and not significantly different from that of the control. Immersion chilling was judged by a sensory panel to produce darker lean and whiter rind, although there were no significant differences between instrumental measurements.

Ice bank chilling

Another way of reducing weight loss is to increase the humidity of the air in the chilling system. In the early stages of chilling when the surface of the carcass is still much warmer than the air in the room, humidity has little effect. However, during the later stages of cooling and in subsequent storage its effect can be substantial.

Ice bank refrigeration systems produce high humidity air at a steady temperature close to 0°C and have proven advantages in storage of fruit and vegetables. Such systems use refrigerated coils or plates to cool tanks of water and then build up ‘banks’ of ice. The chilled water/melting ice is used to cool and humidify air, by direct contact, which is in turn used to cool the product. The ice bank is energy and cost effective because it uses smaller compressors operating at full power and hence high efficiency. It can also be run overnight on off-peak electricity to build up the bank of ice for use the next day. This bank can then be used to overcome the high heat loads that are initially produced when the hot pigs are loaded into the chill room. In experimental trials pork carcasses were chilled in ice bank air at 2.5°C, 1.5m/s and 99% RH for 1.2 h, then ice bank air at 2°C, 0.4m/s and the same

humidity for a further 70.8 h. They were compared with controls chilled conventionally in air at 2°C, 0.7m/s and 90% RH for 72 h.

In the first 24h the carcasses in the ice bank room lost 0.4% less weight than those chilled in the conventional chill room (Table 4). Over the subsequent 2 days in storage the pigs in the ice bank room lost little additional weight, while those in the conventional chill room lost an additional 0.9%.

Table 4: Cooling time and weight loss in ice bank trials compared with control.

Treatment	Time to 7°C	Percentage weight loss at		
	(h)	24 h pm	48 h pm	72 h pm
Ice bank	11.90_a	1.94_a	2.02_a	1.92_a
Control	9.60_b	2.37_b	2.79_b	3.31_b

Means within columns with different subscripts are significantly different (P<0.05).

The only problem encountered experimentally was that of surface texture, with experienced butchers subjectively judging that the ice bank chilled carcasses were less firm and more slippery. In commercial use ice banks would require more space than conventional chilling systems. To reduce 250 pork carcasses with an average weight of 63kg from 35 to 7°C would require over 4.5 tonnes of ice.

Spray chilling

In spray/evaporative cooling a small amount of water is sprayed on the surface of the carcass at intervals to replace water lost by evaporation. The surface remains wet giving maximum mass transfer and evaporative cooling effect, with no penalty in increased weight loss. Spray chilling has been successfully applied to beef, pork and lamb carcasses.

Brown et al., (21) developed two spray-chilling treatments to improve appearance and reduce weight loss during lamb chilling. The first treatment was an intermittent spray, 8 sprays of 250 ml at 10°C at 20 minute intervals, during the first 3 h of chilling. The second consisted of two sprays, one at 2 and the second at 10 h post mortem. These treatments were compared to a conventional two-stage process, with air at 10°C, 1 m/s for the first 10 h, followed by air at 0°C, 1 m/s for 14 h.

Both treatments significantly reduced weight loss after chilling and this advantage was retained during four further days of storage (Table 5).

Table 5: Mean weight losses and standard deviations () from conventional and spray-chilled lamb carcasses.

Treatment	Weight loss (%)		
	8 h	24 h	5 days
Conventional	1.16^a (0.25)	2.20^a (0.24)	3.97^a (0.55)
Multiple-spray	-0.01^b (0.25)	0.86^b (0.15)	2.97^b (0.27)
Double-spray	0.78^c (0.21)	1.20^c (0.28)	3.19^b (0.42)

Values within columns with different superscripts are significantly different (P<0.05)

There were small (<1 h) but significant reductions in the cooling rates of spray-chilled loins and legs due to sustained evaporative cooling of the wetted surfaces. No effects on texture or drip loss and only slight effects on surface lean and fat colour were found. No significant differences in bacterial numbers were found between treatments after chilling and storage. There were small but significant increases (<1 log cycle) on all diaphragms and on the breasts of double-sprayed carcasses. Commercial spray chilling plants for pork have now been installed in France and the Netherlands but none currently operate in the UK.

Conclusions

The factors which control the cooling of a meat side or carcass are the rates at which heat can be conducted from the innermost tissues to the surface, and from the surface to the circulating air in the chill room. The former is often rate controlling because of the very poor conductivity and considerable thickness of the meat, although this fact is still not appreciated by many of those engaged in refrigeration design. Attempts by contractors to meet continuing commercial pressures for increased rates of cooling are therefore confined to changing the parameters which affect surface heat transfer, i.e. temperature, air velocity and humidity. There is often confusion about the relevant importance of these factors and their effect on yield and quality. Other critical issues, such as product heat load, and its change with time, are given scant consideration, although they are crucial to efficient plant design and operation.

Basic design information is available on the effect of air temperature, velocity and carcass size on the chilling time of beef sides, and lamb and pork carcasses which can be used in the specification of conventional chilling systems.

Data are also available on more novel chilling systems. Ultra-rapid and immersion chilling provide substantial gains in reduced weight loss and increased operational efficiency but would require large capital investment in purpose built plant, and can produce quality problems. Ice bank and spray are easier to integrate with existing systems and can produce significant weight savings without textural problems but still require an overnight chilling time.

It is clear that the abattoir operator and/or the refrigeration engineer should carefully consider the advantages and disadvantages of these systems in relation to any proposed chilling operation, and the market destined for the product (fresh, frozen or processed), before simply opting for a 'standard' chilling system.

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Utilisation of dairy proteins, polysaccharides and blends in the manufacture of reformed and comminuted meat products

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Abstract

Commercial non-meat proteins, such as high gelling whey protein concentrates (WPCs), polysaccharides (iota-, kappa-carrageenan or potato starch) and WPC/polysaccharide blends were used in the manufacture of both comminuted and reformed low-fat porcine meat products. Results from cured reformed meat studies showed that the addition of high gelling 35, 55 and 75% protein WPCs, significantly ($P \leq 0.05$) increased cook yields, water binding capacity (WBC) and colour of final test hams, as well as improving sensory scores for flavour, colour, juiciness and meat bind compared to controls. Control stress rheology further demonstrated that the addition of high gelling WPCs and high gelling WPC/polysaccharide blends to test hams increased the elasticity of meat exudates on heating from 20 to 80°C at 1°C/min and cooling to 20°C at 1°C/min compared with controls. Morphological assessment of test meat exudates supported rheological data showing large changes in the microstructure of test meat gels. Low-fat (<3%) comminuted fresh pork sausage products were also evaluated using high gelling 35 and 55 and 75% WPCs in combination with polysaccharides (tapioca starch, carrageenan and gums). Response surface methodology showed that addition of 20-25% preformed protein gel as a direct replacement for pork backfat produced fresh pork sausages with similar textural characteristics to full-fat (20%) control products. Low-fat fresh pork sausages containing preformed gel as a 100% fat replacer gave similar cook loss, water holding capacity (WHC), mechanical texture values and organoleptic scores for juiciness, flavour and texture when compared to the full-fat controls. Addition of carrageenan to preformed gels improved the strength and water holding of final gels. However, while starch addition further enhanced the quality of low-fat products, the use of gums had a negative effect on the quality of final sausages.

Keywords: WPC, protein, polysaccharide, low-fat, ham, sausage, gel, functionality

Introduction

Protein functionality may be defined as, ‘those physical and chemical properties which affect the behaviour of proteins in food systems during processing, storage, preparation and consumption’ (Kinsella, 1981). The structural, rheological, as well as other physicochemical properties of food systems are determined largely through protein-protein (Ziegler and Foegeding, 1991), polysaccharide-polysaccharide (Morris, 1986) and/or protein-polysaccharide (Ledward, 1995) interactions. Consequently, the type of functional property required in a protein or a protein mix varies depending on the particular type of food system in question. Moreover, the mode of ingredient addition will similarly influence its overall performance.

The growing consumer awareness for the need to reduce levels of dietary fat have led to a demand for low-calorie products which provide all the textural and flavour characteristics of full-fat products. Moreover, the links between dietary fat and cardiovascular diseases and obesity have led to

recommendations that diets should contain no more than 30% of their calories in the form of fat (Pearson and Gillet, 1995). Dry addition of proteins, polysaccharides and protein/polysaccharides powders as fat replacers can reduce the total caloric content of meat products, however, they may also adversely affect the sensory properties of these foods (Kerry and Buckley, 1996; Lyons *et al.,* 1998). Thus, the mode of ingredient addition, the processing conditions employed, as well as the quality of the meat cuts utilised, will ultimately dictate the quality and acceptability of the final low-fat product processed.

The current advances in dairy ingredients technology have led to the development of a range of novel functional high gelling whey protein concentrates (WPCs) as well as WPC/polysaccharide blends containing starches and carrageenans, for use in processed meat products. These non-meat proteins and blends have been successfully employed as potential binding, extending, texture modifying and/or nutritional enhancing adjuncts in both reformed and comminuted porcine meat systems (Kerry *et al.,* 1998c).

Results

Reformed meat trials

Cook yield is generally accepted as one of the most important test criteria for evaluation of protein functionality in reformed meat products (Thomsen and Pedersen, 1993). Data presented in Table 1 shows that the water binding capacity of non-meat proteins increased on cooking at a temperature of 80 *versus* 70ºC. Test hams containing either 35 or 75% high gelling WPCs (4% residual powder), increased cook yields by 5-6% over controls on processing at 80ºC, both in the presence and absence of 0.5% residual Na tripolyphosphate. These results are in agreement with Thomsen and Pedersen (1993) and Haylock and Sanderson (1991) who similarly reported that the WPC addition to reformed meats improved product cook yields, as well as product texture and flavour. However, the use of low-temperature long-time cookery (70°C) increased cook yields compared *with* products processed at 80°C. Cook yield data also showed that processing at lower cooking temperatures decreased protein performance which may be explained through a failure of test proteins to reach their gelation point (70-75°C) during thermal processing (Tables 1 and 2.). Addition of Na caseinate at a residual powder level of 1% to test hams showed no significant increases in cook yield, or WBC, however its addition significantly ($p<0.05$) decreased Hunter 'a' values, organoleptic scores and meat bind values on processing at either 70 or 80ºC. This is in agreement with data cited by Siegal and Schmidt (1979a,b) who similarly reported that Na caseinate had a negative effect on ham quality and ultimate meat bind. Protein isolates were added to test hams at a maximum residual powder level of 1% due to unacceptable increases in brine viscosity and excessive foaming on mixing at higher residual powder levels. Compositional analysis showed that addition of non-meat proteins significantly ($p <0.001$) increased the protein concentration of test batches by as much as 3-4% over controls containing no added protein binders. Addition of soya isolate and egg albumen at residual powder levels of 1%, WPCs at a residual level of 2-4%, as well as tripolyphosphate at a level of 0.5%, significantly ($p <0.001$) increased cook yields and WHC values (Table 2). The type and concentration of non-meat protein used was also shown to have a significant ($p <0.05$) effect on ham quality. Addition of high gelling 35% WPC gave the highest scores for visual colour and Hunter 'a' values compared to controls (Table 2). Similarly, the addition of tripolyphosphate enhanced the water binding capacity of hams and moreover, did not interfere in the functionality of added non-meat proteins. Addition of high gelling WPCs in combination with either iota-, kappa-carrageenan or

starch also produced optimum blends for use in reformed meats.

Control stress rheology demonstrated that the addition of test WPCs increased G' (Pa) values of test meat exudates (extracted on massaging) over controls (Figs 1.a and b). Addition of non-meat proteins, especially the high gelling WPCs and soya isolate at 1-3% and 1% residual powder levels, respectively, had a positive influence on meat bind. β-Lactoglobulin (WPC C) showed the largest increases in G' values for the proteins assessed and was the only WPC to show two transition zones on heating. Na caseinate at a 1% residual powder level was the only non-meat protein found to give G' values lower than the control in agreement with processing data. While a significant ($P<0.05$) increase in protein concentrations of 1-3% for test exudates was observed, rheological data showed a five to six-fold increase in G' values. The differences in the G' profiles between control and test exudates indicated the potential variations which exist in their gelling abilities when used in model reformed meat system (Kerry *et al.,* 1998a).

Addition of *iota*-carrageenan, potato starch on their own or *iota*-carrageenan, potato starch and LM pectin in combination with WPCs, especially the thermally modified high gelling WPC powders, enhanced exudate gelation and ultimate meat bind (Table 3). Polysaccharide/WPC blends using WPC C (β-Lactoglobulin) gave the largest increases in G' values. Na alginate and carrageenan blends containing locust bean gum were shown to interfere with protein gelation. The addition of *i*-carrageenan on its own or in combination with WPCs, especially the thermally modified high gelling WPC powders A, B and C, enhanced exudate gelation and ultimate meat bind producing a five six-fold increase in G' values. Blends using WPC C (β-Lactoglobulin) produced the largest increases in G' (Pa) values (Table 4). The differences in the G' profiles between control and various test exudates demonstrates the complexity of multicomponent gel systems, both in terms of their structure and chemistry, and the influence which various ingredient combinations can have on the potential gelling properties of reformed meats.

Morphological analysis of the gelled control meat exudate (Fig. 2.a.) showed that it had a very open microstructure, possessing pores of a larger diameter than samples containing test ingredients (Figs.2.b.-2.d.). Hermansson, (1986) reported that where protein gels displayed an open microstructure and/or possessed large pore sizes or voids within the matrix, their water holding capacity was greatly reduced. The morphological analysis of the control meat exudate was in agreement with rheological data, where control meat exudates produced the lowest gel rigidity (as expressed by G') *versus* test exudates, with the exception of the Na caseinate. The gel surface of control exudate was coarser than that observed in meat exudates containing test ingredients. The occurrence of polygonal or 'stick-like' structures (myofilaments) on the gel surface of control exudates was not observed in test exudates, which all possessed a smoother surface. The microstructure of gelled test exudate containing 1% soya protein isolate had a more compact microstructure with smaller pore sizes compared to the controls (Fig. 2.b.).

These changes in gel morphology agreed with the observed increases in G' (Pa) values for test exudates containing soya isolate over control values. The morphology of the test exudate containing high gelling 55% ß-Lactoglobulin WPC, at a 2% residual level showed that it was more open in structure, possessing a fine gel network and smaller pore size (Fig. 2.c.). This morphology again compared favourably with rheological data, where the high gelling 55% WPC produced the largest increases in G' values for test exudates assessed. A further example of a protein/polysaccharide blend using 55% high gelling WPC in combination with potato starch is presented in Figure 2.c. The

interaction between starch and protein within the meat system in this example could be categorised as a complex interpenetrating gel network or multicomponent gel type system as described by Zeigler and Foegeding (1990).

Comminuted meats

Lyons *et al.,* (1998) recently reported on the utilisation of functional high gelling WPCs is the manufacture of low-fat (<3%) fresh pork sausages using 20-25% addition of a whey protein preformed gel (10% protein) as 100% fat replacer (Fig. 3). Preformed gels containing blends of high gelling 35% WPC (8-10%) and carrageenan (1.5-2%), in combination with dry additions of tapioca starch (1.5-2%), significantly ($p < 0.05$) improved the final texture of low-fat sausages. The 'cakey' texture often associated with the addition of dairy ingredients as fillers was not evident when incorporating the preformed gel at a 20% addition rate, however, higher levels of preformed gel addition did result in a drier final low-fat sausage. Use of the preformed gel as a fat substitute resulted in a more homogenous, juicier product similar to the full-fat commercial control and this was achieved through efficient water entrapment and ability of gel to produce a more open texture in the low-fat product. Combinations of preformed gel and 3% tapioca starch resulted in a low-fat sausage with similar mechanical and organoleptic characteristics to those of full-fat controls. Response Surface Methodology was found to be an effective method for the determination of optimum levels of the test ingredients required to produce low-fat sausages (<3% fat) with similar properties to those of commercial controls (20% fat).

Table 1: Delta (test – control) values for hams cooked at 80°C to a core temperature of 68°C containing added non meat protein powders (Pow) at residual powder levels of 1 and 4% used in ham manufacture. in the presence and absence of phosphate (Phos). Results are presented for Water binding capacity (WBC), Hunter 'L' and 'a' values).and % protein levels (P).

Pow	**Conc**	**Phos**	**CY**	SD	**WBC**	SD	**'a'**	SD	**'l'**	SD	**P**	SD
1	1	+	0.45	0.11	0.15	0.02	0.96	0.07	0.42	0.02	0.26	0.06
1	1	-	0.98	0.04	0.24	0.02	0.86	0.05	0.71	0.21	0.56	0.02
2	1	+	0.70	0.20	0.17	0.05	0.39	0.18	0.12	0.11	0.40	0.11
2	1	-	1.02	0.03	0.43	0.03	0.34	0.01	0.25	0.06	0.59	0.02
3	1	+	0.47	0.02	0.23	0.03	0.46	0.04	0.28	0.04	0.27	0.01
3	1	-	0.64	0.04	0.51	0.03	0.49	0.04	0.31	0.07	0.37	0.02
4	1	+	-0.28	0.04	0.05	0.02	-0.12	0.40	1.16	0.08	0.16	0.02
4	1	-	-0.43	0.02	0.01	0.04	0.05	0.02	1.18	0.05	0.25	0.01
5	1	+	2.47	0.05	3.13	0.03	-0.75	0.33	2.22	0.17	1.41	0.03
5	1	-	3.15	0.05	3.14	0.02	-0.04	0.34	2.35	0.14	1.81	0.03
6	1	+	2.34	0.03	3.39	0.04	0.35	0.02	2.22	0.03	1.41	0.03
6	1	-	2.26	0.02	3.38	0.04	0.36	0.11	2.37	0.03	1.81	0.03
1	4	+	5.55	0.08	6.57	0.05	3.24	0.12	2.20	0.20	3.18	0.04
1	4	-	4.22	0.06	7.13	0.05	3.29	0.05	2.21	0.12	2.42	0.03
2	4	+	5.50	0.08	7.16	0.03	1.24	0.09	3.22	0.29	3.15	0.05
2	4	-	6.05	0.03	7.57	0.04	1.26	0.04	3.36	0.22	3.47	0.02
3	4	+	6.28	0.08	7.65	0.05	1.21	0.04	3.45	0.24	3.62	0.06
3	4	-	6.45	0.04	7.94	0.02	1.24	0.03	3.44	0.12	3.70	0.02

Table 2: Listing of commercial non-meat powders used

No.	Powder Type	Protein. Conc (%)	Supplier
1	(A) High gel WPC	35.0	Dairygold Co-op, Cork Ireland.
2	(B) High gel WPC	75.0	M.D. Foods, Holland.
3	(D) Regular WPC	76.5	Alacen 132 N.Z. Dairy Board
4	Na caseinate	88.5	DMV Veghel, Holland.
5	Soya isolate	90.0	Protein International, Leper, Belgium.
6	Egg albumen	75.0	Lactosan, London, U.K.
7	(C) High gel WPC (β-lactoglobulin)	55.0	Dairygold Co-op, Cork Ireland.
Low Methoxy (LM) Pectin			Copenhagen Pectin, Denmark
Carrageenans			FMC Corporation, Belgium
Potato Starch			National Starch U.K.

Table 3: Mean (n=30) organoleptic scores and standard deviations (SD) for hams cooked at 80°C to a core temperature of 68°C containing added non-meat protein powders (P) at residual powder levels of 1 and 4%, in the presence and absence of phosphate (Phos). Results are presented juiciness (J), flavour (F), non-meat flavour (NMF), salt intensity (SI), Bind (B), colour (C), and meat odour (O).

Powder	Conc	Phos	J	SD	F	SD	NMF	SD	SI	SD	B	SD	C	SD	O	SD
Con		+	6.57	0.51	3.52	0.68	1.10	0.30	4.95	0.59	3.57	0.60	4.05	0.50	1.05	0.22
Con		-	5.62	0.67	3.86	0.79	1.05	0.22	4.90	0.77	3.38	0.59	3.62	0.59	1.19	0.40
1	1	+	5.67	0.73	4.29	0.72	1.10	0.30	4.14	0.36	3.38	0.50	4.67	0.58	1.05	0.22
1	1	-	5.67	0.48	4.52	0.68	1.05	0.22	4.19	0.40	4.29	0.72	4.52	0.60	1.05	0.22
2	1	+	5.76	0.44	5.76	0.70	1.57	0.51	4.19	0.40	4.67	0.66	5.33	0.66	1.62	0.50
2	1	-	5.95	0.67	5.76	0.94	1.38	0.50	4.57	0.60	4.86	0.48	5.76	0.44	1.29	0.46
3	1	+	5.57	0.51	5.48	0.98	1.14	0.36	4.48	0.51	4.67	0.58	5.10	0.62	1.71	0.46
3	1	-	5.67	0.73	5.76	0.77	1.14	0.36	4.62	0.59	4.43	0.51	5.24	0.62	1.33	0.48
4	1	+	3.10	0.30	3.43	0.51	4.57	0.51	3.19	0.51	1.95	0.38	1.76	0.83	4.48	0.60
4	1	-	3.14	0.36	3.38	0.67	5.19	0.60	3.24	0.44	1.95	0.80	2.81	0.51	5.05	0.74
5	1	+	4.10	0.30	3.81	0.68	4.76	0.70	3.76	0.89	1.29	0.46	4.71	0.72	3.90	0.83
5	1	-	4.05	0.50	4.67	0.58	4.71	0.85	2.90	0.54	4.48	0.51	4.81	0.51	3.76	0.83
6	1	+	4.05	0.22	5.14	0.79	4.90	0.77	3.90	0.62	4.76	0.77	4.38	0.50	4.24	0.89
6	1	-	3.71	0.46	5.52	0.81	4.48	0.51	4.71	0.46	4.71	0.56	5.38	0.74	4.24	0.94
1	4	+	5.43	0.51	4.29	0.56	1.05	0.22	3.81	0.51	5.67	0.48	5.62	0.97	2.43	0.51
1	4	-	5.48	0.60	4.67	0.48	1.10	0.30	3.10	0.44	5.00	0.71	6.62	0.50	2.33	0.48
2	4	+	4.48	0.51	3.38	0.50	3.81	0.40	2.71	0.64	4.67	0.48	3.76	0.83	4.48	0.51
2	4	-	4.57	0.60	3.43	0.51	3.71	0.56	2.71	0.56	4.62	0.74	4.00	0.84	4.24	0.94
3	4	+	4.19	0.40	3.33	0.48	4.14	0.48	2.86	0.48	5.14	0.48	3.62	0.74	4.19	0.87
3	4	-	4.05	0.22	3.00	0.00	4.38	0.50	2.52	0.51	4.19	0.51	3.57	0.75	4.76	0.83

Table 4: The means and standard errors of the means for storage modulus (G') values (n = 6) on final heating to 80°C x 30mins at 1°C/min, with subsequent cooling to 20°C at 1°C/min. for control exudates and test exudates containing residual powder levels of 2% iota-, kappa-carrageenan, carrageenan blend, LM pectin, modified starch or 0.5% Na alginate on their own or in combination with 55% High gelling β-lactoglobulin WPC at a 2% residual powder level.

Protein/ Polysaccharide Powders*	**Mean Storage Modulus G'(Pa) values**					
	20° C	41° C	55° C	70° C	80° C	20° C
WPC	544 ± 38	2,107 ± 25	3,363 ± 25	11,068 ± 52	12,430 ± 58	40,058 ± 51
WPC + *Pectin*	115 ± 27	229 ± 30	1,948 ± 20	5,418 ± 29	14,528 ± 23	63,325 ± 32
WPC + *Alginate*	665 ± 30	457 ± 21	134 ± 24	979 ± 23	4,471 ± 26	16,898 ± 46
WPC + *Starch*	153 ± 30	390 ± 20	3,283 ± 29	102,369 ± 32	137,040 ± 31	41,460 ± 40
Pectin	232 ± 19	337 ± 23	2,397 ± 23	6,193 ± 19	7,026 ± 27	9,994 ± 31
Alginate	505 ± 25	490 ± 25	150 ± 26	451 ± 23	1,535 ± 26	10,121 ± 36
Starch	122 ± 21	144 ± 20	1,699 ± 30	2,013 ± 35	7,925 ± 35	18,980 ± 33
WPC + *i*-Carr	939 ± 33	2,623 ± 26	2,623 ± 30	5,138 ± 34	23,527 ± 36	179,579 ± 45
WPC + *k*-Carr	1,113 ± 36	10,610 ± 28	10,610 ± 26	14,643 ± 32	19,398 ± 38	93,211 ± 41
WPC + Carr mix	935 ± 33	5,816 ± 22	5,816 ± 26	9,113 ± 28	12,127 ± 31	17,350 ± 42
Iota-Carrageenan	817 ± 25	985 ± 19	4,304 ± 31	11,936 ± 35	7,925 ± 35	18,577 ± 40
Kappa-Carrageenan	825 ± 26	921 ± 28	2,105 ± 23	5,361 ± 28	5,584 ± 31	10,121 ± 36
Carrageenan mix	838 ± 23	997 ± 27	3,149 ± 25	7,706 ± 29	4,725 ± 29	9,751 ± 38
Control	538 ± 24	1,299 ± 41	1,790 ± 16	4,513 ± 44	4,233 ± 51	9,999 ± 19

Details of non-meat protein powders and polysaccharides used are presented in Table 2

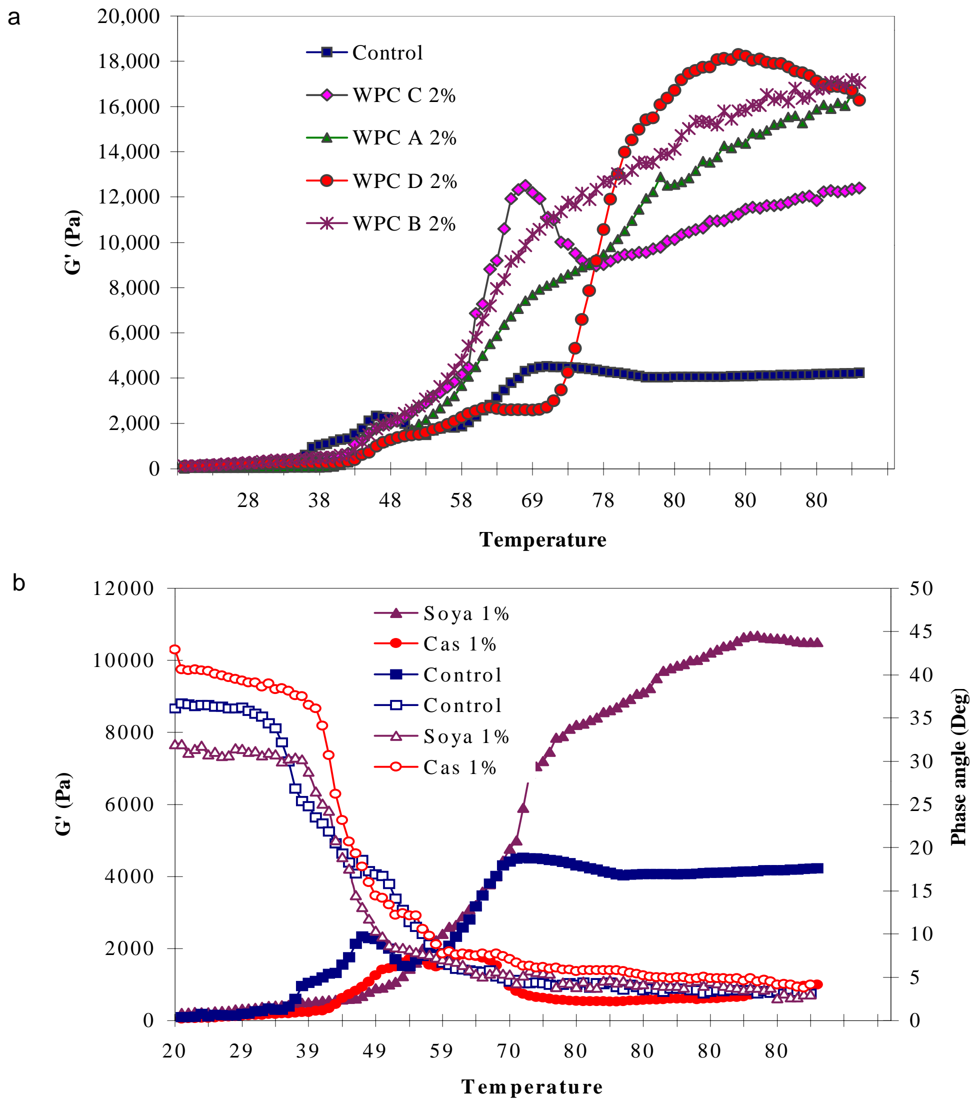

Figure 1: Plot of G' (Pa) (closed symbol) and Phase angle (open symbol) *versus* increasing temperature (°C) for control meat exudate and test meat exudates containing WPCs at a 2% residual powder level (a), as well as soya isolate and sodium caseinate at a 1% residual powder level (b).

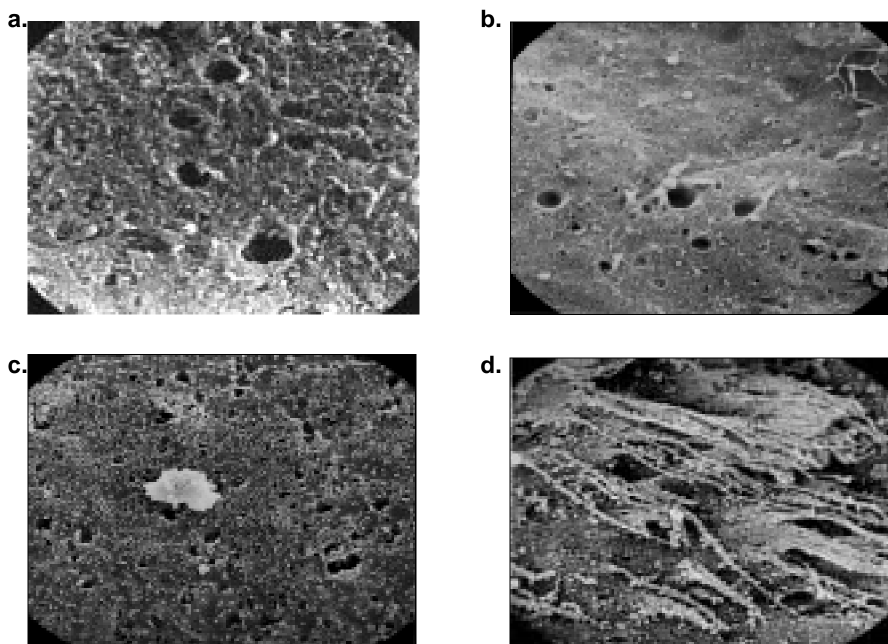

Figure 2: Scanning electron micrographs of control (a) and test meat exudate gel containing soya isolate at a 1% residual powder level (b), 55% high gelling WPC at a 2% residual powder level (c) and high gelling 55% WPC at a residual powder level of 2% in combination with 1% iota carrageenan. Exudate were heated to 80°C at a rate of 1°C/min held at 80°C for 30min and cooled to 20°C at 1°C/min (Microscope magnification, 3000 fold).

Conclusions

The use of non-meat proteins, especially WPCs, as meat, fat and water binders and/or texture modifying ingredients for the manufacture of low-fat meats shows significant commercial potential. The type of protein, concentration, thermal processing temperatures and mode of protein addition, as well as combinations with other binders (polysaccharide or protein sources) can all influence their performance in low-fat meat systems. Thus an understanding of the potential mechanisms and interactions occurring between these added ingredients and meat proteins is very important to ensure optimal non-meat protein performance.

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Inhibition of mutagen/carcinogen MeIQx by SH - containing substances in aqueous and meat matrix based model systems

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Summary

The influence of 8 various substances (ascorbic acid, proline, thryptophan, cysteine, N-acetyl-cysteine, glutathione, tannic acid and Tween 80 ®) on the formation of mutagen/carcinogen MeIQx in an aqueous model system of creatinine, glycine and glucose was investigated. This screening based on a statistic model using a 16-run, 8-factor experimental design with 2 blocks, resolution 4, and was performed with SAS/QC® software. The organo-sulfur compounds cysteine, N-acetylcysteine and glutathione showed the strongest negative and most significant ($p < 0.05$) effect on MeIQx formation. In addition, the dose dependent inhibiting potency of these substances has also been studied in this model system. The highest concentration of ascorbic acid, cysteine, N-acetyl-cysteine and glutathione (0.05 mmol/mmol creatinine) lead to a MeIQx reduction of 70.8 %, 80.4 %, 71.5 %, and 91.4 %, respectively.

To check the results from this aqueous model system we used the organo-sulfur compounds and ascorbic acid in meat matrix based model systems. As expected, cysteine, N-acetylcysteine and glutathione showed negative effects on MeIQx formation. To our surprise we found higher levels of MeIQx with increasing content of ascorbic acid. A response surface analysis with 3 factors (cysteine, glutathione and ascorbic acid) also performed with SAS/QC® software, confirmed the effects of cysteine and glutathione at a significant level. A higher concentration of ascorbic acid lead in this model system to a lower level of MeIQx reduction which is in agreement with the results we obtained from the dose-dependent meat matrix model. Therefore we conclude that ascorbic acid could not be used to decrease MeIQx content in meat. On the other hand, both experiments show that SH-containing compounds could play an important role as inhibitors of mutagen/carcinogen formation in heated meat. More work is needed to determine the reactions behind this inhibiting effect and to explore the role of ascorbic acid and its interaction with the meat matrix in more detail.

Introduction

Thermal processing of meat often enable products to be made edible, make them more appetizing or provide microbiological stability during storage. However, the heating process could also lead to substances with a toxicological relevance on consumer's health like Nitrosamines, Polycyclic Aromatic Hydrocarbons (PAHs) or Heterocyclic Amines (HAs). Heterocyclic Amines are produced when Maillard reaction between amino acids, reducing sugars and creatine/ine takes place, and have been shown to be potent mutagens and carcinogens. For this reason the International Agency for Research on Cancer (IARC) has recommended the reduction of human exposure to HAs [1]. One of the most common representatives of HAs is 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) which can be found in cooked beef, pork, chicken, meat extracts and bacon as well as in cooked fish [2].

Aqueous model systems containing the HA precursors creatin/ine, amino acids and sugars are a useful tool to study the inhibiting potential of substances on HA formation because HAs isolated from such model systems were identical to mutagens isolated from cooked meat. The aim of this work was to determine the inhibiting potential of various substances on HA formation in a comparative study performed by statistical experimental design with SAS/QC® software. Furthermore, the dose dependent inhibition potency of most effective substances has been studied in the aqueous and in meat matrix based model systems.

Materials and methods

Statistical analysis

All experimental designs and statistical analysis were carried out using SAS/QC® software and documentation [3].

For the screening experiment, a two-level fractional factorial design with blocking was chosen. The factors which affected the content of MeIQx (as the response variable), were the following 8 substances: ascorbic acid; proline; tryptophan; cysteine; N-acetylcysteine; glutathione; tannic acid and Tween 80®. For each factor a high level of 0.3 mmol and a low level of 0.03 mmol per mmol creatinine was selected, except for tannic acid and Tween 80®. For these substances 0.03/0.003 mmol and 500/50 mg, respectively, were chosen. The screening experiment was a 16-run, 8-factor experimental design (see Table 1) with 2 blocks and resolution 4 (main effects not confounded with each other or with two-factor interactions).

The response surface model for the response variable MeIQx is a function relating to the 3 factors ascorbic acid, cysteine and glutathione. Therefore, we selected a Box-Wilson design with uniform precision. Uniform precision means that the variance of the predicted response is the same for all points near the centre of the design. This design contains 20 runs with 6 centre points.

Table 1: Weight in [mmol/mmol Creatinine], except Tween (screening experiment)

Run No.	Asc.	Cys.	N-Ac.	Glu.	Tan.	Pro.	Try.	Twe.
1	0.03	0.03	0.03	0.3	0.03	0.3	0.3	50 mg
2	0.03	0.03	0.3	0.03	0.03	0.3	0.03	500 mg
3	0.03	0.3	0.03	0.03	0.03	0.03	0.3	500 mg
4	0.03	0.3	0.3	0.3	0.03	0.03	0.03	50 mg
5	0.3	0.03	0.03	0.03	0.003	0.3	0.3	500 mg
6	0.3	0.03	0.3	0.3	0.003	0.3	0.03	50 mg
7	0.3	0.3	0.03	0.3	0.003	0.03	0.3	50 mg
8	0.3	0.3	0.3	0.03	0.003	0.03	0.03	500 mg
9	0.03	0.03	0.03	0.03	0.003	0.03	0.03	50 mg
10	0.03	0.03	0.3	0.3	0.003	0.03	0.3	500 mg
11	0.03	0.3	0.03	0.3	0.003	0.3	0.03	500 mg
12	0.03	0.3	0.3	0.03	0.003	0.3	0.3	50 mg
13	0.3	0.03	0.03	0.3	0.03	0.03	0.03	500 mg
14	0.3	0.03	0.3	0.03	0.03	0.03	0.3	50 mg
15	0.3	0.3	0.03	0.03	0.03	0.3	0.03	50 mg
16	0.3	0.3	0.3	0.3	0.03	0.3	0.3	500 mg

Aqueous model system

The aqueous model system contained the HA precursors creatinine, glycine and glucose in molar concentrations of 14 mmol, 14 mmol and 7 mmol, respectively, dissolved in 50 mL water. The 16 samples containing the substances (see Table 1) dissolved in model system solution, were thermally treated at 135 ± 0.1 °C for 1 h in a 170 L laboratory autoclave (Fedegari, Italy). For the dose dependent study 0.05, 0.04, 0.03, 0.02, 0.01, 0.005 and 0.002 mmol per mmol creatinine, of ascorbic acid, cysteine, N-acetylcysteine and glutathione were weighed in and dissolved with 50 mL of the model system solution. Thermal processing and chemical analysis were identical to the screening experiment.

Meat matrix based model system

For this model system we used very finely chopped beef with the following analysis data (for methods see [4]): 73.6 % water, 20.2 % protein, 5.2 % fat, 1.1 % ash. The content of creatine (0.466 %) and creatinine (< 0.006 %) was analysed with an enzymatic test kit [5].

For the dose dependent study from each of the 4 substances, ascorbic acid, cysteine, N-acetylcysteine and glutathione, 0.014 mmol, 0.07 mmol, 0.28 mmol and 0.42 mmol were weighed in separately, dissolved with 4 mL dest. water, and mixed carefully with 70 g of the meat matrix. For the weight in of ascorbic acid, cysteine and glutathione to run the response surface analysis see table 2, the substances were similarly treated as in the dose dependent study (dissolved in bidest. water and mixed carefully with 70 g of the meat matrix).

Table 2: Weight in [mmol/70g meat matrix], (response surface analysis)

Run No.	Asc.	Cys.	Glu.
1	0.14	0.14	0.14
2	0.14	0.14	0.42
3	0.14	0.42	0.14
4	0.14	0.42	0.42
5	0.42	0.14	0.14
6	0.42	0.14	0.42
7	0.42	0.42	0.14
8	0.42	0.42	0.42
9	0.044549	0.28	0.28
10	0.515451	0.28	0.28
11	0.28	0.044549	0.28
12	0.28	0.515451	0.28
13	0.28	0.28	0.044549
14	0.28	0.28	0.515451
15	0.28	0.28	0.28
16	0.28	0.28	0.28
17	0.28	0.28	0.28
18	0.28	0.28	0.28
19	0.28	0.28	0.28
20	0.28	0.28	0.28

After the heat processing step (135 ± 0.1 °C, $2^1/_2$ h) dest. water was added to the samples up to 70 g, to avoid inaccuracy due to unequal loss of water. For the extraction procedure the samples were mixed with an Ultra Turrax (Fa. Janke und Kunkel, Staufen, Germany) and were stored at -20 °C until analysed.

Sample extraction and HPLC separation

Sample clean up was carried out using the principle method of Gross and Grüter [6] with some modifications. The Extraction procedure was carried out using a series of 8 samples which could be treated in the same way. For the dose dependent study spiking of the samples wasn't necessary, because incomplete recovery was corrected by a control sample which was part of each extraction procedure. SAS planned experiments were repeated in duplicate, the dose dependent studies in triplicate.

Results and discussion

Figure 1 shows the influence of the different substances on MeIQx content in the screening experiment. All substances in the survey do have a negative effect on MeIQx formation. This effect is, however, only significant ($p < 0.05$) for cysteine, N-acetylcysteine and glutathione. Glutathione has the strongest negative effect (-2.34456) on MeIQx formation. This means that changing the concentration of glutathione from a low level of 0.03 to a high level of 0.3 mmol/mmol creatinine decreases the MeIQx content by 4.68912 ng/mL in the model system. Tween 80 is known as an agent for oil in water emulsions and was tested for further experimentation with nonpolar substances like α-tocopherol and β-carotene. The screening experiment shows that there is little evidence that a change of the amount of Tween 80 has a significant effect on MeIQx formation. Therefore it could be used as an oil in water emulsifier which provides stable emulsions even at high temperatures [7].

The 3 substances (cysteine, N-acetylcysteine and glutathione) which showed the strongest and most significant reduction on MeIQx formation were chosen for the dose dependent study. Furthermore, we used ascorbic acid in the dose dependent study although its effect was not significant in the screening experiment. On the other hand, ascorbic acid also shows a markable effect on MeIQx content and is widely used in the meat processing industry. As shown in Figure 2, these substances decrease the content of MeIQx in the aqueous model system. As presumed by the screening experiment, the highest reduction could be achieved by the addition of glutathione. With the highest concentration (0.05 mmol of ascorbic acid, cysteine, N-acetylcysteine and glutathione per mmol creatinine) a MeIQx reduction of 70.8 %, 80.4 %, 71.5 % and 91.4 %, respectively, could be achieved. This means that adding these substances in a ration of only 1:20 to creatinine content results in a markable reduction of mutagen formation.

Figure 3 shows the effects of ascorbic acid, cysteine, N-acetylcysteine and glutathione on MeIQx content in the meat matrix based model system. It could be seen that the trend of reduction by SH-containing substances from the aqueous model system will continue in the meat matrix based model system. Glutathione shows again the highest inhibiting potential, followed by cysteine and N-acetylcysteine. To our surprise we found higher levels of MeIQx with an increasing content of ascorbic acid. This is not easy to understand.

The results of the response surface analysis are visualised in figures 4a, b, c. We observed that the overall model statistic accounts for 89.9 % of the observed variation in MeIQx reduction with a high significance ($p<0.001$).

The fitted MeIQx response surface for figure 4a and b could be described as:

$$\begin{aligned} \text{Red. of MeIQx [\%]} = \; & 36.75 - 70.24x\bullet ASC + 33.62\bullet CYS + 114.88\bullet GLU \\ & + 64.54\bullet ASC^2 - 49.81\bullet CYS\bullet ASC + 162.40\bullet CYS^2 \\ & + 97.64\bullet GLU\bullet ASC - 200.06\bullet GLU\bullet CYS - 38.00\bullet GLU^2 \end{aligned}$$

Joint tests for each factor (ASC, CYS and GLU) showed that cysteine and glutatione, as well as all interaction terms involving cysteine and glutathione, have a significant ($p=0.0017$ and $p=0.0008$, respectively) effect on the response variable MeIQx. The influence of ascorbic acid is not significant at all ($p>0.05$) and must be checked in further experimentation designs.

Both figures, 4a and b, show a higher reduction of the MeIQx content by an increasing concentration of cysteine or glutathione. Also it could be seen that a higher content of ascorbic acid lead to a lower level of MeIQx reduction. This result is in agreement with the data of our previously performed dose dependent study shown in figure 3. Here could also be seen that a higher concentration of ascorbic acid result in a higher content of MeIQx. We should however, also keep in mind that the influence of ascorbic acid is not significant, and therefore more work had to be spent to clear this in detail. The standard error of the fitted response surface model is displayed in Figure 4c. In the marginal areas of the model the standard error is much higher than in the middle, which is due to the fact that there are less data points then in the centre of the model.

SH-containing substances have previously been reported to inhibit non-enzymatic browning of heated amino acid/glucose mixes [8]. To the best of our knowledge, this is the first study reporting the influence of SH-containing substances on MeIQx formation. The mechanism of this inhibition is mostly not well understood, and therefore efforts have be made for a better understanding of the reactions behind the formation. The influence of ascorbic acid on MeIQx formation has been examined in other studies before. Johansson and Jägerstad [9] found that high concentrations of ascorbic acid (1000 ppm) in aqueous creatinine, glycine, glucose model system, reduced the MeIQx formation by 84%, while low concentrations (10 and 100 ppm) had no effect. The results of our survey are in agreement with these findings. With the highest level of ascorbic acid, 0.05 mmol/mmol creatinine (corresponding to 2465 ppm), we observed a MeIQx reduction of 70.8 % (see figure 2). Chen [10] also showed ascorbic acid to inhibit mutagen formation during the frying of beef.

At present we are studying the synergistic effects of the organo sulfur-compounds with ascorbic acid in meat matrix models and minced meat products prepared with household cooking practices.

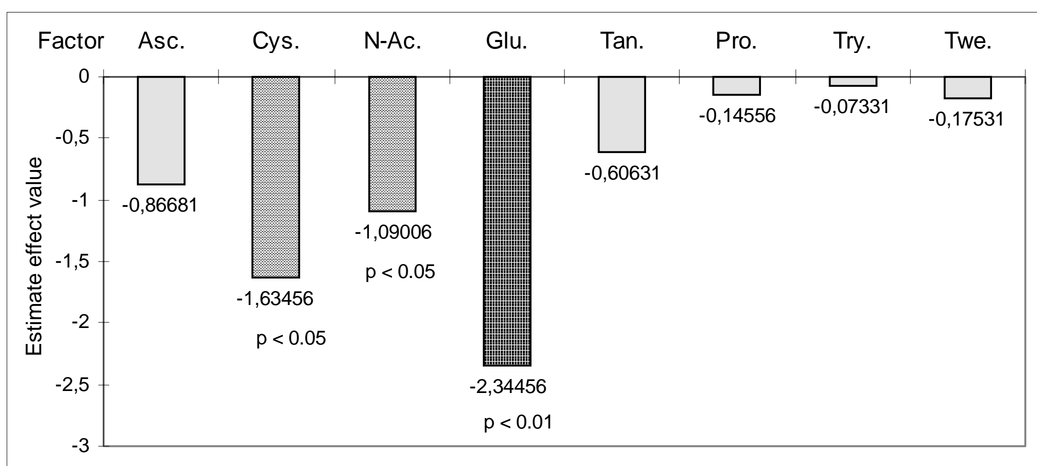

Figure 1: Influence of substances on MeIQx content in aqueous model system

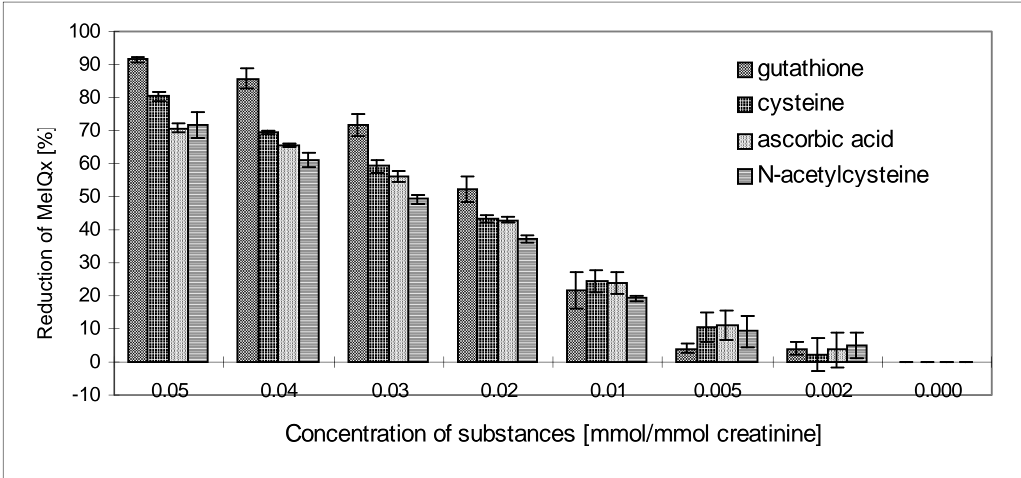

Figure 2: Reduction of MeIQx by different substances in aqueous model system

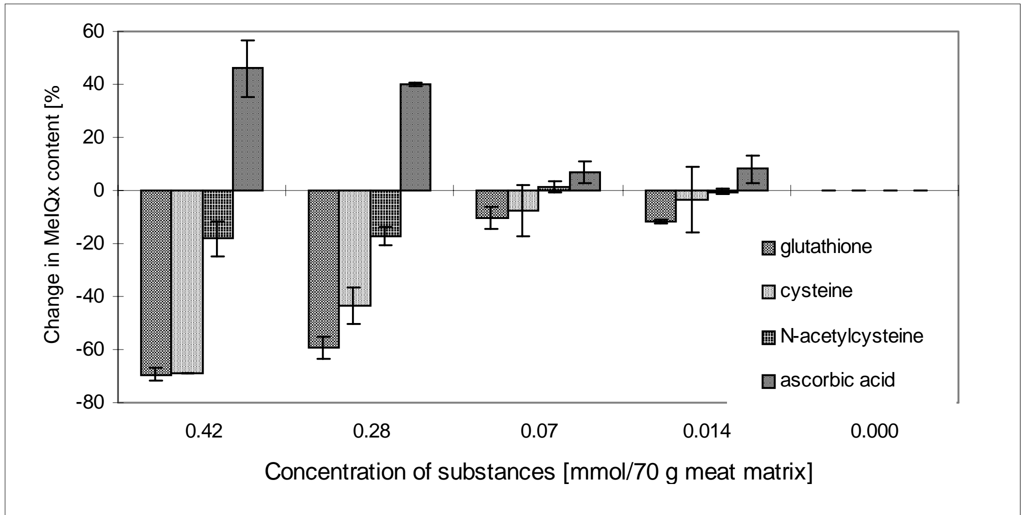

Figure 3: Effects of different substances on MeIQx content in meat matrix model

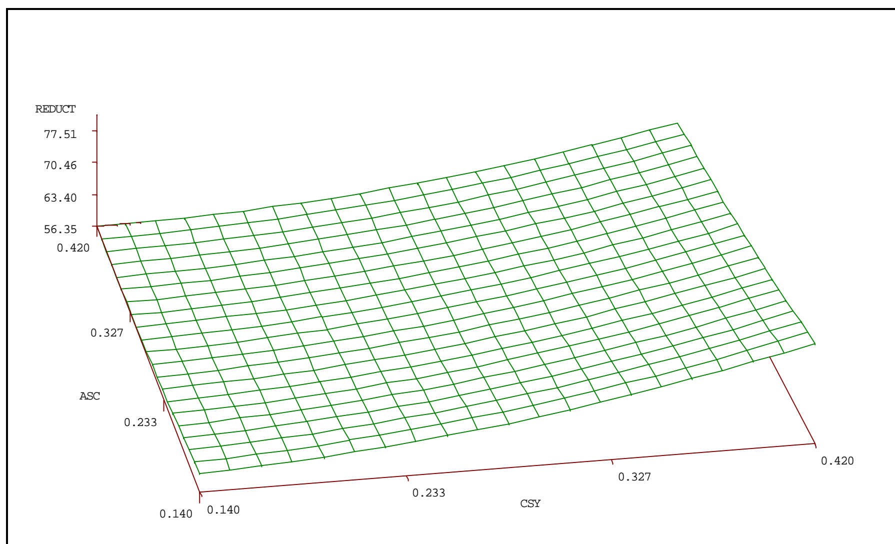

Figure 4a: Effect of cysteine* and ascorbic acid* on MeIQx reduction [%] (meat matrix model)

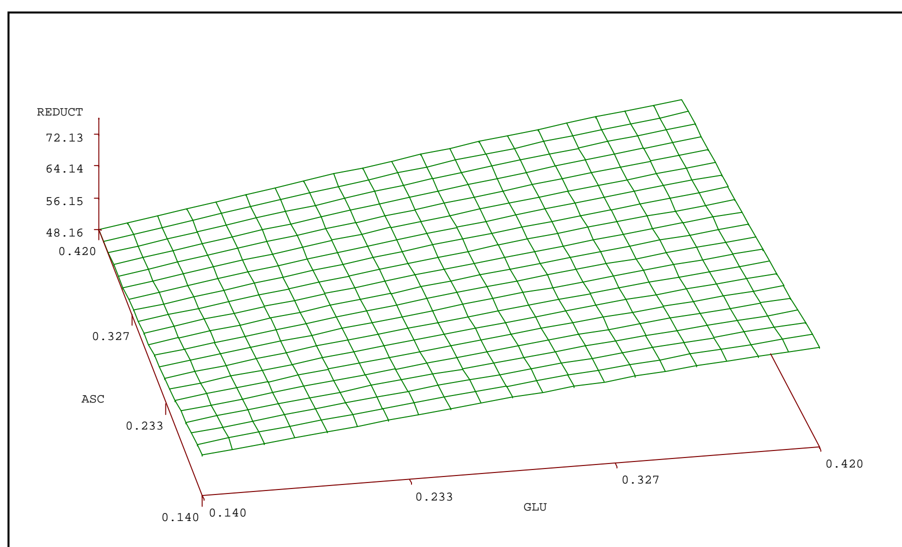

Figure 4b: Effect of glutathione* and ascorbic acid* on MeIQx reduction [%] (meat matrix model)

* the scaling for ascorbic acid, cysteine and glutathione axes are [mmol/70 g meat matrix]

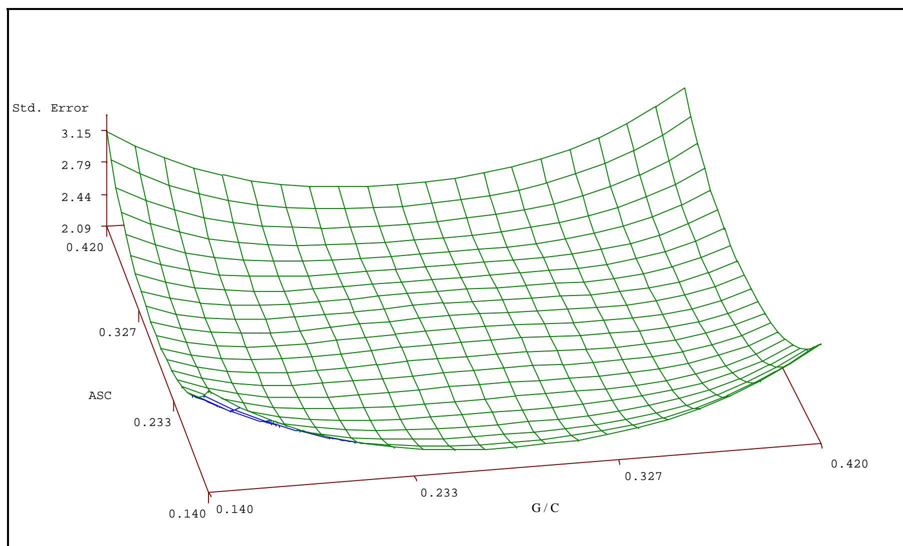

Figure 4c: Visualisation of standard error for the response surface experiment

Conclusions

As shown in the present study, the formation of MeIQx can be significantly reduced by the addition of SH-containing substances like cysteine, N-acetylcysteine or glutathione. The addition of ascorbic acid to the meat matrix based model systems results in higher quantities of MeIQx which is in contrast with the data obtained from the aqueous model system. Glutathione showed the strongest reduction on MeIQx formation in aqueous as well as in meat matrix based model systems. Therefore, glutathione could be tested as a promising additive to improve the toxicological quality of heat processed meats. More work is needed to determine the reactions behind this inhibiting effect and to explore the role of ascorbic acid and its interaction with the meat matrix in more detail.

Acknowledgements

We would like to thank Mrs Silvia Lasta and Mrs Erika Hustede for excellent technical assistance and the Department of Biotechnology for the generous use of their laboratory equipment.

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Control of proper heating of meat-and-bone meal by ELISA-test

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Introduction

The disastrous BSE epidemic amongst cattle („Mad cow Disease") in England was caused by feeding with inadequately heat-treated meat meals. That is why compliance with and monitoring of the heating regulations now in place throughout Europe for all animal carcass disposal plants is particularly important. The actual effect of this treatment on the material in the sterilizer can be checked using the ELISA-species test for pork (supplier CORTECS DIAGNOSTICS, England). The content of material from pig should no be less than 10 percent.

The principle of „meat meal" production

According to EU regulation (in effect since 1.4.1997) the material (animal bodies and meat disposals) has to be heated at least for 20 minutes at 133 °C under a pressure of 3 bar. Normally a so called pre-cooker is used (Fig. 1). The aim of this strong heat treatment is to kill all micro-organisms including the agents of BSE („Mad Cow Disease").

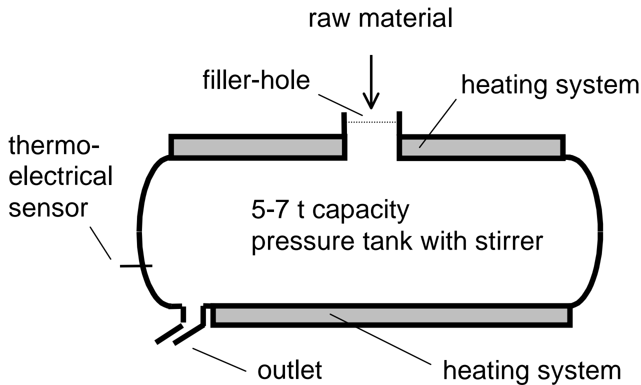

Fig. 1: Principle of „meat meal" production in a sterilizer („pre-cooker")

„Meat meal" inspection

For the inspection of „meat meal" production the physical data of heating, temperature, pressure and time have to be registrated (so called paper control). Furthermore bacteriological investigations have to be carried out. Recently also the effectivity of heating can be established by using the „ELISA-heating-test" which was developed recently in our laboratory (HOFMANN, 1996, 1997). These test has already been used with success for the control of proper heating of meat meals by several commercial producers themselves and by the official service control as well. The reproducibility and reliability of the test has been confirmed by a interlaboratory trial organised in Germany with the participation of 15 laboratories (HOFMANN, 1998).

The principle of the ELISA-heating-test

In the case of proper heating with the heat-damaged meat antigens only a weak immunoreaction can be observed, whereas meat meals insufficiently heated give a strong positive ELISA-test reaction resulting a green colour. The influence of heat-treatment on the immune response in the microwave module for the ELISA-test is shown in Fig. 2 and 3.

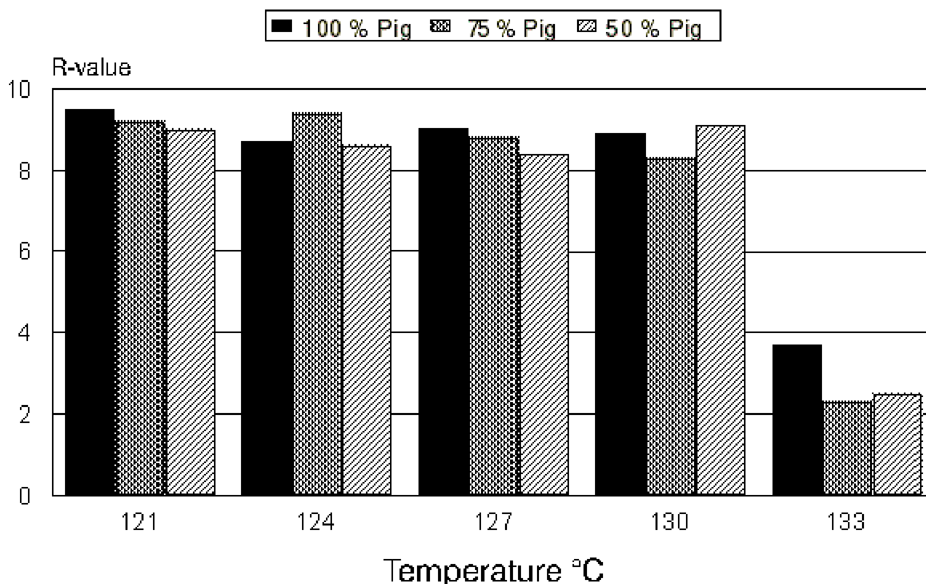

Fig. 2: Influence of heat treatment of meat on the ELISA-test. R-value: Measure of the immune reaction. All meat samples were heated for 20 min

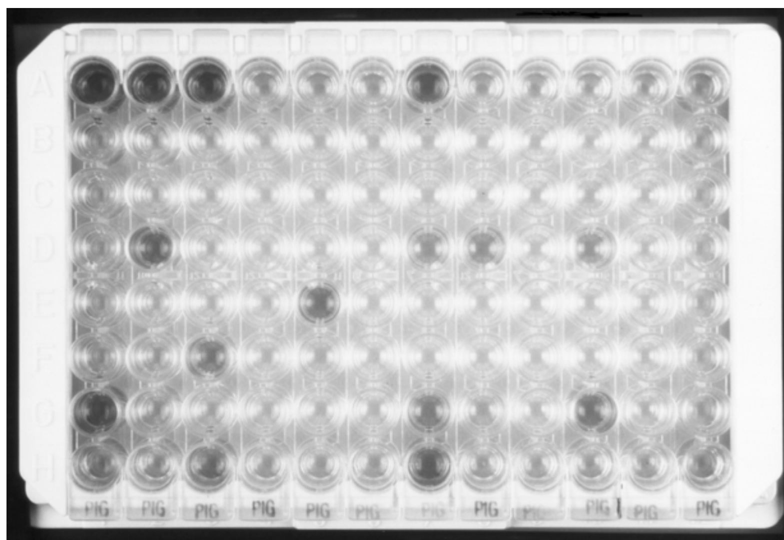

Fig. 3: Microwave module to carry out the ELISA-test. The development of a green colour (in the Figure: grey) means insufficient heating

The procedure

The sample preparation and the ELISA test were already described in earlier papers (HOFMANN at al 1996, HOFMANN 1996-1998) and shall be summarised shortly in the following:

6,25 g of the dry material is weighed into a 250 ml Erlenmeyer flask an then blended with 18,75 g of distilled water to compensate for water withdrawal. The soaking time allowed is 15 minutes. Then 100 ml physiological salt solution (0,9 % NaCl) is added, and the mixture is heated in a simmering water-bath for 15 minutes under stirring or shaking.

Having cooled down to ambient room temperature, the mixture is passed through a 15 cm diameter flute filter. A slight turbidity of the extract is of no concern (don’t repeat the filtering process!). In the next step a pipette is used to fill the undiluted filtrate to the test strip cavities of the microwave module. Afterwards, the ELISA test is performed as described in the supplier’s instruction and in those of the German dealer (TRANSIA, Ober-Mörlen). In the latter also the calculations and tolerance limits for evaluating the results (Fig. 4) are presented.

Meat and bone meal evaluation limits

R: 3,1 | 4,0

PK: 20,4 | 40

Regulatory Heating | Grey Area | Insufficient Heating

$$\text{R - value} = \frac{\text{sample value}}{\text{negative control}} \qquad \text{PK - value} = \frac{\text{sample value} - \text{negative control}}{\text{positive control} - \text{sample value}} \times 100$$

Fig. 4: Calculation of the measures (R- and PK value) and the limits to evaluate the heating status of meat-and bone meal

Evaluation

The limits for the „Thermal-Evaluation-Test“ (ELISA-TEST) are the result of numerous examinations of meat-and-bone meal products from different producers and of model samples specifically prepared for this purpose. For the evaluation of the results both values, R- and PK-values (see Fig. 4), are used.

A meat-and-bone meal product may be regarded as sufficiently (regularly) heated if both indicator limits are below their assigned maxima, i.e. $R < 3,1$ and $PK < 20,4$. On the other hand values of

R > 4 and PK > 40 indicate that the product has not undergone sufficient heat treatment. Between the two limits there is a range („Grey Area, see Fig. 4) in where it is not possible to make a decision as to whether the feed has been sufficiently or insufficiently heated.

A value of R = 2,5 which is based on experience with animal feeds heated to higher temperatures (UNGLAUB, 1997) cannot be accepted as a general limit value. According to general experience, even with an animal feed manufactured at the minimum stipulated (20 min at 133 °C, 3 bar), the R-value can be over 2.5.

Summary

In order to increase the safety of proper heating of meat-and-bone meals being important to minimise the BSE problem a test procedure, based on the immune reaction of meat antigens, has been developed. The test is based on an ELISA-species test for heated meat (CORTECS DIAGNOSTICS). The antigens of meat become inactivated under the heating conditions prescribed for „animal meals“ (20 min at 133 °C, 3 bar). As in most countries the raw material for the animal meal production consists of material form swine, the ELISA-test for pig is used. Limits of the evaluation have been determined by way of investigations carried out on meat-and-bone meals and on model samples specifically tailored for the purpose of this exercise. The ELISA-test provides a valuable tool for verifying the effectivity of heat-treatment of the final product. Therefore this method can be used for both the official control as well as for monitoring the sterilisation process in the producers own interest.

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Session 4:

Technological Methods to Improve Food Quality

Protein hydrolysis during the ripening of salted anchovy (*Engraulis encrasicholus* L.). An objective method to assess the ripening process.

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Abstract

The protein hydrolysis and proteinase activity during the ripening of salted anchovy were studied. A rapid, simple and inexpensive microassay method for determining the protein hydrolysis by trinitrobenzenesulfonic acid (TNBS) has been developed. A linear relationship was observed between proteolysis determination by TNBS method and ripening time in the fish muscle (r= 0.998) and in the brine (r= 0.995). A linear relationship also was observed between the ratio non-protein nitrogen and total nitrogen (NPN/TN) and ripening time (r=0.98). Proteolysis by TNBS method and NPN/TN determination could be considered as objective methods to follow and assess the ripening process of anchovy. A value of proteolysis by TNBS method of 270 mM of leucine in the fish muscle and/or 240 mM of leucine in the brine would indicate the ripening point.

Introduction

Ripened semi-preserved anchovies are prepared from fish of the *Engraulis encrasicholus* (L) species, belonging to C*lupeidae* family, by a process of salting and ripening. The ripening process takes at least two months for *E. encrasicholus* of Mediterranean sea (Campello, 1985), and 8-12 months for *E. anchoita* (Filsinger, et al. 1982) at a temperature between 16-20ºC. Ripening involves a series of complex biochemical processes, which can be grouped broadly into proteolysis, lipolysis and lipid oxidation. The physical and chemical changes that occur during ripening determine the overall organoleptic qualities of salted anchovy. Some attempts have been made to develop a suitable method such as using the total ester index (Filsinger et al., 1982), the total volatile basic nitrogen (TVBN) (Filsinger et al., 1984), the ratio of free amino acids to total amino acids (Baldrati et al., 1977), the ratio non-protein to total nitrogen (Campello, 1985; Durand 1982; Perez-Villarreal and Pozo, 1992) or the estimation of free fatty acids (Roldan et al., 1985). However, these parameters have not been very successful in predicting the quality of the final product or the stage at which anchovy develops optimum flavour and should go to market. As a result, visual inspection and tasting are still the usual methods of following the process.

This investigation was undertaken mainly to study the protein hydrolysis and to study the possibility of using some of these parameters as an objective method to follow and assess the anchovy ripening process.

Material and methods

Preparation of anchovy samples

Fifteen batches of ripened semi-preserved salted anchovies (*Engraulis encrasicholus var. mediterraneus*) were analyzed. Fresh anchovy were purchased from the local landing centre (Barcelona, Spain). These fish samples were caught in the Northeastern Spanish Mediterranean coast. After being caught, the fish were held with ice for at least 24 h before they arrived at laboratory where they were prepared following traditional method as described below. The fish samples were manually beheaded, partially gutted in the same operation and packed in 500 cc cans. A layer of salt was first put in the container then a layer of fish and so on until the container was filled with alternate layers of salt and fish finishing with a layer of salt. Cans were stored for ripening at 20ºC for 9 weeks. Samples were removed every week from the same processing batch until the ripening phase was finished.

Chemical analyses

Determination of degree of hydrolysis of protein by trinitrobenzenosulfonic acid (TNBS)

Basically, TNBS method is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines. The reaction takes place under slightly alkaline conditions (Adler-Nissen, 1979). TNBS also reacts slowly with hydroxyl ions whereby the blank reading increases, this increase is stimulated by light (Fields, 1971).

Preparation of samples

The fish muscle samples were solubilized as follows: 10 g of fish muscle were homogenized with 90 ml of buffer (borax buffer: 2% (w/v) of sodium dodecyl sulfate (SDS), 0.477 (w/v) $Na_2B_40_7.10\ H_2O$, pH 8.9 adjusted with HCl) and were shaken thoroughly in screwed capped 100 ml-flasks in a thermostatic bath at 75ºC for 15 min (to prevent proteolytic degradation) and then at 60ºC for 2 h. The brine samples were obtained by filtration through a No. 1 Whatman filter paper.

Microtiter plate assay

The following reagents were used: 0.2125 M phosphate buffer (0.2125 M NaH_2PO_4 is added to 0.2125 M Na_2HPO_4 until pH is 8.2 ± 0.02), 0.1% TNBS solution (covered with aluminium foil; the solution must be prepared immediately before use); leucine standard in borax buffer or distilled water. Fresh standards should therefore be prepared regularly.

The reaction was started by addition of: 10 µl of a sample containing between 0.25 and 4 mM or standard solutions, 80 µl of phosphate buffer and 80 µl of 0.1% TNBS solution. After incubation at 42ºC, the absorbance at 405 nm was directly read using a microtiter plate reader 340 ATTC (STL Labinstruments, Salzburg). Incubation time was studied for optimization of proteolysis quantification. Readings were realized every 5 min during 1 h. All the experiences were repeated 8 times.

Nitrogen compounds

Total nitrogen (TN) was determined on 0.5 g of fish muscle samples and 3 ml of brine using the macro-Kjeldahl method (Anonymous, 1985). Non-protein nitrogen (NPN) was extracted using trichloroacetic acid (TCA).

Statistical analyses

Analysis of variance and Kruskal-Wallis analysis were performed, previously verifying normality and variance homogeneity (Domenech and Riba 1990) to determine the differences between the experimental periods of storage. Regression analysis was performed to obtain the correlation between variables. All statistical analysis were performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc. Chicago, IL).

Results and discussion

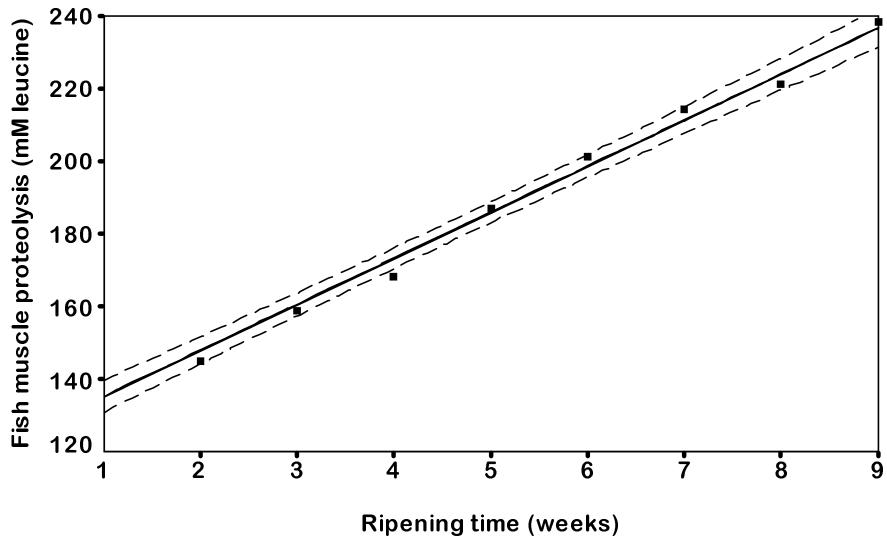
Proteolysis by TNBS method

Proteolysis results in the formation of a new "-amino groups, which can be measured by the reaction of anchovy extracts with TNBS reagents. This method is specially suitable for checking of blocking or unblocking of amino groups in proteins and peptides (Fields, 1971; Hatakeyama et al., 1992). An assay for proteolysis determination using TNBS has also previously been reported by Adler-Nissen, (1979). This author performed the reaction of TNBS for 60 min at 50ºC and the absorbance was measured at 340 and 420 nm. In our study, it was concluded that a period of at least 60 min at 42ºC should elapse to ensure reproducible results. Moreover, measurements were done at 405 nm since this is one of the wavelengths available for the microtiter plate reader that we used, and absorption of the products is comparably high enough to be determined at that wavelength. On these conditions, the linearity of the test was observed between 0.25 and 4 mM of leucine and the correlation was very significant after a regression analysis of the variance ($r= 0.9997$ and $p <0.01$).

The protein hydrolysis of fish muscle decreased slightly ($p<0.05$) from 135 to 133 mM of leucine during the first week of the ripening process, because nitrogenous substances chiefly of low molecular weight diffuses from the fish into the brine. After the first week, protein hydrolysis of fish muscle and brine increased gradually ($p<0.05$) throughout the ripening time. A higher correlation was obtained between fish muscle proteolysis and time ($r= 0.991$; $p<0.01$). However, this correlation is higher ($r= 0.998$; $p<0.01$) when it was only considered the ripening process between the 1st and 9th week (Figure 1). Moreover, a linear relationship was also observed between the proteolysis of the brine and ripening time ($r= 0.995$; $p<0.01$) (Figure 1). Consequently, proteolysis determination by TNBS method in the fish muscle and in the brine would allow to follow and assess the ripening process of anchovy. A value of 270 mM of leucine in the fish or 240 mM of leucine in the brine would indicate the ripening point. Proteolysis determination in the brine would allow to obtain the samples by not destructive methods. Moreover, the use of microtiter wells allows large numbers of samples to be assayed, the amounts of the samples and the reagents are small, and the following measurement of absorbance can be rapidly and simultaneously done by using a microtiter plate reader. The following expression relates the proteolysis quantification (x) of fish muscle (1) and brine (2) to the time (y):

$$y = 13.256\,x + 111.07 \qquad r= 0.998 \quad (1)$$

$$y = 13.574\,x + 79.65 \qquad r= 0.995 \quad (2)$$



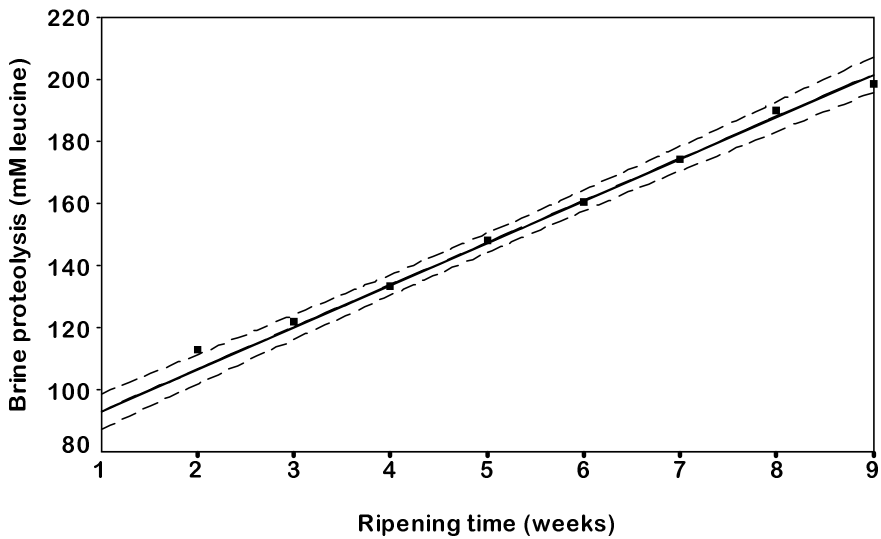


Figure 1: Protein hydrolysis determined by TNBS method in fish muscle and brine *versus* time during the ripening of salted anchovy. The area between the dashed lines is the 95% confidence limits of the regression estimate.

Nitrogen compounds

The protein nitrogen (PN) content in the fish muscle decreased markedly ($p<0.05$) after 6 weeks and then gradually to 2.85% after 9 weeks. However, the PN content in the brine remained constant after six weeks and then decreased appreciably until the 9th week. After 9 weeks, the 88% of the total nitrogen of the brine were NPN compounds. Protein gradually loses their colloidal properties and diffuses from the fish into the brine, where they undergo further changes. In spite of a considerable protein degradation the ripened anchovy maintains its structure and is easily cut into fillets.

The non-protein nitrogen compounds (NPN) of fish muscle decreased appreciably ($p<0.05$) from 0.68% to 0.53%, during the first week of the ripening process. Thereafter, the NPN increased markedly ($p<0.05$) until the sixth week and then it remained constant. A similar evolution was observed in the NPN content of the brine. During salting, the exchange of matter in the system is accomplished mainly by the movement of salt molecules; but during the ripening period nitrogenous substances, mainly of low molecular weight (NPN compounds), diffuses from the fish into the brine (Voskresensky, 1965). In this study, during the first week of the ripening, the 75.4% of total nitrogen of the fish muscle that diffuses into the brine are NPN compounds.

The correlation between NPN/TN in the fish muscle and time observed was $r= 0.621$ ($p< 0.01$). However, a higher correlation was obtained between anchovy NPN/TN and time ($r= 0.987$; $p<0.01$) when it was only considered the ripening process between the 1st and 9th week (Figure 2). Moreover, a linear relationship was also observed between the NPN/TN of the brine and ripening time ($r=0.979$; $p<0.01$) (Figure 2). NPN/TN determination in the fish muscle and in the brine would also allow to follow and assess the ripening process of the anchovy. The NPN/TN determination in the brine has the advantage that samples could be obtained by not destructive methods, but their correlation coefficient is lower than correlation coefficient of fish muscle. The following expression relates the NPN/TN content (x) of fish muscle (3) and brine (4) to the time (y):

$$y = 0.898\,x + 12.96 \qquad r= 0.987 \quad (3)$$

$$y = 1{,}544\,x + 74.61 \qquad r= 0.979 \quad (4)$$

The ratio non-protein and total nitrogen have already been proposed as an objective method to follow and assess the ripening process of the anchovy (Campello, 1985; Durand, 1982; Perez-Villarreal and Pozo, 1992). These authors observed a high correlation between NPN/TN of fish muscle and ripening time and they proposed a value of NPN/TN between 33 and 40% as indicative of the ripening point. However, in our work, a value of NPN/TN of 23% in the fish muscle and/or 91% in the brine would indicate the ripening point.

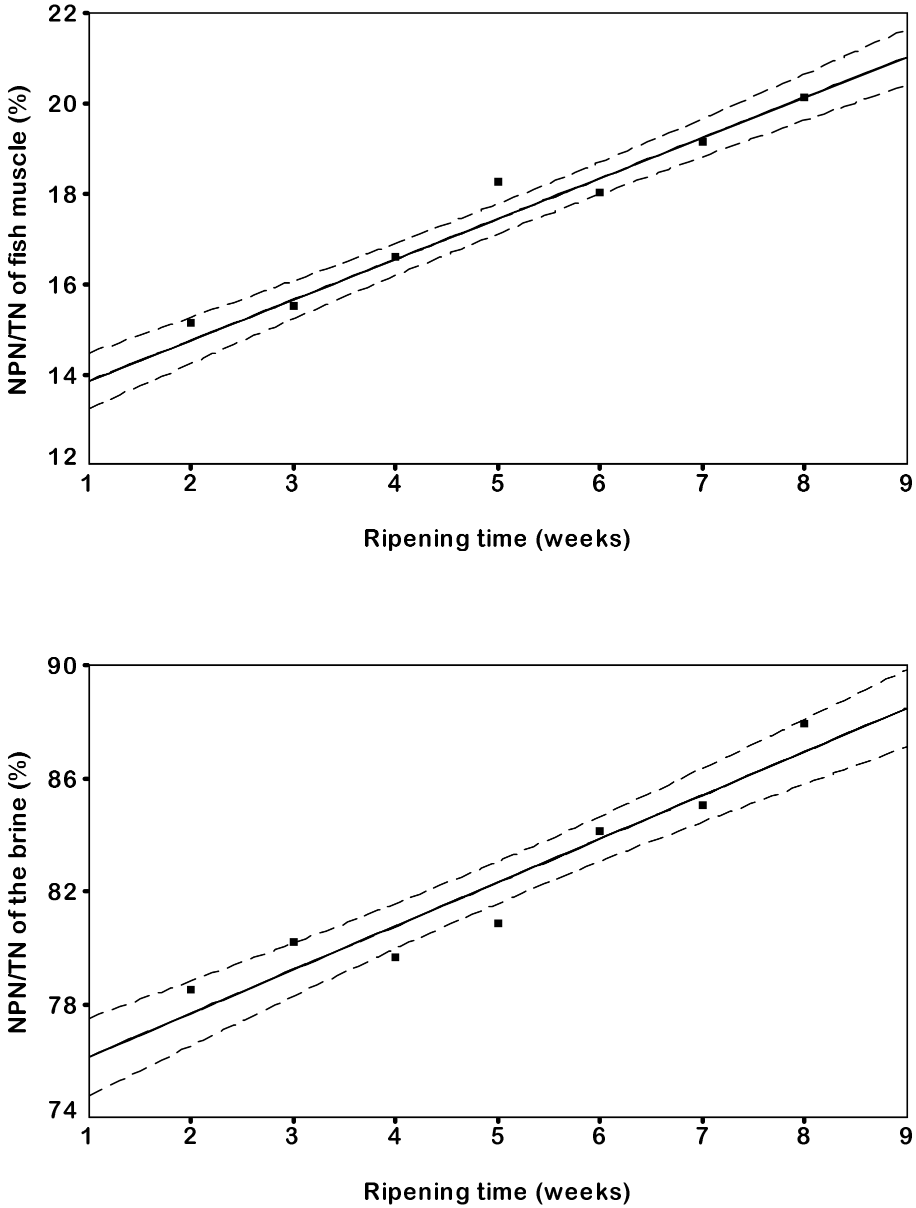


Figure 2: Ratio non-protein nitrogen and total nitrogen (NPN/TN) in fish muscle and brine *versus* time during the ripening of salted anchovy. The area between the dashed lines is the 95% confidence limits of the regression estimate.

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Hazard Analysis and Critical Control Point system in apple juice processing

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Introduction

The "New Regulation Sanitarium of Food", effective in Chile since November 1997, clearly shows the need of the country of being modern regarding the international approaches contents in Codex Alimentarius (elaborated by FAO and the OMS jointly), that are fundamental facilitate as much the exports as the imports.

In spite of the great advance that this regulation represents, there are some aspects that remain unregulated which make necessary the support of the Codex. The Codex Alimentarius proposes specific systems for food control, like the Hazard Analysis and Critical Control Point System (HACCP), that at the present time, is the system used in Chile by big international exporters (as Cargill and Nestlé), who have become a model for the incipient Chilean food industry.

Chile is a large agroindustrial exporter, providing almost 6,9% of the world juice market, mainly for countries as USA, Japan, Canada, Argentina and Germany.

Fig.1 shows the production and export of apple juice in different countries, in 1997 (Carreño and Lazo, 1998).

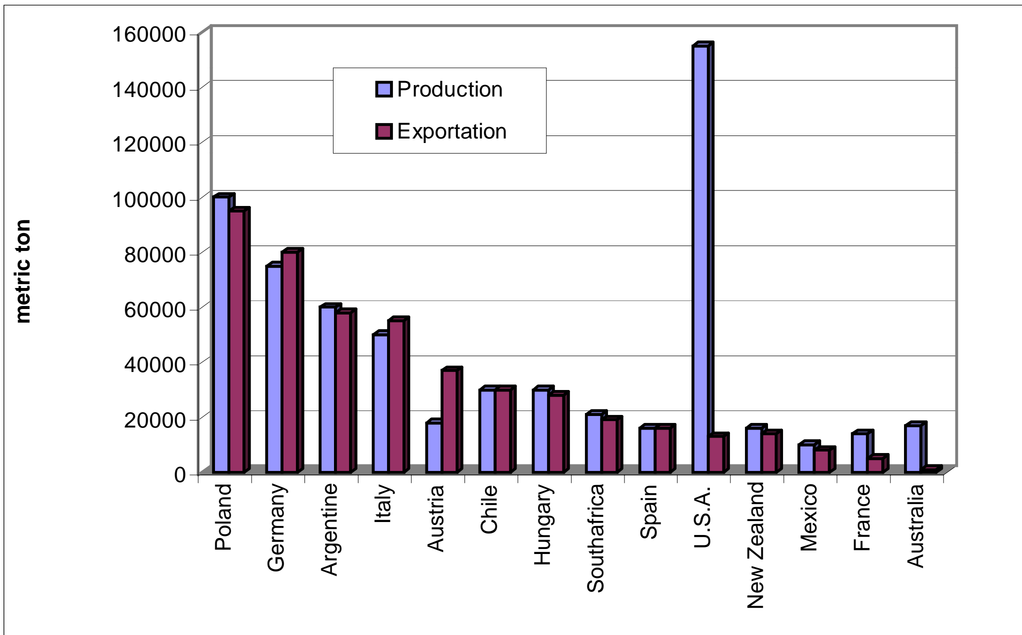
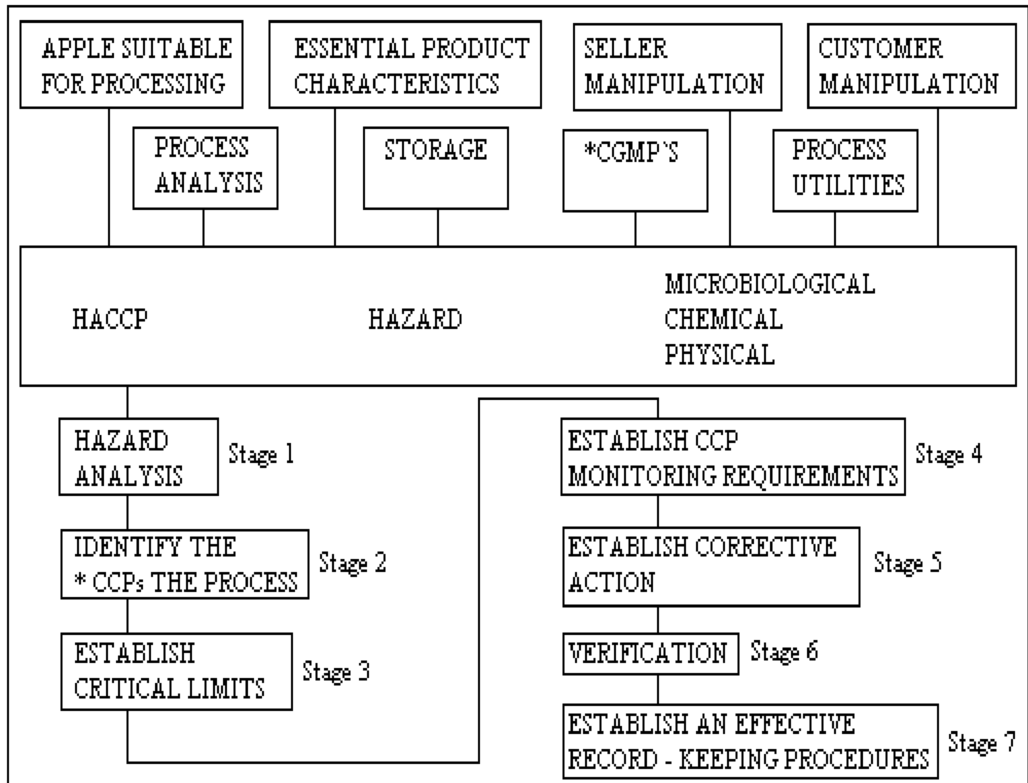


Fig. 1: Production and export of apple juice.

In this study we will implement HACCP system to an obsolete apple juice processing defining a new process, the steps at which control can be applied, and giving the relevant aspects to consider in juice safety hazard. Based on the new techniques applied to control and/or to eliminate a microbiological, chemical or physical hazard. We established the implementation procedure of the first three stages of the system, since each step involves a very extensive study, according to the scheme as shown in Fig.2 (Mortimore,1994).



*CGMP'S: Current good manufacturing practices, CCPs: Critical control points

Fig. 2: Stages in HACCP analysis

The Hazard Analysis and Critical Control Point (HACCP) system is an effective, proactive approach to assuring the safe production of food products. The basis premise of HACCP is that of prevention rather than inspection or end-product testing. Although the HACCP concept is applicable to all stages of a food production process (including agricultural practices, equipment used, growing location, degree of animal contact, product handling, distribution, sale, storage and customer consumption), its strength is in its suitability to be applied to specific operations and to specific products.

Stage 1

Hazard Analysis

Hazard analysis deals with all aspects of a food chain and includes production, distribution, sale, storage and customer consumption. It includes determination of the hazard , assessment of risks and effects of each hazard as determined using a process flowsheet.

To identify hazards the fundamental questions are:

1. Does a source of damage exist?
2. Who (or What) could be damaged?
3. How could the damage occur?

From the discussion of risk factors and critical control points it has been shown that instrumentation and measurement of various control factors is only possible in a limited number of cases. Many important control points rely on the knowledge and implementation of good manufacturing practice guidelines. These are based on expert knowledge and experience of operating systems; they cover every eventuality which can possibly occur.

The following questions are some of the necessary requirements to be considered in juice processing:

1. Food process equipment
   - Is the plant correctly assembled?
   - Has it been hygienically designed?
   - Is the plant internally clean?
   - Does the control system work consistently and have the sensors been assessed for accuracy, reproducibility and calibration?

2. Aseptic filling system
   - Is the system cleanable to the required microbiological standards?
   - Can it be separated from the processing plant by a sterile barrier?
   - Does the control system work reliably and have the sensors been checked?
   - Can the system be cleaned adequately prior to use?
   - Is asepsis being consistently achieved in the filling zone?

3. Packaging material
   - Is the packaging material supplied consistent with specifications?
   - Is the final package consistent with specification?
   - Can the packaging material be adequately decontaminated?

To start with the Hazard Analysis of the process we need the flow diagram shown in Fig.3.

Next, we establish the main possible risks in each stage for the production of Cloudy Juice using the flow diagram.

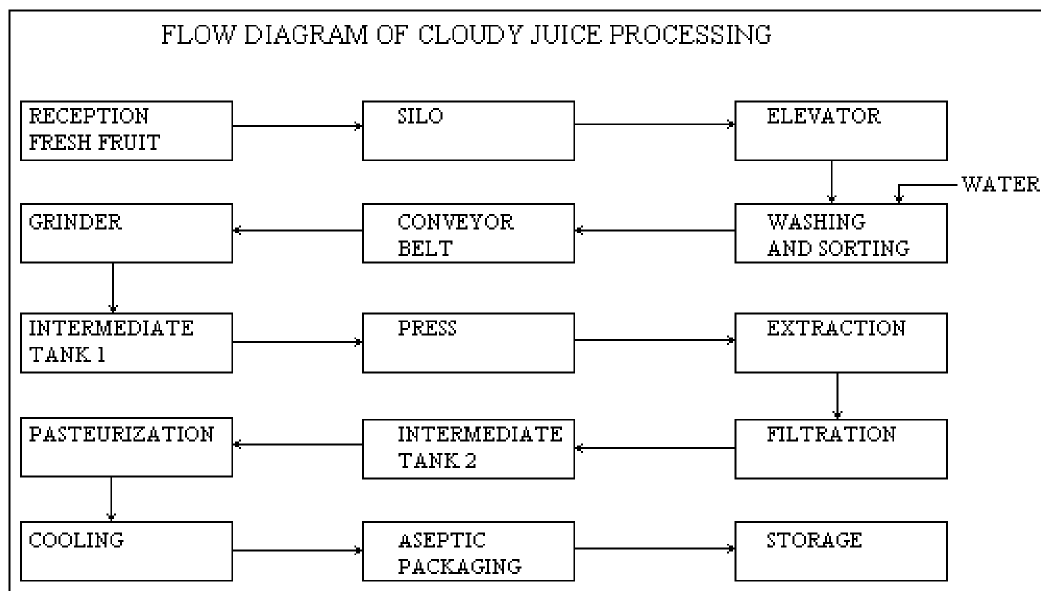


Fig. 3: Flow sheet of Cloudy Juice Processing

Practical Hazard Control for couldy apple juice processing

PROCESSING STEP	HAZARD
Reception of fresh fruit	- Agricultural practices (Pest infestation) - Growing locations of raw material affects acidity and another properties of the juice - Insect and their less - Rotten fruit - Place to storage (patio, refrigerator or the fruit need immediate processing) - Storage conditions - Growth of pathogens due to temperature abuse (Hold-up prior to processing should be minimized in order to avoid excessive growth) - Contamination by rodents - Mixture of varieties and with other fruits. This affects acidity and ºBrix of final juice
Silo	- Mechanical damage - Mixture of varieties - Extraneous material (metallic objects, glass and miscellaneous objects intentionally or unintentionally present). These should be absent from the raw materials since they could cause damage to equipment).
Washing and sorting	- Rotten fruit - Insect contamination - Employed distraction - Contamined water - Physical contamination (stalks, stones, leaves, insects, plastic, metals). These should be absent from the raw materials since they could cause damage to homogenizer or pumping equipment and cause blockages in the system.

Grinder or crushing	- Contamination by rodents - Equipment contamination - Material of construction of the equipment - Chemical residues of detergents and sanitizants
Intermediate tank 1	- Air contamination (Air is contaminated with micrococci, yeast and moulds which are often to with dust particles and small droplets of water. The sizes of the microorganisms range from 1-10 μm for yeasts and moulds, 0.1-5μm for bacteria and <0.1μm for viruses and they are generally present in quantities between 100 and 1000 per m^3. The number of yeast or mould spores is usually about 200 which is ten times that of the bacteria present in air (Taubert,1972;Wallhäusser, 1987). - Long residence time - Temperature alterations - Reactions occur during storage in the tank - Development of off-flavours - Contamination by sodium hydroxide sanitizing agent used for cleaning - Contaminations by birds, rodents and insects - Miscellaneous objects intentionally or unintentionally present - Chemical contamination from surface contact
Filtration	- Chemical contamination arising from cleaning and desinfecting materials, impure diatomite earth, intentional or unintentional - Foreign body contamination not removed
Intermediate tank 2	- See Intermediate tank 1
Pasteurization	- Microbiological - Sedimentation of constituents - Crystallization of food ingredients - Chemical reactions including degradation due to elevated temperatures - Protein denaturation, starch decomposition and undesirable product interaction which occur at the localized high temperature in the laminar sub-layer. - Fouling of heat transfer surfaces by deposits of thermal degradation products (deposits from minerals present in the product) - Organoleptic losses - Residues of chemicals, used in Cleaning in Place systems - Degradation of nutritional value - Faulty thermometer (instrumentation failure) - Residence time
Cooling	- Microbiological. Outgrowth of spores due to slow cooling.
Aseptic packaging	- Employee distraction - Miscellaneous objects intentionally or unintentionally present - Improper personal hygiene - Nonsanitary conditions of the environment and equipment
Storage	- Losses of organoleptic characteristics - Losses of shelf-life - Wrong temperature of storage - Storage time

General Hazards

1. Contact with cooper pipe fittings or other metals that can cause chemical reactions with the product.
2. Pieces of glass or plastic presents in bottles.
3. Customer handling practices.
4. Personnel practices.
5. Piping, elbow, fittings and other dead spaces in process equipment that can cause microbiological growth.
6. The cloudy apple juice contains a large quantity of fine cellular material, so it is essential to take precautions to prevent oxidation. The process is, therefore, continue with the juice never being exposed to the air.

Stage 2

This stage includes the process analysis for the identification of critical control points

PROCESS STEP	CCP-type
Reception of fresh fruit	CCP2
Silo	CCP2
Washing and sorting	CCP1
Grinder or crushing	CCP2
Intermediate tank 1	CCP2
Filtration	CCP2
Intermediate tank 2	CCP2
Pasteurization	CCP1
Cooling	CCP1
Aseptic packaging	CCP1
Storage	CCP2

CCP1 Reduces contamination physical, chemical or microbiological. The control is not completely efficient.

CCP2 The last opportunity to eliminate contamination by physical, chemical or microbiological means. The control must be completely efficient

Stage 3

To establish critical limits it must know the characteristics of the final product, therefore, we give basic information about Apple Juice Composition and some typical agents involve in microbiological alteration.

Composition of apple juice

Apple juice contains a considerable portion of soluble constituents of the original apple (Table 1, Carreño and Lazo, 1998), for example, sugar, acids, other carbohydrates and minerals. The water content of the fruit affects the quality and composition of the juice, because its affects the percentage of soluble solids. The pectin and "pectin-like" compounds have a marked effect on the

"body" or viscosity of the juice. The presence of these latter substances influences the easy or difficulty encountered when the juice is filtered and also affect the stability of the juice packed without being brilliantly filtered.

The predominant sugar in apple juice is levulose with small amounts of sucrose and glucose. (Hulme, 1970).

Typical alteration agents of the juice

Among the fermentation producing yeasts are found: Saccharomyces cerevisiae, Saccharomyces uvarum, Saccharomyces florentinus, Saccharomyces bailii, Brettanomyces naardensis, Torulopsis stellata, Candida parapsilopsis

Among the bacteria, the main alteration agents are: lactic acid bacteria and Leuconostoc type.

Potential Agents of alteration of the juice

Essentially aerobic microorganisms like Hansenula, mushrooms like Mucor and Oosporas

Observed alterations for these microorganisms

Modifications of aspect, turbidity, appearance of floculates, sediments, gelification, strange smell, decrease of turbidity in cloudy juice (pectolitic organisms), fading of natural pigments of the juice (by yeasts or bacterias). (U.Chile, 1997)

Some pathogens microorganisms found in apple juice (FDA, 1998).

E. Coli O157:H7 Salmonella typhimurium Yeast	Causing severe gastrointestinal illness and death of children

Table 1: Chemical Composition of Apple (100g edible part)

Calories	56 g
Humidity	84.2 g
Proteins(N*6,25)	0.3 g
Lipids	0.3 g
Carbohydrates (by difference)	14.5 g
Crude Fiber	0.5 g
Ash	0.2 g
Ca	7 mg
P	10 mg
Fe	0.2 mg
Na	1 mg
K	108 mg
Thiamin	0.04 mg
Riboflavin	0.07 mg
Niacin	0.2 mg
Total Ascorbic Acid	5.6 mg

Some of the critical limits established, for this process are:

Pasteurization: 90-92ºC; and 15-20s

Total aerobic microorganisms

- Incubated at 37ªC, 24 h: Max 10 colonies/ml
- Coliformes: absent in 100 mL
- Escherichia Coli: absent in 100 mL
- Streptococcus D de Lancefield: absent in 100 mL
- Clostridium perfringens: absent in 100 mL
- Pathogenic microorganisms and parasites: Total absence

It is recommended to make a total count of moulds and it is recommended that it should not exceed 10 colonies per ml (Instituto Nacional De Sanidad,Valencia, 1993)

Storage temperature: 0°C

Finally we propose some preventive measures to consider in Cloudy Juice Processing

Preventatives measures

Microbiological Hazard

Raw materials:

- Specification for organism and/or toxin
- Evidence of control during supplier process
- Testing
- Certificate of analysis
- Visual inspection
- Application of FIFO (First in-first out)

Personal

- Hand and wash procedures
- Covering cuts/wounds, etc.
- Occupational health procedures
- Management control of food handlers
- Build up during process
- Control of temperature to avoid growth of microorganism in products

General

- Design of process equipment to minimize dead volume
- "Clean as you go" procedures
- Uses of a new method of cleaning validation System. This systems consists of a luminometer and swab devices to detect food residues by ATP, and provides immediate feedback to guide the cleaning process.
- Secure building (roof leaks, ground water, etc.)
- Logical process flow, including where necessary: segregation of people, clothing, equipment, air, processing area, direction of drain and waste disposal

- Control of harvesting and storage to prevent mould growth and mycotoxin formations in raw materials
- Control of heat treatment(Temperature and Time)
- Pasteurization Critical limits: 90-92ºC, 15-20s
- Use of filtered water

Physical Hazard

- Metal detection-sensitivity appropriate for the product, calibrated (3-monthly) and checked (hourly)
- Visual inspection
- Sieving
- Magnets
- Stone traps
- All persons entering food handling areas remove jewellery and other objects which may fall into or otherwise contaminate food. Jewellery, including wedding bands and medical alerts which cannot be removed, are covered.

Chemical Hazard

- Use of non-toxic, food-compatible cleaning compounds
- Safe operating practices and written cleaning instructions
- Separate storage for cleaning reagents
- Label and cover container for all chemicals
- Specification to include supplier compliance with maximum legal dosage levels

Conclusions

- HACCP including aseptic processing and packaging has been developed as a very viable and versatile method for food cloudy apple juice packaging in Chile. The process requires a high degree of engineering expertise in order to produce juice, with a high degree of microbiological integrity. Provided that the food processor and marketer observes the requirements of the total process then the public can have a very high degree of confidence in de product.
- To apply the system HACCP, knowledge of the total process is required, including all the points of the chain until arriving to the consumer.
- It is fundamental the motivation, the control and the training of the manipulators of foods for their effective implementation.
- The implementation of HACCP is translated in competitive advantages with regard to the competition but it is a system that involves a high investment.
- The National Academy of Sciences of USA, National Advisory Committee for microbiological criteria for food, and the Codex Alimentarius have endorsed HACCP as the best process available today and this method can be applied to any kind of food.

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Controlling temperature during distribution and retail

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Summary

Substantial advances have been made in the design, construction and operation of containers for the long distance transportation of chilled and frozen food. Care needs to be taken to cool the food to the correct temperature before loading and to establish the required air gap between the food and the walls of the container. If both these criteria are met then food temperatures can be maintained to within ±0.5°C of the control point during long distance, sea or land, transportation.

Maintaining the temperature of chilled foods during short distance distribution to retail and catering outlets is a much more difficult problem. The refrigeration system has to cope with a vary variable heat load due to door openings, changing ambient conditions, entry of people, etc. To help designers, constructors and operators of such vehicles a user friendly computer program ‘CoolVan’ has been written to allow the effect of the interaction of the variables to be studies.

Maintaining food temperatures under retail display conditions is an equally difficult problem due to the marketing requirements which require easy access to the food. This makes it difficult for cabinets to be designed and operated in a manner which maintains the food at its optimum temperature.

Introduction

Developments in frozen transport in the last century established the international food market. Further developments in temperature controlled transportation systems for chilled products have led to the rapid expansion of the “fresh” food market. The sea transportation of chilled meat from Australia to European and other distant markets and road transportation of chilled products throughout Europe and the Middle East, is now common practice. Air freighting is increasingly being used for high value perishable products such as strawberries, asparagus and live lobsters (1). However, foods do not necessarily have to fall into this category to make air transportation viable since it has been shown that “the intrinsic value of an item has little to do with whether or not it can benefit from air shipment, the deciding factor is not price but mark-up and profit” (1).

In a survey carried out in 1970-71 of vehicles used to transfer chilled meat from small abattoirs to shops almost 70% were unrefrigerated and 20% had no insulation (2). Since that time the intensifying demands from legislation and retailers for lower delivery temperatures, has put increasing pressure on fleet operators to improve temperature control. However, there are substantial difficulties in maintaining the temperature of chilled foods transported in small refrigerated vehicles that conduct multi-drop deliveries to retail stores and caterers. The vehicles have to carry a wide range of products and operate under diverse ambient conditions. During any one delivery run, the chilled product can be subjected to as many as fifty door openings, where there is heat ingress directly from outside and from personnel entering to select and remove product. The design of the refrigeration system has to allow for extensive differences in load distribution, dependent on different delivery rounds, days of the week and the removal of product during a

delivery run. All these problems combine to produce a complex interactive system in local delivery which is not present in long distance transportation.

Retailers are discovering that considerable quality and economic advantages can be derived from maintaining chilled products at temperatures far closer to their initial freezing point. Maintaining such temperatures during retail display is another difficult operation. A critical element is the need for food to be at, or below, the required temperature before loading. Consequently, fleet operators will be required by retailers to deliver chilled food at temperatures between 0 and 2°C.

Long distance transportation

Overland

Most long distance overland transport of chilled and frozen foods is carried out in 12 m long mechanically refrigerated containers.

Loading with warm product and inadequate air distribution are the principle causes of product deterioration and loss of shelf life during transport. Conventional forced air units usually discharge air over the stacked or suspended products, either directly from the evaporator or through ducts towards the rear cargo doors. Because air takes the path of least resistance, it circulates through the channels which have the largest cross sectional area. These tend to be around rather than through the product. If products have been cooled to the correct temperature before loading and do not generate heat, then they only have to be isolated from heat ingress. Surrounding them with a blanket of cooled air achieves this. Care has to be taken during loading to stop any product touching the inner surfaces of the vehicle because this would allow heat ingress during transport. Many trucks or containers are now being constructed with an inner skin that forms a return air duct along the side walls and floor, with the refrigerated air being supplied via a ceiling duct.

Gill and Phillips (3) found that the deep temperature in beef sides and quarters at the time of their loading into transport vehicles, in three USA plants, ranged from 6 to 18°C. Maximum surface temperatures were also high and ranged from 0.5 to 6.5°C. In rail wagons, the surface temperature declined during the first 24 h and was subsequently maintained at a temperature of 0±1°C. In the road vehicles, the surface temperature fell slowly during the whole journey and had not attained a steady minimum value when unloaded. On average the deep temperature of sides in rail wagons reached 1°C after 72 h. Temperatures in quarters in road vehicles were still above 2°C after 120 h.

Sea transport

Recent developments in temperature control, packaging and controlled atmospheres have substantially increased the range of foods that can be transported around the world in a chilled condition. With conventional vacuum packing it is difficult to achieve a shelf life in excess of 12 weeks for beef and 8 weeks for lamb (4). However, a shelf life of up to 23 weeks at -2°C was achieved in cuts of lamb that were individually packed in evacuated bags of linear polyethylene, and then placed in foil laminate bags that was gas flushed and filled with a volume of CO_2 approximately equal to that of the meat (5). In the late 1990's similar storage lives are being achieved with beef primals transported from Australia and South Africa to the European Union (EU). Heap (6) stated that assuming good standards of preparation and prompt cooling, the times given in Table 1 could be used as approximate guidelines for long distance meat shipment.

Table 1: Guidelines for storage life of meat during shipment (6).

	Vacuum pack 0°C	Vacuum pack -1.5°C	CO_2 -1.5°C
Pork	6 weeks	8 weeks	-
Lamb	7 weeks	10 weeks	>12 weeks
Beef	10 weeks	14 weeks	-

These times rely on the meat being at or below the storage temperature before loading. The 2 to 4 week advantage of transporting meat at -1.5 rather than 0°C will be lost if the meat is loaded at a temperature above 0°C. Cooling in the centre of a load of meat is very slow and the meat will be well into its journey before the desired temperature is achieved.

Most International Standard Organisation (ISO) containers for food transport are either 6 or 12 m long, hold up to 26 tonnes of product and can be 'insulated' or 'refrigerated' (7). The refrigerated containers incorporate insulation and have refrigeration units built into their structure. The units operate electrically, either from an external power supply on board the ship or dock, or from a generator on a road vehicle. Insulated containers either utilise plug-in type refrigeration units or may be connected directly to an air-handling system in a ship's hold or at the docks. Close temperature control is most easily achieved in containers that are placed in insulated holds and connected to the ship's refrigeration system. When the containers are fully loaded and the cooled air is forced uniformly through the spaces between cartons, the maximum difference between delivery and return air can be less than 0.8°C. All the product in a container can be maintained to within ±1.0°C of the set point.

Refrigerated containers are easier to transport overland than the insulated types, but have to be carried on deck when shipped because of problems in operating the refrigeration units within closed holds. On board ship, they are therefore subjected to much higher ambient temperatures and consequently larger heat gains, which makes it far more difficult to control product temperatures.

For bulk transportation of frozen meat refrigerated cargo ships are commonly used (6). Frozen meat is generally stored and transported at -18°C or below. The storage life of unprocessed meat increases as the storage temperature is lowered and microbial growth will not occur at temperatures below -12°C. Small temperature changes during loading and unloading can therefore be tolerated with frozen meat.

Air transport

Although air-freighting of food offers a rapid method of serving distant markets, there are many problems because the product is unprotected by refrigeration for much of its journey. Up to 80% of the total journey time is made up of waiting on the tarmac and transport to and from the airport. During flight the hold is normally between 15 and 20°C. Perishable cargo is usually carried in standard containers, sometimes with an insulating lining and/or dry ice, but is often unprotected on aircraft pallets (1).

Sharp's studies in Australia have led to the following recommendations for air transport of chilled foods:

1. Insulated containers should always be used to reduce heat gain.
2. Product should always be pre-cooled and held at the required temperature until loading.
3. With products that deteriorate after any surface freezing, dry ice should not be used.
4. Containers should be filled to capacity.
5. A thermograph should accompany each consignment.

Short distance transportation

The majority of chilled products are delivered to retail stores by sales vans. These are small to medium size refrigerated vehicles which are loaded with products in the morning and travel around to a series of retail outlets, selling to each in turn. They therefore have a large number of stops when the doors are opened and food is removed from the van. Sometimes, food which has passed its sell-by-date and empty trays are returned from the shops to the vans. The insulation, door protection and refrigeration plant fitted to the vans have sometimes proved inadequate to maintain food temperature as cold as required. Operators of the vans need to know in advance whether a particular van, on a particular round, under given ambient conditions, will be able to deliver food at the correct temperature.

A computer program, ‘CoolVan’, is now available to aid the design and operation of small delivery vehicles. The heart of the model is the temperature of the air inside the van. This air exchanges heat with the outside environment by the movement of air into and out of the van whilst the doors are either opened or closed. Heat is conducted into the van through the insulation from the outside air and from solar radiation on the outside of the van. Inside the van the racking, fittings, trays and food exchange heat with the van air. Evaporator coils or eutectic plates cool the van air to maintain its temperature.

At each stage in the program’s development, it was tested against measured data. The program was able to predict the mean temperature of the food in the vehicle with an accuracy better than 1°C at any time throughout the journey. However, food temperatures within the van actually varied by more than 5°C at any one time, due to the uneven temperature distribution within the vehicle. The composition of each side of the van is modelled separately and heat flow through each side assessed independently. Each side can be modelled as up to three layers, an inner and outer skin and insulation. The thermal properties of new insulation are entered. The year of manufacture and the ageing rate are also entered and the program calculates the reduced thermal properties of the aged insulation.

The structure of the program also allows different external heat transfer coefficients to be entered for each side of the vehicle. By default these are calculated values based on the vehicle’s speed, but the default values can be over-ridden by the input of measured values or values taken from different calculations.

Solar radiation onto each surface of the van is modelled separately. The direction in which the van is moving (heading) is input as data in the initial program. A complex journey can also be modelled in which the van’s heading changes during each stage of the journey.

The infiltration of outside air into the van is dependent on the van structure, the degree of maintenance and the speed of the vehicle. This was measured empirically in several vans, all of which exhibited the same characteristics. There is a minimum vehicle speed below which the air infiltration rate does not vary with speed, usually less than 4.5 ms^{-1}. Above this speed the rate of infiltration increases linearly with speed.

Infiltration of air into the vehicle during door openings is much greater. This is a function of air exchange when the door is opened and closed and is independent of time and temperature. Once the door is opened the amount of air infiltrating the vehicle increases linearly with time. The amount of air exchanged during this period is dependent on the area of the opening, its height, any barriers that are put in the way and on the temperature difference between the air inside and outside the vehicle.

Transparent plastic strip curtains, inner lightweight sliding doors or shielding are sometimes used on doors in order to limit the air exchange through them. To accurately model the air exchange in a particular van it must be measured empirically. However, measurements of the air exchange rate on several vans showed that a reasonable approximation was that it varied as a function of the height and width of the door only. An improvement was found when air from the evaporator or cooling system was blown directly towards plastic strip curtains placed over the door. The momentum of the air counteracted the tendency of warm air to enter through gaps at the top of the strip curtains. A full description of the program is given in Gigiel (8).

Retail display

Retail cabinets are designed to display either chilled packaged food, chilled unpackaged food or frozen packaged food. A 'perfect' retail display cabinet would have its refrigerated air form a closed loop much like a domestic refrigerator when the door is closed. In reality, warm moist air from the surrounding store entrains with refrigerated air from the air curtain, causing a loss of refrigerated air into the store and a gain of warm air and moisture into the cabinet.

Developing a cabinet can be a very lengthy process. The cabinet temperatures are not steady with time, as the cabinet's coil ices up and then defrosts. Any movement in front of the cabinet will have an effect on the air curtain and product temperatures. Any changes made to the cabinet may not have an immediate effect on product temperatures, therefore making a number of small changes to a display cabinet can be a timely and hence costly process.

Computational fluid dynamics (CFD) is becoming widely accepted as a tool to aid the development of display cabinets. CFD allows the user to make changes to a computer model of the cabinet and see its effect before trying out the real thing. If computing resources allow, a number of changes can be made to a computer model relatively quickly and the best case tried on a real cabinet. For example CFD has been used to show the effect of removing shelves from a retail display cabinet (9). A two dimensional model showed quite clearly that when shelves are removed, pressure differences between the cold cabinet and the store cause the air curtain to bend inwards. This causes more mixing between the cold and warm air, increasing product temperature, reducing store temperatures and increasing energy consumption.

Display of packaged chilled foods

Air movement and relative humidity (RH) have little affect on the display life of a wrapped product, but the degree of temperature control can be important especially with transparent, controlled atmosphere packs. During any control cycle, the cabinet temperature rises, heat enters the pack, the atmosphere inside the pack warms with consequent reduction in (RH) and increase in the surface temperature of the product. As the surface temperature rises so does its saturation vapour pressure (a factor controlling evaporation) and more water evaporates into the sealed atmosphere of

the pack. If the cabinet temperature stabilised then evaporation would continue until the atmosphere became saturated. However, in practice the cabinet air temperature cycles and as it is reduced the wrapping film is cooled. If it reaches a temperature below the dew point of the atmosphere inside the pack, then water vapour will condense on the inner surface of the pack. This film of water can obscure the product and consequently reduce consumer appeal. As the cycling process continues the appearance of the product deteriorates.

To maintain product temperatures close to 0°C, the air off the coil must typically be -4°C and any ingress of humid air from within the store will quickly cause the coil to ice up. Frequent defrosts are often required and even in a well maintained unit the cabinet temperature will then rise to between 10 and 12°C and the product by at least 3°C (10). external factors such as the store ambient temperature, the siting of the cabinet and poor pre-treatment and placement of products substantially affect cabinet performance. Warm and humid ambient air and loading with insufficiently cooled products can also overload the refrigeration system. Even if the food is at its correct temperature, uneven loading or too much product can disturb the air flow patterns and destroy the insulating layer of cooled air surrounding the product. An in-store survey of 299 pre-packaged meat products in chilled retail display cabinets found product temperatures in the range -8.0 to 14.0°C, with a mean of 5.3°C and 18% above 9°C (11). Other surveys (12, 13) have shown that temperatures of packs from the top of stacks were appreciably higher than those from below due to radiant heat pick up from store and cabinet lighting.

It has also been stated that products in transparent film overwrapped packs can achieve temperatures above that of the surrounding refrigerated air, due to radiant heat trapped in the package by the ‘greenhouse’ effect. However, specific investigations failed to demonstrate this effect (14).

Display of unpackaged chilled foods

Changes in appearance are normally the criteria which limit display of unwrapped products rather than microbiological considerations. Deterioration in the appearance of unwrapped meats has been related to the degree of dehydration (Table 2) which makes the product unattractive to consumers (14). Weight loss on its own can not only be a measure of performance but also has important economic considerations to the retailers. In the UK, the direct cost of evaporative weight loss from unwrapped products in chilled display cabinets has been estimated to be in excess of 6.25m ECU per annum.

Table 2: Relationship between evaporative weight loss and appearance of sliced beef topside after 6 h display

Evaporative loss ($g.cm^{-2}$)	Change in appearance
up to 0.01	Red, attractive and still wet; may lose some brightness
0.015-0.025	Surface becoming drier, still attractive but darker
0.025-0.035	Distinct obvious darkening, becoming dry and leathery
0.035-0.05	Dry, blackening
0.05-0.10	Black

The rate of dehydration is a function of the temperature, velocity and RH of the air passing over the surface of the food. James and Swain (15) found that changes in RH had a substantial effect with a reduction from 95 to 40% increasing weight loss over a six hour display period by a factor of between 14 and 18. The effect of air velocity on weight loss was confounded by that of RH. Raising the air velocity from 0.1 to 0.5 ms^{-1} had little effect on the weight loss at 95% RH, however, the magnitude of the effect increased as RH decreased producing maximum changes at 40% RH. Changing the temperature from 2 to 6°C had a far smaller effect on the weight loss than the changes in RH or air velocity.

Evans and Russell (16, 17) found that at a RH of 40% the effect of surface drying became apparent after approximately 100 min. At 85% RH the products could be displayed for between 4 to 6 h before surface drying was noticed. The overall weight loss at 40% RH was approximately three times that at 85% RH.

In the same work they also found that changing the lighting combination of 50 W sons and 100 W halogen spot lights to 100 W sons and a colour 83 fluorescent significantly increased the weight loss. The increase was similar in magnitude to that produced by a 20% reduction in RH. On average the rate of weight loss under the combination of 50 W sons and 100 W halogen spot lights was approximately 1.4 times less than the 100 W sons and colour 83 fluorescent lighting (Figure 1).

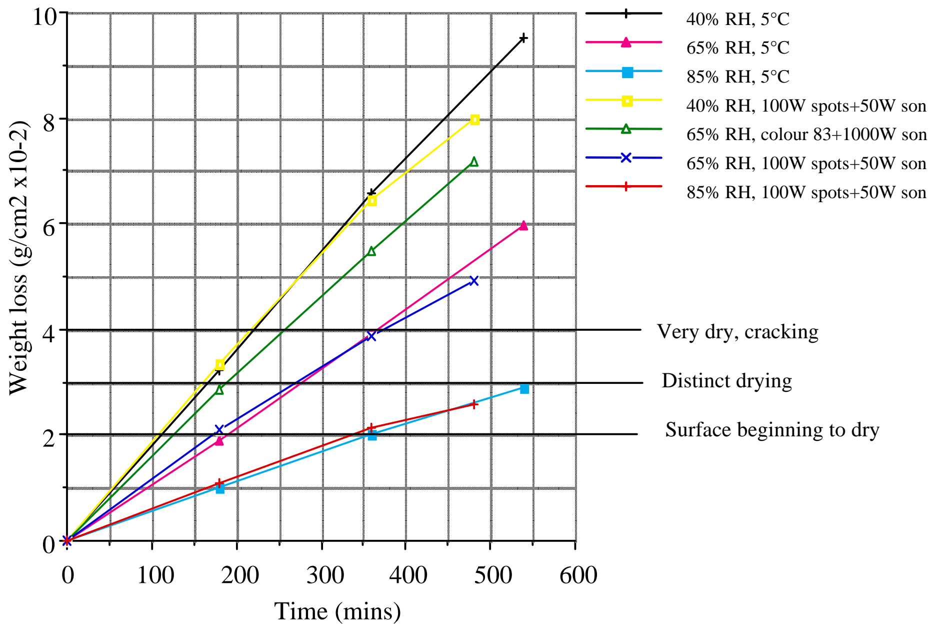

Figure 1: Comparison of mean weight loss at different relative humidities and lighting regimes for delicatessen products.

Frozen food display

In frozen display as long as the food is maintained below -12°C its bacterial state will not deteriorate. Its taste, texture and appearance are the main quality factors that can deteriorate during frozen display. During display, temperature, temperature fluctuations and packaging are the main display parameters which control quality.

Temperature fluctuations can increase the rate of weight loss from wrapped meat. Cutting and Malton (2) reported that a retail cabinet operating at -15°C produced greater product dehydration than another cabinet operating at -8°C. This was shown to be due to the much greater fluctuations in air temperature in the -15°C cabinet, ranging from -5 to -21°C compared with ±1.5°C in the -8°C cabinet. Successive evaporation and condensation (as frost) caused by such a wide temperature differential resulted in exaggerated in-package dehydration.

The extent of temperature fluctuations will be dependent upon the air temperature over the product, the product packaging and the level of radiant heat. Retail display packs have a relatively small thermal mass and respond relatively quickly to external temperature changes. These can be from store and display lighting, defrost cycles and heat infiltration from the store environment. In products where air gaps exist between the packaging and the meat, sublimation of ice within the product leads to condensation on the inside of the packaging, resulting in a build up of frost. This dehydration causes small fissures in the surface of the food, allowing the ingress of any packaging gases into the food. This can aid the acceleration of oxidative rancidity within the product. Minor product temperature fluctuations are generally considered to be unimportant, especially if the product is stored below -18°C and fluctuations do not exceed 2°C.

The purpose of air flow over the products is to provide an effective barrier to warm ambient air and to provide limited heat extraction from the product surface. The major thrust in cabinet design has been to reduce the warming of the refrigerated air as it flows over the product. This has resulted in the development of devices for air movement to ensure a uniform distribution of air across the cabinet length. The most popular methods use axial or propeller fans. Variations in the velocity of the air ‘curtain’ will increase shear with ambient air and induce localised mixing. This is exaggerated by differences in product loading height, merchandising labels, restriction in air flow by icing and frosting of heat exchanger coils. Cabinets should be designed to use air flows as low as possible to maintain cabinet air temperatures at the desired levels. This will minimise ambient air mixing, ideally the air should ‘roll’ over the product, typically at velocities of 0.5 ms^{-1}. FRPERC are using CFD modelling techniques to look at the design of air flows in retail display cabinets (18).

The development of low radiant energy transmission glass and high insulation techniques has encouraged the adoption of more glass to increase product visibility. This has necessitated the use of anti-mist heating to keep the glass clear, which increases energy use, unless heat can be recovered from another part of system.

Conclusions

There are few problems in the long distance transportation of chilled and frozen products provided they are fully cooled before loading and the air distribution is designed and operated so that the cargo is surrounded by a blanket of refrigerated air.

Multi-drop short distance distribution of chilled product is much more difficult. ‘CoolVan’ is a powerful computer simulation tool to improve temperature control. The software package is benefiting refrigeration unit manufacturers, van body manufacturers and food companies in a number of ways. Food companies are able to exert greater control over food temperatures during their distribution operation. Food temperatures can be predicted quickly and easily with the program. Depot managers can use it to optimise their delivery operation, ensuring that temperature legislation is met. Refrigeration unit manufacturers are able to size their units for customers more accurately and

with more confidence. Van body manufacturers are able to try out different scenarios to see the effect of improving elements of their van body design.

Food temperature control in retail display will always be problematical. The marketing requirements for the product to be visible and easily accessible make it difficult to shield consumer packs from radiant, convective and conductive heat sources. In unpackaged food, conflicting needs to reduce weight loss whilst maintaining the food at the correct temperature cause more problems. The use of CFD and other modelling techniques are providing a greater understanding of the problem and identify methods of improving temperature control in retail stores.

Acknowledgements

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Modelling of food refrigeration systems

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Summary

As the speed of computers has increased and prices have dropped, an increasing number of models to predict food refrigeration processes have been developed. These models now cover the whole of the cold chain from primary chilling through freezing, thawing, frozen and chilled storage, transport to retail display.

The methods used have ranged from analytical solutions based on equations derived by Newton or Plank, for example, to numerical finite difference and finite element codes and recently computational fluid dynamics (CFD).

Most of the modelling has concentrated on the prediction of time temperature data for an individual process of interest. Some have also attempted to model weight loss, rates of heat release or multiple refrigeration operations. In the case of primary or secondary chilling modelling has been used in general to extend experimental studies and produce design charts for industrial use. Most freezing and thawing predictions rely on the identification of a correlation between some parameter, i.e. weight or thickness, of the object being modelled and the dimensions of a regular body i.e. slab, sphere or cylinder. Heat flow within the regular body can be handled mathematically.

Models are now becoming much more user friendly but many suffer from the lack of sufficient experimental verification and knowledge of boundary conditions and thermal property data.

Introduction

Refrigeration has been the predominant method of food preservation in the 20th century. Research has shown that rates of reducing and subsequently maintaining the temperature of food has important consequences in terms of microbiological safety, eating quality, appearance, weight loss and overall economics of the processing chain. A clear understanding of the factors that influence and control the rate of heat and mass transfer, both within and from food, is required before the design of any process in the cold chain can be optimised. Predictive methods are having a growing influence on the understanding of these factors and as an aid to optimisation of the design and operation of refrigeration systems.

During the 1990's as part of a process to harmonise legislation throughout the European Community, regulations, that before only covered export or intervention purchase of foods, were modified and applied to internal trade. Among these modified regulations are a number that specify the maximum internal temperature of food after initial chilling or freezing, maximum surface temperatures during thawing, chilling rates and temperatures during storage and display. The legal requirements to attain and maintain specific temperature criteria have created an increased demand for data on the relationship between environmental conditions and the temperature history of food. The low profit margins of the food industry are also applying pressure to maximise yield and minimise weight loss during refrigeration processes. At the same time, eating quality and microbiological safety need to be maintained.

Problems caused by biological variability make it very time consuming and expensive to use practical experimentation to produce all the data required. Increasingly predictive modelling techniques, usually in combination with limited experimentation, are being used to generate the data and optimise refrigeration processes. Initially analytical solution based techniques were used, but as the speed of computers increased and prices have dropped then numerical methods have taken over.

What needs to be modelled?

The food refrigeration processes that need to be modelled fall into two groups. The first type are processes, such as primary and secondary chilling, freezing, tempering and thawing, where a change in product temperature is required. The second type are refrigeration processes such as chilled and frozen storage, transport, retail display and domestic storage where the requirement is to maintain food temperature.

In most food refrigeration processes a primary aim is to minimise weight loss from the product. Excessive weight loss reduces yield and is usually detrimental to the eating quality of the food being produced. However, in certain circumstances, maintaining a high water activity can increase the rate of bacterial spoilage and in some refrigeration processes such as freeze drying weight loss is a primary aim.

In all food refrigeration processes there is a need to be able to model heat transfer from and into the product and in many instances a further requirement to model mass transfer.

What are the problems?

Andrew Cleland (1) stated that there are four factors that currently limit the accuracy of freezing and chilling time prediction. In general the same factors limit the accuracy of predicting any food refrigeration process.

The four factors are:

1. Imprecise knowledge of the conditions surrounding the product in the system.
2. Imprecise thermal and diffusional data for the product.
3. Extrapolating a method beyond its range of applicability.
4. Shortcomings in the prediction method.

He states that in every case the observed difference between a measured freezing or chilling time and that predicted is the net effect resulting from the interaction and accumulation of the four factors.

What has been achieved?

Primary chilling and secondary chilling

Chilling is the first and probably the most important process in the cold chain and the optimum modelling process would provide data on the rate of cooling at the thermal centre, the temperature at the surface, and the rate of weight loss from the product. Many foods are highly inhomogeneous, have temperature dependent thermal properties and are very irregular in shape. These factors cause problems and the authors, for example, knows of no attempt to try to directly model heat and mass transfer in a meat carcass. Most of the modelling of chilling has been carried out for meat and Daudin and Van Gerwen (2) have produced an excellent review of progress in this area.

In general some large experimental programmes have been carried out to investigate the effect of air temperature, air velocity and relative humidity on cooling rates and weight losses. Mathematical methods have then been used to extend the range of cooling data outside of that covered experimentally. The methods are based on the recognition that, after an initial lag, the temperature at a set depth within food decreases in an exponential fashion.

It can be shown theoretically, under constant cooling conditions and when there is no water evaporation, that the logarithm of the dimensionless temperature $Y = (t-t_f)/(t_i-t_f)$, at any point of a product is a linear function of time after a sufficiently long lapse of time. Where t = meat temperature, t_i = initial meat temperature and t_f = air temperature. The chilling rate is the slope of the linear part of the logarithmic plot of Y against time. It is related to the air velocity and the product shape, size and thermal conductivity. For example, for pork hindquarters, it increases by 35% when the carcass weight decreases from 100 to 50 kg and by 25% when the air velocity increases from 0.5 to 2 ms^{-1} (3).

Since the chilling rate is, in principle, independent of air temperature, experimental results obtained at one air temperature can be interpolated at another one. This methodology has been used to provide design charts for beef (4), goat (5), mutton (6) and pig (7) chilling.

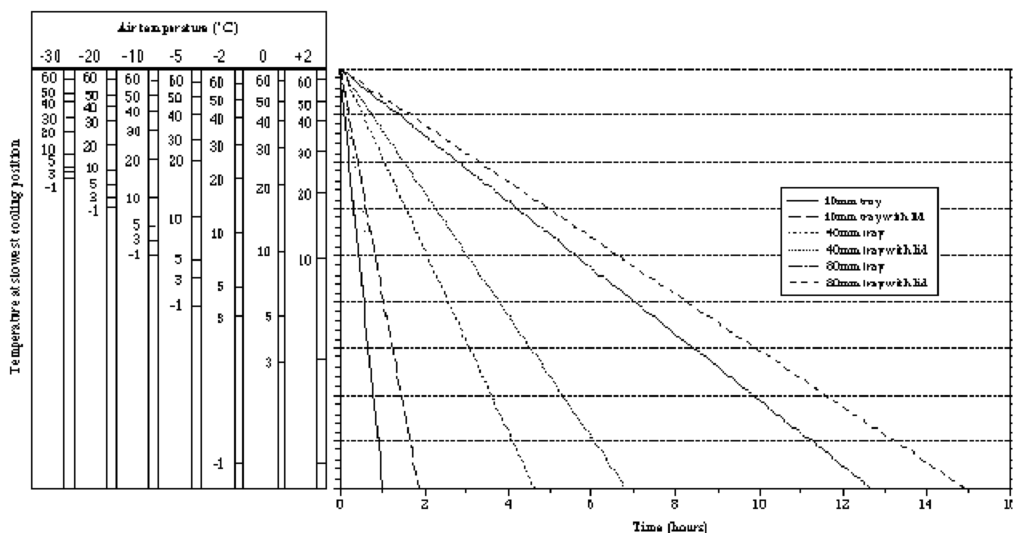

Figure 1: Cooling times of hot recipe dish meals in air at 3.0 m/s.

Many countries in Europe have guidelines or recommendations for the cooling of cooked products. A similar modelling technique to that used for primary chilling has been used to produce design charts (Figure 1) for the cooling of hot recipe dish meals (8).

Finite Element Methods (FEM) have the potential to handle the complex shape of a food. Using FEM the food is initially divided into several hundreds or thousands of elements. The centre of an element is called a gridpoint. The temperature of all gridpoints is then calculated, assuming a certain temperature distribution within a grid element, and known equations for heat flow between the gridpoints.

Moerman (9) and Van Gerwen (10) used a 3-dimensional cubic grid to simulate the evolution of temperature distribution in pork carcasses in the cold chain. A frozen carcass was cut into 50 mm thick slices to determine the nature of each cube. A 46 kg pork carcass corresponded to 224 cubes of lean meat, 89 cubes of fat, and 25 cubes of bone. This approach was then applied to veal, turkey and chicken carcasses to simulate temperature and weight loss changes (Van Gerwen et al., 1991). These models were used in association with computational fluid dynamics techniques that calculated air flow patterns and air velocities around carcasses in chillers.

Freezing

In freezing a number of equations, most based on the initial work of Plank (12), have been developed for the prediction of freezing times of slabs and cylinders. With experience they can be used to estimate the freezing time of 'homogeneous' foods under constant conditions with a high degree of accuracy. They are also very quick methods of indicating the sensitivity of freezing time to small changes in product thickness, media temperature, etc. Complex cases in which the ambient temperature, the heat transfer coefficient and the physical properties of the solid may vary can be handled using finite difference techniques based on the numerical method described by Dusinberre (13).

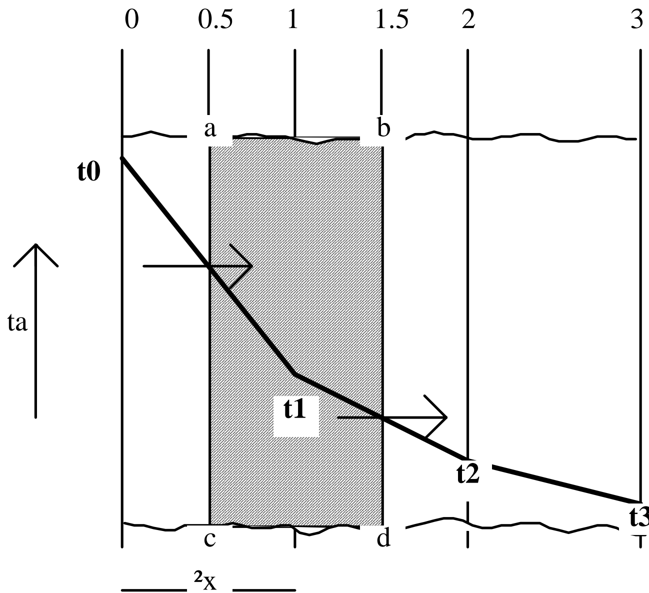

Figure 3: Dusinberre diagram of one-dimensional transients in a slab.

The approach taken using the numerical method is very simple. It is impossible to directly calculate the heat flow through the complicated structure of a real food. The food is therefore equated to a geometric solid that can be treated mathematically. Figure 3 shows a cross-section of a large slab of thickness x having a uniform cross-sectional area *A*; the solid is divided into a number of equal finite slices of thickness Δx by temperature-reference planes. A heat balance is written on the cross-hatched zone abcd; the slope -d*t*/d*x* at plane ad is approximately equal to the chord slope $(t_o - t_1)/\Delta x$; similarly the slope -d*t*/d*x* at plane bc is replaced by $(t_1 - t_2)/\Delta x$; the temperature at plane 1 approximates to the average temperature of the cross-hatched zone. The resulting heat balance is

$$\frac{KA(t_0 - t_1)}{\Delta x} - \frac{KA(t_1 - t_2)}{\Delta x} = \frac{(A\Delta x)(\rho C_p)(t_1' - t_1)}{\Delta T} \qquad (1)$$

where t_1' is the new temperature at plane 1, after the elapse of a finite time increment ΔT, K is the thermal conductivity, ρ is the density and C_p the specific heat. Upon replacing the thermal diffusivity $K/\rho C_p$ by α and the dimensionless ratio $(\Delta x)^2/\alpha\Delta T$ by the modulus M, equation (1) becomes

$$t_1' = \frac{t_0 + (M-2)t_1 + t_2}{M} \qquad (2)$$

which is the equation for conduction through the interior of the block. Using a similar process equations can be derived for heat flow at the surface and centre of the slab (14).

An infinite slab is not a good geometric analogue to many food products in which heat flow is two or three dimensional. However, it can be argued that the same principles used for the slab apply to an infinite cylinder and sphere where the heat flow is totally radial. Code has therefore been developed for spheres and cylinders.

The numerical method has been used to predict the relationship between freezing time and air temperature for meat blocks in different packaging configurations (15), beef quarters (16) and mutton carcasses (17).

In the 1990's interest has developed, especially in New Zealand, in the simulation of total freezing systems (18). Models have been developed to predict the changes in rate of heat release from products during freezing (19, 20) and integrated with those of plant performance (21). These produces powerful tools to be used in the design of new freezing plant. Attempts are also being made to use predictive models to control freezing operations (22).

Thawing

Thawing is the reversal of the freezing process, but the three-fold difference in thermal conductivity between frozen and unfrozen material, in meat and other high water content food, increasingly restricts the rate of heat penetration during thawing. If bacterial numbers are to be maintained at an acceptable level then limitations must also be applied to the maximum surface temperature that can be tolerated. Thawing is therefore a more difficult operation than freezing and research has been directed to methods of increasing the surface heat transfer coefficient without attaining high surface temperatures. Predictions of air thawing have taken into account the changes in the surface heat transfer coefficient with surface temperature. At surface temperatures below 0°C water sublimes to ice on the surface of the food enhancing the heat transfer coefficient as a result of the phase change.

Little data are available on surface heat transfer coefficients in food processing in general and for thawing in particular and values from different sources can differ substantially. Investigations have been carried out to measure surface heat transfer coefficients during air thawing at different relative humidities (23). These revealed that in conventional air based thawing systems the contribution to the coefficient resulting from the condensation of water vapour was substantial. At 30°C and a dew point of 28°C, which would be considered to be saturated air in a practical thawing system, the coefficient at 3 ms^{-1} varies from 70 to a peak value of 115 $Wm^{-2}K^{-1}$ over the surface temperature range -20 to +26°C (Figure 4).

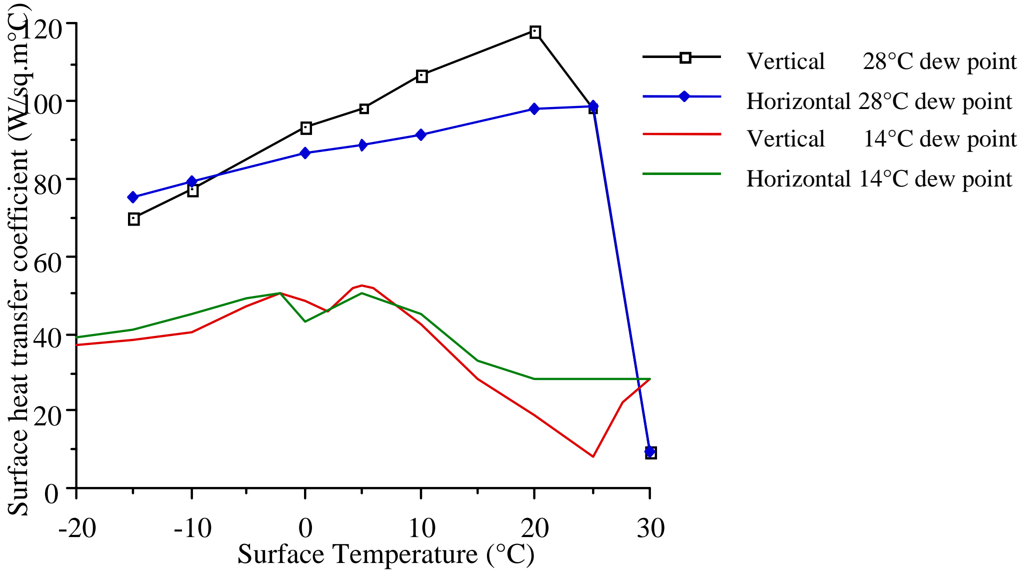

Figure 4: Surface heat transfer (h) on vertical and horizontal surfaces in air at 30°C, 3 ms^{-1} and dew points of 28 and 14°C.

The numerical methods described for freezing have been used in conjunction with experimental investigations to produce data on the thawing time of pork legs (14) , beef quarters (24), lamb (25) and mutton (17) carcasses, and meat blocks (26). Spheres, infinite slabs and cylinders were used as geometric analogues in these investigations.

Transport

There are substantial difficulties in maintaining the temperature of chilled foods transported in small refrigerated vehicles that conduct multi-drop deliveries to retail stores and caterers. The vehicles have to carry a wide range of products and operate under diverse ambient conditions. During any one delivery run, the chilled product can be subjected to as many as fifty door openings, where there is heat ingress directly from outside and from personnel entering to select and remove product. The design of the refrigeration system has to allow for extensive differences in load distribution, dependent on different delivery rounds, days of the week and the removal of product during a delivery run. A refrigeration system's ability to respond to sudden demands for increased refrigeration is often restricted by the power available from the vehicle. All these problems combine to produce a complex interactive system.

With so many interacting variables it was not cost effective, by purely experimental means, to obtain the depth of understanding required to design and operate vehicles to maintain the required food temperatures. A research project was therefore set up to provide a predictive model which would assist fleet operators in specifying the design of, and the equipment for, small delivery vehicles. The resulting vehicles should be capable of maintaining all food products at temperatures within the ranges 0 to 2°C or 0 to 5°C, allowing for the relative influence of operating conditions peculiar to the fleet in question.

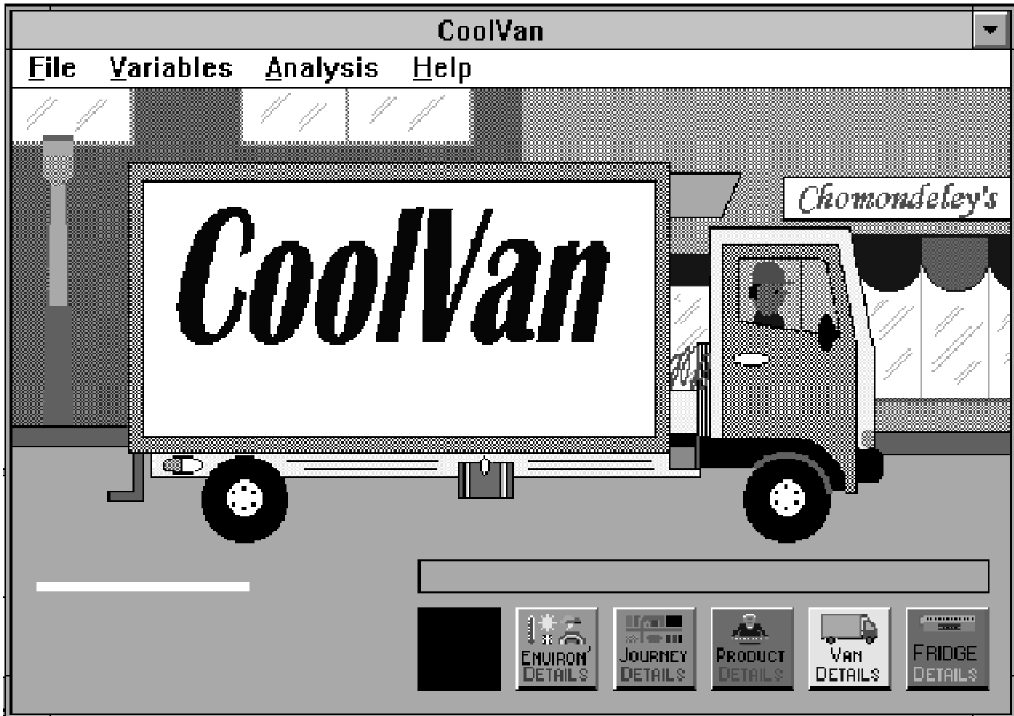

Figure 4: Initial input window for 'CoolVan'.

The model was developed from individual modules which were verified either in processing halls or on the road (27). The temperature of the air inside the van forms the core of the prediction model. When the doors are opened or closed this air exchanges heat with the outside environment by the infiltration of air into and out of the van. In addition, heat is conducted into the van through the insulation from the outside air and from solar radiation on the outside of the van. Staking systems, fittings, trays and food inside the van exchange heat with the van air. The evaporator coil or eutectic plates cool the van air to maintain its temperature.

The heat transfer equations are solved with a backward or implicit finite difference technique. The resultant set of linear equations define the future temperature at each node in the network, the current temperature at that node and the future temperature at each adjacent node. The set of linear equations is solved simultaneously to give the future temperature at each node using a matrix inversion method.

The resulting program, 'CoolVan', is a unique tool for vehicle operators and manufacturers (28, 29). Much effort was put into making the program very user friendly and easy to use (Figure 4). There has been very favourable feedback from the many companies involved who now use the program and it is now intended to release it as a commercial product.

Storage and display

In storage and display most of the predictive modelling has concentrated on weight loss from unwrapped products, although recently, attention is being paid to the use of Computational Fluid Dynamics (CFD) to model storage situations.

The display life of unwrapped products was shown clearly in the mid 1980's to be determined by colour changes caused by evaporation of water from the surface of the food. Experimental investigations clearly demonstrated the substantial effect of changes in the relative humidity of the air on weight loss from chilled meat under retail display conditions (30, 31). The investigations indicated that mean conditions around unwrapped products were more important than their degree of control. Computer predictions have since substantiated this view (32, 33). However, substantial problems were encountered in the latter work in obtaining a relationship between heat (h) and mass (k) transfer coefficients that would allow accurate predictions to be made of heat and mass transfer from the foods. A series of experimental investigations have since been carried out to simultaneously measure h and k (34).

The control of air movement within retail display cabinets and the interaction between refrigerated and ambient air within the store has always been a critical factor in cabinet performance. In the late 1990's CFD modelling expertise is being used to study flow within and around cabinets. The design and position of shelves has been shown to have a large affect on spillage of refrigerated air into a retail store (35). Current research studies use CFD and in store investigations to reduce both air spillage and the resulting 'cold feet' phenomena which is commonly found in the isles between refrigerated cabinets in supermarkets.

What needs to be done in the future?

A more rigorous testing and evaluation of existing models in controlled investigations is required, together with the development of measuring systems for critical parameters in commercial situations. The development of models that accurately predict the rates of heat and mass transfer that occur simultaneously in processes such as the chilling of meat carcasses and the display of unwrapped food is still in its infancy.

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Food freezing and thawing time prediction with new simple calculation formulas application

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Abstract

There were developed new methods for freezing and thawing time prediction analysing the effects of water behaviour and product properties. Introduction of a characteristic freezing temperature resulted in the elimination of cryoscopic temperature out of the calculations. Hence, both models got more universality. The models had been comprehensively verified and their high accuracy and full practicability was confirmed.

Nomenclature

A	- area, [m^2]
Bi	- Biot number
c	- specific heat capacity, [J/kg K]
d	- characteristic dimension, [m]
E	- prediction error, [%]
h	- heat transfer coefficient, [W/m2 K]
ΔH	- enthalpy difference, [J/m3]
k	- heat conductivity, [W/m K]
L	- latent heat of freezing, [J/kg]
R	- correlation coefficient
t	- time, [s, h]
T	- temperature, [K, °C]
V	- volume, [m^3]
ρ	- density, [kg/m^3]
ω	- degree of water freezing out, %

Indices

c	- centre, end
kr	- cryoscopic
n	- unfrozen
o	- medium
p	- initial
rz	- experimental
z	- frozen

Introduction

Particularly significant effects observed at food freezing-thawing processes are freezing (crystallization) - water melting in a product structure. The general character of water freezing out kinetics is most often described by logarithmic dependencies (Fig.1) [1]. Regarding the complexity of the changes from both, physical and biochemical aspect as well as their determination of process course (freezing, thawing) and product quality, the effects were and still have been the objects under investigation in the food technology and engineering and some fields related with them (i.e. cryobiology, cryosurgery). From an engineering and technological aspect the most significant fact is a way in which a process of water freezing out- ice melting in a product and product properties formed due to these and other factors operation affect the very process and product quality following the treatment. Thawing is an ultimate operation in a cooling chain performed directly before further industrial treatment or product relay for market.

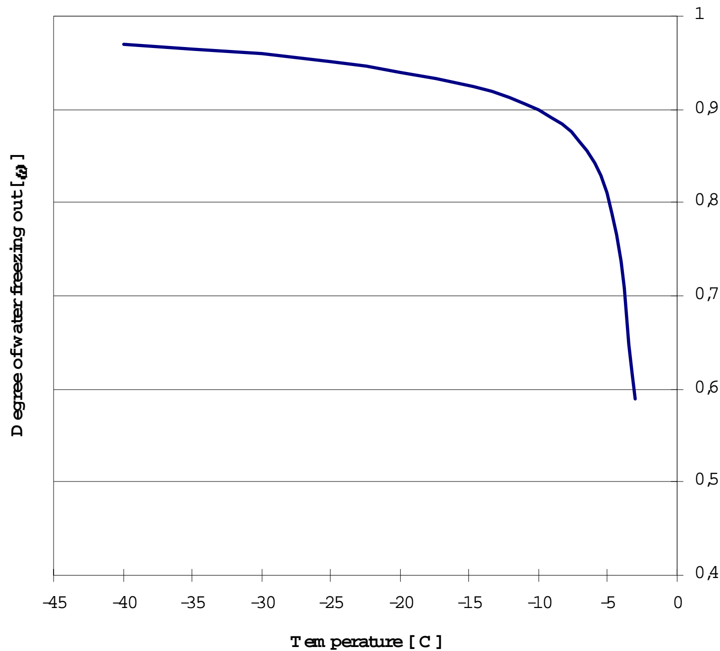

Figure 1: Dependency of water freezing out degree (ω) on product temperature (T).

This process aims at bringing the products to a state suitable for further use, in a maximal degree approaching a product natural state. Possibility for freezing and thawing time prediction allows, to form advantageous process course, to facilitate fixing effects as well as to enable a refrigerating machine design. Considering ice crystallisation and melting processes in a problem fundamental for food freezing and thawing, it was comprehensively examined to work out a new analytical and empirical methods those they would let predict effective freezing and thawing time. So, the objective of the investigation was to work out, simplified models for freezing and thawing time calculation for foods of regular dimensionality.

Development of the models

Some possibility to obtain the new simple freezing time prediction model [7] was found in Mott's method [1]. Calculations acc. to this method consist in the application of criterion numbers:

$$S = \frac{B+1}{G} = A \cdot \frac{d}{V}, \qquad B = \frac{h \cdot d}{2 \cdot \lambda_z}, \qquad G = \frac{t \cdot h \cdot \Delta T}{\rho_z \cdot Q \cdot d} \tag{1}$$

Comparison of the dependencies of Eq. (1) leads to the form:

$$t = \frac{Q}{\Delta T} \cdot \frac{V}{A} \cdot \left(\frac{d}{2 \cdot \lambda_z} + \frac{1}{h} \right) \tag{2}$$

Defining the ratio of product volume V to its surface A as a shape factor K and the ratio of the heat Q removed from the product to its temperature change ΔT as ΔH, the following dependence for the freezing time can be obtained:

$$t = \Delta H \cdot K \cdot \left(\frac{d}{2 \cdot \lambda_z} + \frac{1}{h} \right) \tag{3}$$

Because the thermal conductivity of most frozen food, is about 2.0 [W/m K], it can be assumed that *28_z = 4 [W/mK],* and finally the Eq. (3) becomes:

$$t = \Delta H \cdot K \cdot \left(\frac{d}{4} + \frac{1}{h} \right) \tag{4}$$

Considering the fact, the heat removed during the freezing process consists of the initial product enthalpy surplus *)* I_P in relation to the cryoscopic condition, latent heat of freezing L_F and its enthalpy change *)* I_T in subcooling, *)* H is defined as:

$$\Delta H = \Delta I_P + L_F + \Delta I_T \tag{5}$$

The final form of the dependence (5) is obtained by the experimental methods correcting particular terms and values in the equation because of the minimum error of freezing time calculations for the conditions under which our measurements were performed [4,7].

$$\Delta H = \rho_n c_n \cdot \frac{0{,}74 T_p - T_F}{\frac{T_p}{2{,}4} - T_o} + \frac{\rho_z L}{T_F - T_o} + \rho_z c_z \frac{T_F - 0{,}5\left(T_c + T_o\right)}{T_F - T_o} \tag{6}$$

At the characteristic freezing temperature T_F the amount of the frozen water is 70% of the total water content in product. By some transformation of Riedel's results [1], the dependence of product temperature on the ice fraction can be expressed as:

$$T = -\left[10^{\frac{0{,}31 \cdot b}{1{,}105 - b}} - \left(1 - /T_{kr}/ \right) \right] \tag{7}$$

Hence, the characteristic product freezing temperature is:

$$T_F = -\left[3{,}43 - \left(1 + T_{kr} \right) \right], \tag{8}$$

and because the freezing temperature is often about T_{kr} = -1.0 °C, then

$$T_F = -3.4 \ [^o C] \tag{9}$$

For a rectangular brick or a finite cylinder, the shape factor *K* defined in the Eq. (3), can be obtained directly from the product dimensions. For an infinite slab, an infinite cylinder or a sphere the determined values of *K* are: slab = d/2,75; inf. Cylinder = d/5,6; sphere = d/8,2 respectively. On the basis of experimental researches there was obtained an every product thawing course related to temperature conditioned on process time (Fig.2). The process comprised phases: I (A-B) initial preheating of a product, II (B-C) specific thawing, III (C-D) product reheating up to a temperature required.

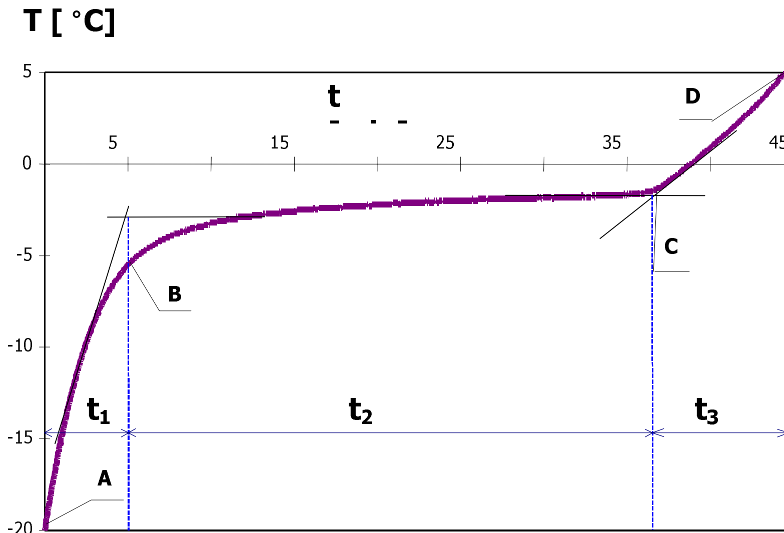

Figure 2: Typical temperature *versus* time profile for thawing a food product

The time of the first thawing phase was determined from the heat balance [3]:

$$V \cdot \rho \cdot c \cdot dT = -A \cdot h \cdot (T_o - T)dt \tag{10}$$

as

$$t_1 = \frac{\rho_z c_z V}{hA}\left(- \ln \frac{T_o - T_F}{T_o - T_p} \right) \tag{11}$$

The II-nd phase time was derived basing on the classical solution of the problem by Plank [1]:

$$t_2 = \frac{\rho_n LV}{hA(T_o + 3{,}4)}\left(1 + \frac{Bi}{2{,}8} \right) \tag{12}$$

Calculation formula for the product reheating time up to required final temperature was derived analogically as a dependence defining the first phase of the process

$$t_3 = \frac{\rho_n c_n V}{hA}\left(- \ln \frac{T_o - T_c}{T_o + 3{,}4} \right) \tag{13}$$

Considering that, the time of the complete thawing process is:

$$t = \frac{V}{hA}\left(C_z B + \frac{\rho_n L}{T_o + 3{,}4} E + C_n D \right) \tag{14}$$

where: C - specific heat capacity, B,D - temperature simplexes respectively, E - Biot number function.

Methods of model verification

The proposed model for freezing time prediction was verified due to its application for freezing time calculations, based on 383 freezing tests published, including six kinds of products shaped in five geometric forms [7]. The same calculations were performed by Cleland's and Pham's methods previously tested [8].

There were considered 232 published cases of the products thawing, the objects of regular shapes and broad range of process conditions. The selection of products was imposed by availability of reliable experimental data, which together with full characteristics of thawing parameters were taken from Cleland's works [5] and Ilicali [6]

The results of test predictions were statistically evaluated by: the characteristics of relative prediction errors, examining of fitting the results distribution to real values distribution, regression analysis of the experimental freezing and thawing time vs. the time predicted and also by one way variance analysis of relative prediction errors.

Results and discussion

Absolute mean value of relative prediction errors of the freezing time calculated by the new method, for the whole sample of 383 tests figures +1.74% (Tab. 1) and is found between the means of results obtained acc. to the Cleland's and Pham's methods.

Table 1: Statistical examination of the relative freezing time prediction errors (sample size 383)

	Average	Median	SD	SE	95% conf. int.
This work	1,74	2,61	12,40	0,64	0,49÷2,99
Pham	0,32	3,09	13,98	0,71	-1,08÷1,73
Cleland	-4,06	-4,54	13,60	0,69	-5,42÷2,69

The linear regression analysis was used to evaluate the convergence of the freezing time values obtained by the new method and the experimental data [Tab. 2] (Fig.3).

Table 2: Regression equations of the freezing time vs. the time predicted.

	this method	Pham	Cleland
Intercept *A*	0.0525	0.0398	0.0478
Slope *B*	0.9877	0.9950	0.9296
Correlation coef.	0.9902	0.9868	0.9896

To examine the distributions fitting of the freezing time values obtained by presented model and the experimental data Kolmogorov-Smirnov's test was used. This test confirmed the hypothesis H_o, that provided distribution compatibility of both variables (Fig.4).

Freezing times values obtained by the proposed method, those by Pham's and Cleland's methods were subjected to the one-way analysis of variance. As an examined parameter the absolute values of relative prediction errors were considered (Tab. 3).

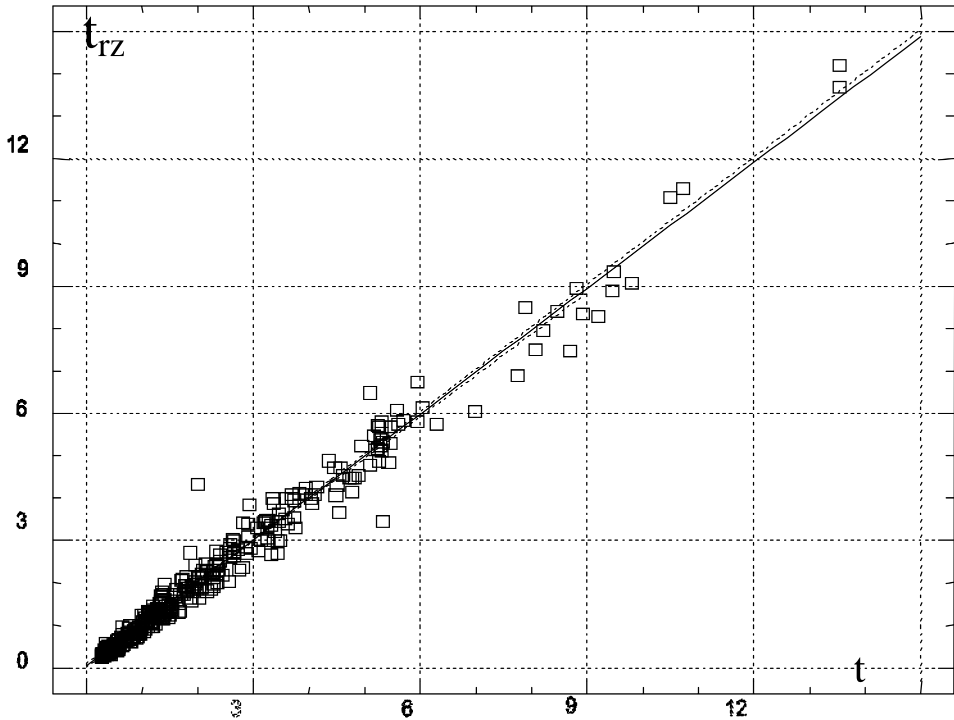

Figure 3: Regression of experimental freezing time vs. time calculated after new model

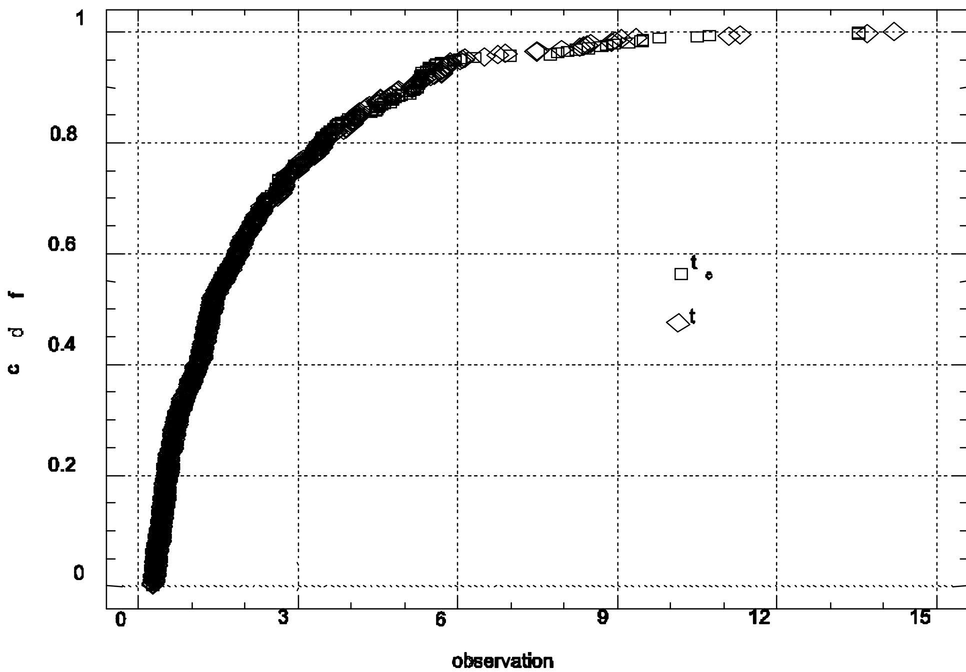

Figure 4: Plot of the cdf of experimental freezing time and time predicted after new model

Table 3: Means and confidence intervals for absolute values of prediction errors

	Sample size	Mean %	Std. Error	95% conf. int. for mean %
New model	383	9.83	0.399	8.923÷10.729
Pham	383	10.59	0.466	9.688÷11.493
Cleland	383	10.13	0.507	9.226÷11.031

Considering the thawing time prediction it can be assumed, that the best results are obtained after the models of: Cleland's (1986, 1987 EHTD, 1987 MCP) and the ours one. The above statement is confirmed by the data in Table 4.

Table 4: Descriptive statistics of thawing time prediction error *E* (sample size: 232)

	Mean	Median	Mode	Var.	SD	SE
Pham	21.4	23.03	20.0	530.7	23.04	1.51
Cleland	3.8	5.03	0	182.3	13.50	0.89
Cleland with EHTD	8.1	6.84	0	130.1	11.41	0.75
Cleland with MCP	7.9	6.40	0	134.3	11.59	0.76
This work	-3.2	-2.64	0	208.0	14.42	0.95

Regression analysis of the experimental thawing time against the values calculated after examined models leads to a statement, that the results after new model are good correlated (Tab. 5).

Table 5: Regression analysis of thawing time t_z vs. the time calculated t

	This method	Cleland EHTD	Pham	Cleland MCP	Cleland
Intercept *A*	0,51	0,12	0,60	0,12	0,07
Slope *B*	0,88	0,91	0,74	0,91	0,94
R	0,9903	0,9980	0,9747	0,9978	0,9981

Summing up, it should be stated that in case of thawing time prediction after the studied models, the use of our new model and Cleland's models, guarantees the results approximating the real ones most.

Conclusions

1. The new simple calculation models for freezing and thawing time prediction were developed, which are characterised by a number of advantages including the fact that the calculations can be performed with a basic hand calculator.
2. Mathematical analysis of freezing and thawing time calculations confirmed high accuracy of the models. The mean relative error of freezing times prediction is 1.74%. In the case of new thawing time prediction model, low average value of error (-6,86%) was proved.

3. The regression equations of the freezing time in relation to the time determined by the new method indicates the significant correlation between both values (R = 0.9902). The same analysis on thawing time shows good agreement with experimental data (R = 0.9903).

4. The new models have got a constant introduced instead of initial cryoscopic temperature, therefore some potential errors can be omitted, that might result from inadequate values of this temperature placed to the model.

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Ice crystal size analysis in frozen model food gels

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The freezing step of the foodstuffs occurs with the change of liquid water into ice crystals. The bigger the crystals are, the more destroyed the food tissue is, and the organoleptic quality is reduced.

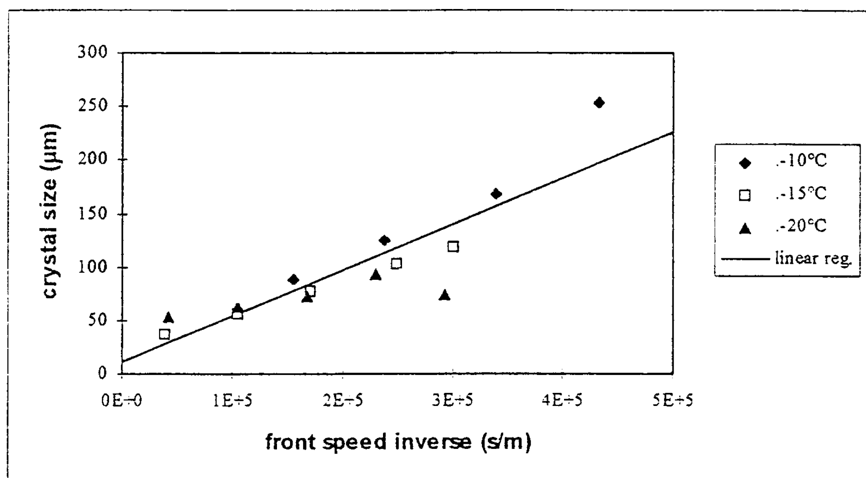
Our work [1] consisted on a qualitative and quantitative determination of the operating conditions on the crystals' size and shape, in order to obtain the best frozen food quality.

The experimental study was carried out with model food gels of well known compositions. The binary gels contained water and a food polymer (gelatin, agar ....); in other systems a solute (NaCl, sucrose,...) was added.

The freezing apparatus was designed to ensure a one-dimensional heat transfer. It was made of a freezing cell in which the gel was poured. At the bottom a copper plate was thermally controlled by two refrigerating devices in order to ensure a temperature step. Thermocouples were regularly placed at different levels of the freezing cell in a parallel way to the isotherms. Initially the gel was maintained at a temperature above the freezing temperature. Suddenly the copper plate was set at a constant temperature under the freezing temperature, and this until all the gel was frozen. The thermograms acquisition of the gel during its freezing allowed to determine the freezing temperature of the water inside the material and the evolution of the freezing front speed versus time.

The methodology of crystalline size determination was decomposed in three steps. Firstly, the frozen gel was left in its freezing cell and was freeze-dried, so that to recover the dried structure of the macromolecular network. Secondly, the freeze-dried sample was cut in thin slices parallel to the copper plate. Then each slice was observed with a stereo-microscope and photographed. Thirdly, each slide was projected on a graphic-tablet controlled by an image analysis software. This allowed to measure the pore size distribution inside the structure. This pore size distribution was assumed to be the same as the correspondent crystal size distribution.

According to the morphology of the growing crystals, among the analyzed parameters, we chose the width as the most representative size of ice crystals. Then the experimental distributions were described by the RRSB distribution function : the characteristic size was the width value at 63.2% of the cumulative distribution. This characteristic size for the binary gels seemed to be proportional to the position in the sample. The preliminary thermal study allowed to determine the relationship between the freezing front position and its propagation speed [2]. Thus, the relationship between the size and the speed of the front was analyzed. As shown on the graph below for the 2% gelatine gel frozen at three different temperatures, we observed a proportional relationship between the characteristic size and the inverse of the freezing front speed.



Characteristic crystal size as a function of the freezing front speed inverse for the 2% gelatin gel frozen at -10, -15 an -20°C

Our work will continue with crystal size analysis of gels containing solutes and of foodstuffs.

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Modelling of quality attribute variations in food under heat treatment conditions. Important aspects.

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1 Quality attributes, major types

Main groups of quality attributes of pasteurised or sterilized food are concentration of living (surviving) microbes, activity of enzymes, concentration of vitamins and other (favourable or disadvantageous) constituents, sensory attributes and related (physical and/or chemical) properties.

Great differences exist among these groups:

Microbial concentration is related to population growth and destruction. Time dependent variation of survival concentration means the simultaneous change in survivals' composition (according to genera, species, variety and biological variability) under heat treatment conditions, as more heat sensitive subpopulations are the first to be destroyed.

Chemical concentration can be treated by some kind of more or less exact reaction kinetics, where uniformity on molecular level and heterogeneity in energy distribution is characteristic.

Sensory attributes differ considerably from concentrations. There is no evidence that a sensory attribute would follow linear mixing law. Linear mixing law means that the attribute intensity of a mixture of two or more homogeneous parts of the same food is equal to the weighted average of the intensities of the original parts (Körmendy, 1990, 1993, 1994b; Miklós, 1990).

In spite of the differences among attribute groups, a tendency of unification could be experienced in variation kinetics from about 1920 up to the mid-seventies or later. The introduction of the enzyme inactivation and cooking values analogously to the sterilization equivalent reveals this tendency (Teixeira *et al.*, 1969; Ohlsson, 1980).

2 Major types of time dependent attribute change equations

Authors use at present the following methods for predictive attribute variation calculations:

2.1 Empirical equations

Earliest applications belonged to the n-th order empirical kinetics group. Most frequently first order, less often zero order or other integer orders appeared in the literature (Ball and Olson, 1957; Horak and Kessler, 1981; Labuza and Kamman, 1983; Dannenberg and Kessler, 1986; Rao and Lund, 1986; Kennedy *et al.*, 1992; Hendrickx *et al.*, 1995). Non-integer reaction orders have been also applied (Körmendy, 1987; Körmendy *et al.*, 1994; Körmendy, 1994a).

A number of equations has been evolved to handle time dependent survival concentration, when initial lag-phase and/or tailing phenomenon appears on a logarithmic concentration vs. time plot. The equations are sometimes named after the authors' name as Gompertz equation (Linton *et al.*, 1995), Consolari model and Whiting - Buchanan model (Garzaroli *et al.*, 1996; Zanoni *et al.*, 1997). Kamau *et al.* (1990) also developed a logistic equation.

Özlingen and Özlingen (1990), Tijskens and Polderdijk (1996) applied simple logistic equations, the first authors for lipid oxidation in food, the latter ones for quality deterioration of agricultural produce. Simplicity and conformity to international usage is important.

2.2 Exact and semi -exact methods

A number of authors applied more or less exact reaction kinetics principles to chemical concentration changes when the reaction under examination was simple enough. Their results were based mostly on stoichiometric relations and the mass action law (Froment and Bischoff, 1990). For example Singh *et al.* (1976), Hermann *et al.* (1978), Eison-Perchonok and Downes (1982) based their equations on the stoichiometric relation A + B -> Product. Körmendy *et al.* (1998) tried to calculate ascorbic acid oxidation in a fruit juice modelling solution according to the 2A + B -> Product relation.

Finite difference methods are very effective for solving either tasks in population dynamics or reaction kinetics. Rate equations, sets of equations can be solved in this way (Sapru *et al.*, 1992; Zanoni *et al.*, 1997; Körmendy *et al.*, 1998). Fitting problems can be overcome by some optimization strategy and the exploitation of extreme high calculational speeds.

Empirical relations of n-th order (see Para 2.1) become exact, when the ratio of components partaking in the reaction are constant or some components' concentrations are kept nearly constant, or the type of reaction is nA -> Product (Eison-Perchonok and Downes, 1982; Froment and Bischoff, 1990).

3 Design of experiments

The most important experimental method to obtain kinetic parameters consists of time dependent attribute intensity measurement at a set of constant temperatures (temperature has parametric values). Non - isothermal methods also exist, e.g. the method of Reichart (1979), but first order inactivation kinetics and low viscosity medium are conditioned here.

Some aspects, for consideration before starting experimental work, will be enumerated in the next paras.

3.1 Sample size

Sample size is mostly as small as possible for other reasons to ensure quick warming up (and cooling down). It is possible to correct for these transient temperature zones on the well known equivalent heat treatment time principle, always taking the fixed experimental temperature as the reference one. The heating up and cooling down time periods are to be replaced here by equivalent time periods (Ball and Olson, 1957; Dickerson, 1969).

3.2 Head - space volume in sample container

If a dissolved gas, oxygen for example, takes part as reactant in the attribute change, then its transition from solute to head - space (and vice - versa) should be regarded. Calculations can be based on Henry's law, Dalton's law and the ideal gas - law (Körmendy, 1982).

3.3 Initial attribute intensity

Initial attribute intensity might have an important role, when empirical relations are fitted to measure data. The case will be treated here, where constant temperature attribute intensity variation can be reduced to an equation, which contains one temperature dependent rate constant. There is always a possibility to transform the non - linear attribute intensity vs. time relation into a linear relation between transformed intensity vs. time (see para 5.2):

$$y(A) = y_i \pm kt, \quad (1)$$

where y(A) symbolizes the transforming function, $y_i = y(A_i)$ is the transformed initial intensity; A, A_i are original intensity and original initial intensity. As time constant is always a time period for obtaining some prescribed change, the linearity concept enables to create a perspicuous time constant as

$$D = \frac{y_i}{k} \quad (2)$$

It is evident (by substituting D for t in Eqn (1), that D is the time to effect a change in y, of which the absolute value is just y_i (doubling the initial transformed intensity, or reducing it to zero; see **Fig. 1**). According to Eqn (2) time constant is proportional to the initial transformed intensity, if rate constant is independent of it and vice - versa (Körmendy, 1991, 1993).

For example, if one wants to fit an n-th order empirical relation to measured data of a chemical concentration change and it seems to be justified by reaction kinetics background, then he must do it on the “rate constant independent of initial concentration” principle. On the other hand, if the heat resistance distribution of a mixed microbial population is independent of cell concentration (i.e. the average distance between cells), an n-th order fitting should be done on the “time constant independent of initial concentration” principle (see **Fig. 2**).

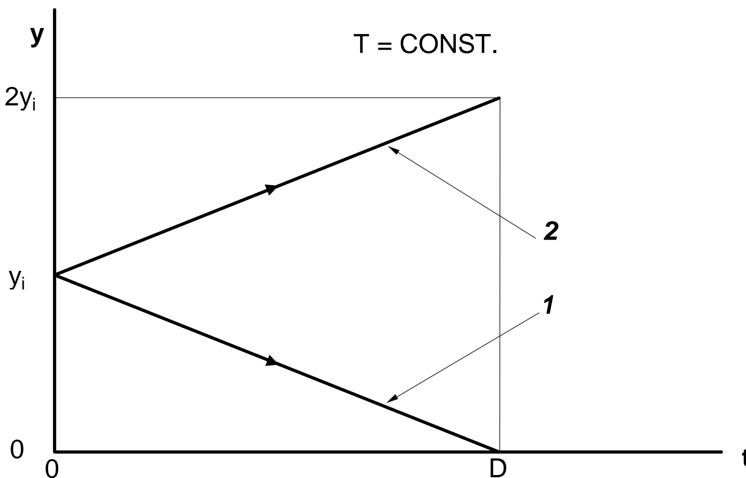

Fig. 1: Interpretation of generalized time constant ($n \neq 1$). *1:* transformed attribute intensity decreases vs. time; *2:* transformed intensity increases vs. time.

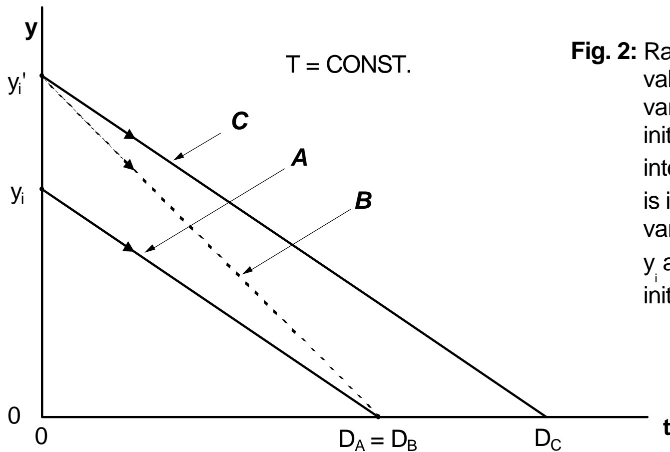

Fig. 2: Rate constant time constant and initial value of transformed intensity. *A:* variation of transformed intensity, when initial value is y_i; *B:* variation of the same intensity, when $y_i' > y_i$ and *time constant* is independent of initial intensity; *C:* variation of the same intensity, when $y_i' > y_i$ and *rate constant* is independent of initial intensity (line *A* and *C* are parallel).

The latter case can be easily understood by realizing, that time dependent relative microbial concentration (N/N_i) of survivals is the same for any N_i, contrary to chemical concentration change (see **Fig. 3**).

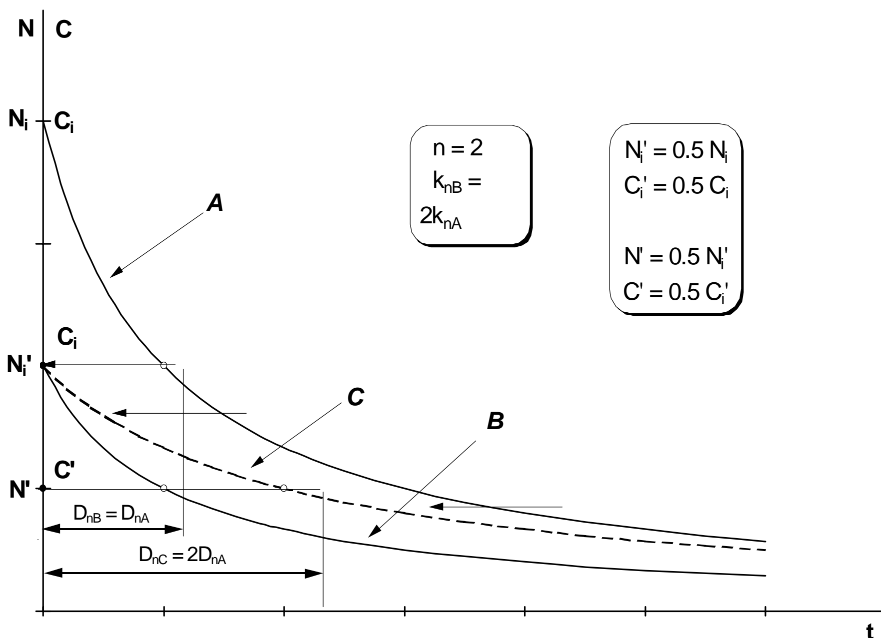

Fig. 3: The same ideas are illustrated as in **Fig. 2**, but in case of n = 2 and using original attribute intensities. *A:* variation of microbial concentration (N) and a chemical concentration (C), when initial concentrations are N_i, C_i; *B:* variation of the same variety as under symbol *A*, but with diluted initial concentration N_i', when time constant is independent of initial concentration (all ordinates of curve *A* have been halved); *C:* variation of the same constituent's concentration as under symbol *A*, but with diluted initial concentration C_i', applying "rate constant is independent of initial concentration" principle (curve *A* has been shifted towards the origo).

In dubious situations experiments are to be repeated at different initial intensities. Horak and Kessler (1981) made presumably a bias, when they evaluated measured data by dividing time dependent thiamine concentrations by the initial one. In this way they changed to the "time constant independent from initial intensity" version (initial concentrations were different at different temperatures).

4 Statistical methods of fitting

A few aspects regarding the fitting method are as follows (Tusnády *et al.*, 1994; Körmendy, 1994a).

4.1 Supposition of normally distributed error is not always justified

The supposition of log - normal error distribution might be occasionally more sensible, specially for intensities in the positive domain, e.g. as concentrations. In case of n-th order equations ($n \neq 1$) intensity transformation results in another log -normal distribution for transformed error. The least squares method has to be changed now, in accordance with the maximum likelihood principle.

4.2 High coefficient of correlation is only one aspect of fitting

Acceptable fitting adequacy has also importance. Inhomogenity of variances needs the use of weighted regression analysis.

5 Attribute intensity change and time dependent temperature

5.1 If attribute intensity variations are available at a set of constant temperatures and proper interpolation formulae are known, the time dependent temperature problems can be always solved on the principle, that the momentary rate of intensity change depends on the actual temperature (T) and intensity (A). Thus, using the reciprocal of intensity rate:

$$d\tau/dA = \partial t[A,T(\tau)]/\partial A \qquad (3)$$

is the differential equation with initial condition $\tau = 0$, $A = A_i$ for solving a task. The essence of Eqn (3) has been demonstrated in the publication of Körmendy and Körmendy (1997) by applying a step - wise time dependent temperature variation. Eqn (3) results from diminishing time intervals and infinitum.

5.2 It has been also demonstrated (Körmendy and Körmendy, 1997), that the use of equivalent heat treatment time calculation is justified only, if relation among attribute intensity (A), time (t) and temperature (T) meets a special condition. This condition appeared in different forms in the literature:

General condition	$t(A,T) = f(A) \cdot g(T)$	Körmendy (1966); Körmendy and Körmendy (1997)
Linear variation of transformed intensity concept	$y(A) = y_i \pm kt$ (see para 3.3.)	Has been applied since about 1981
Index of Deterioration, quality function concepts	$f(A) = \pm kt$	Saguy and Karel (1987); Taoukis and Labuza (1989)

(Tijskens and Polderdijk (1996) based their keeping quality concept on the quality function idea).

It must be realised that the calculation of equivalent heat treatment time is only the first step to obtain attribute intensity at the end of the actual time dependent temperature variation. Naturally, the constant reference temperature relation is to be applied for this purpose, substituting the reference rate constant (k_r) into it.

Rules under para 3.3. are also to be regarded here. In case of “rate constant independent of initial attribute intensity” and different initial intensities, the proper form of the lethality ratio is $L = k/k_r$, while in case of “time constant independent of initial intensity” $L = D_r/D$; reminding that equivalent time is the definite integral of L according to time.

5.3 Local attribute intensity, extreme values and averages

Heat treatment is effected on food either in a food container (can, jar, etc.), or in units of a flow-through type apparatus. In the first case one might speak of local attribute intensities, extreme values and averages. In many industrial processes the concentration of survivals is greatest in the central part of the container (cold point) and lowest in the surface layer at the end of heat treatment. Average concentration or intensity has also importance (Ohlsson, 1980; Silva *et al.*, 1994) and is calculated by a volume integral. No average intensity can be calculated in lack of linear mixing law (see Chapter 1). The previous concepts can be easily transferred to flow - through type heat treatment in case of **macro-mixing**. At the exit of a constant temperature unit (e.g. holding tube) extreme intensity values are related to food elements residing with minimum or with maximum residence time periods. Average intensity can be calculated now by integrating according to time dependent intensity weighted by residence time density (frequency) function (Körmendy, 1994b, 1996).

6 Heat resistance distribution, survival function and first order rate constant distribution

Heat resistance is understood as the lifetime of an individual microbe at constant temperature measured from start of exposure to the said temperature. Its (cumulative) distribution function (F(t)) gives the concentration of already inactivated microbes (N_i - N) as a fraction of initial concentration of living cells (N_i) vs. lifetime, accordingly:

$$F(t) = 1 - \frac{N(t)}{N_i}, \tag{4}$$

id est one minus survival ratio is equal to the (cumulative) distribution function of lifetime (Vas, 1961).

In case the population under heat treatment is a mixture of a large number of subpopulations, which follow first order destruction kinetics (and no microbial growth is present in the mixture), the survival ratio can be approximated as:

$$\frac{N}{N_i} = \int_0^\infty e^{-k_1 t} f(k_1) dk_1 \tag{5}$$

where $f(k_1)$ is the density (frequency) function of the first order rate constant (k_1). Eqn (5) means that survival ratio is the Laplace transform of $f(k_1)$, as $k_1 > 0$. Naturally the cumulative distribution

function of heat resistance is one minus Laplace transform of $f(k_1)$. Thus it is possible to find $f(k_1)$ from a survival function by applying inverse Laplace transformation to survival ratio vs. time relation. In case of n-th order empirical relations ($n \neq 1$, see **Fig. 4**):

$$\frac{N}{N_i} = \left[1 + \frac{t}{D_n}\right]^{-m} \tag{6}$$

and

$$f(k_1) = \frac{D_n^m}{\Gamma(m)} k_1^{m-1} e^{-k_1 D_n} \tag{7}$$

where m = 1/(n - 1).

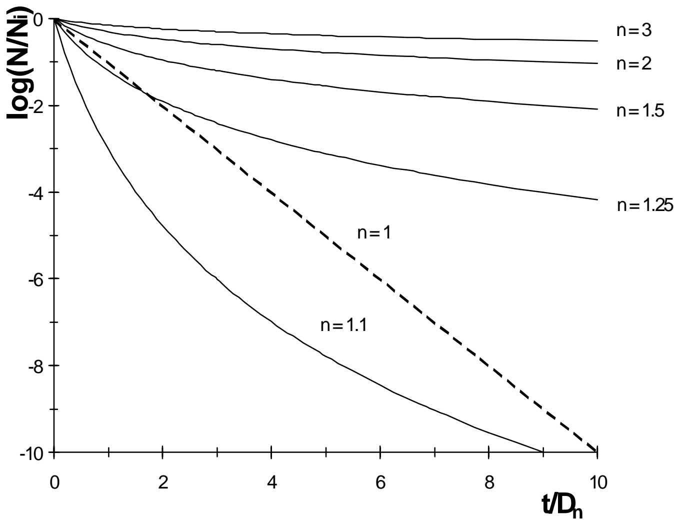

Fig. 4: Logarithms of survival ratios vs. dimensionless time for a number of reaction orders.

Eqn (6) is well-known from literature (perhaps with n-th order rate constant instead of time constant), while the gamma distribution of Eqn (7) has been obtained by using existing Laplace transformation tables (Carslaw and Jaeger, 1959). **Fig. 5** illustrates distribution (frequency) functions according to Eqn (7), but using dimensionless variable $\lambda = k_1 D_n$.

It is possible to decompose a mixed population into subpopulations of first order kinetics in this way (Körmendy *et al.*, 1998).

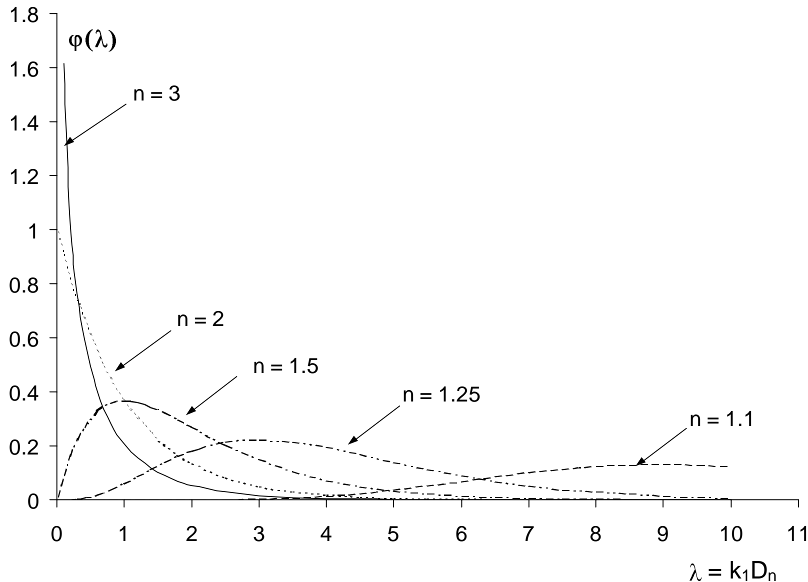

Fig. 5: Dimensionless first order rate constant distribution (frequency) functions.

7 List of symbols

A; A_i	Quality attribute intensity; its initial value
C; C_i	Chemical concentration; its initial value
D; D_r	Time constant; time constant at the reference temperature (s, min)
D_n	Time constant in n-th order empirical relation (s, min)
$f(A)$	Function depending on attribute intensity
$f(k_1)$	Distribution (frequency) function of k_1 (s, min)
$F(t)$	Cumulative distribution function of heat resistance distribution
$g(T)$	Function depending on temperature
k; k_r	Rate constant; rate constant at reference temperature, T_r ([y]/s, [y]/min)
k_1	First order rate constant (1/s, 1/min)
L	$= k/k_r = D_r/D$, lethality ratio
m	$= 1/(n - 1)$
n	Reaction order
N; $N(t)$	concentration of surviving microbes; survival function ($1/cm^3$)
N_i	Initial concentration of living microbes ($1/cm^3$)
t	Time at constant temperature (s, min)
$t[A,T]$	Attribute intensity vs. time relation at constant temperature, explicit for time (s, min)
T; T_r	Temperature; reference temperature (°C, K)
$T(\tau)$	Temperature as function of time
y; $y(A_i)$	Transformed attribute intensity; its initial value
$\Gamma(m)$	The value of the gamma function for m
λ	$= k_1 D_n$, dimensionless first order rate constant
τ	Symbol of time, in case of time dependent temperature change (s, min)
$\varphi(\lambda)$	Dimensionless distribution function of λ

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Validation of food pasteurisation treatments using an amylase time-temperature integrator

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Abstract

A process validation method was described that used 20 μL of an α-amylase time-temperature integrator injected into the centre of a silicone cube. The kinetic factor (z-value) for the amylase was 9.7 ± 0.3 C°, appropriate to values for bacterial spore destruction. Case studies are presented to validate pasteurisation processes for 400 kg yogfruit batches of commercial strawberry and pineapple & passion fruit products where the target P-value at the fruit centres was equivalent to 5 minutes at 85 °C (T_{ref} = 85 °C and z = 10 C°). Silicone cubes of 10 mm size were used to represent heating rates of the strawberry and pineapple pieces. After processing, residual amylase activity was assayed and converted to P-values that gave a minimum of 8.3 minutes for the strawberry and 6.0 minutes for the pineapple. This method can be applied to other products/technologies, for example in-pack rotary processing of foods containing particles, cooking in continuous hot air ovens or in continuous belt fryers (e.g. poultry, chicken nuggets, burgers, bread), cook-chill products cooked in agitated vessels (e.g. ready meals, soups, cook-in-sauces, yogfruits), and continuous heat exchangers for products with particulates (e.g. cook-in-sauces, yogfruits).

Introduction

The application of time-temperature integrator (TTI) systems for validating thermal processes was investigated during an EU funded project led by the Katholieke Universiteit Leuven (Van Loey et al, 1997). From the range of TTIs studied, CCFRA chose an α-amylase based TTI and further developed it as part of a MAFF LINK funded project on continuous thermal processing. This application was to the pasteurisation of food products containing discrete particles in sauces (Tucker, 1998a; 1998b), an important industry sector with the market for cook-in-sauces, soups and preserves expanding and the competition between brands intense. The consumer demand is for products of ever increasing quality to compete with chilled foods but with the advantage of a long shelf life. To consistently deliver products that are safe to eat, have a long shelf life and are of high quality, it is essential to accurately measure the pasteurisation achieved at the centre of the largest and/or slowest heating particle. This becomes a critical control point for establishing the thermal process times and temperatures, and is where the benefits of this new method can be realised over conventional methods.

The key attributes of the α-amylase that make it suitable for validating pasteurisation treatments are given in table 1.

Table 1: Key attributes of the α-amylase system

Amylase source	*Bacillus amyloliquefaciens*
Operating principle	Reduction in enzyme activity
Measurement method	Enzyme assay
Active temperature range	60-100 °C
Kinetic factor, or z-value	9.7±0.3 C°
Decimal reduction time	$D_{80.7}$ = 18.7 minutes
Process value	'pasteurisation-value'
Sample size	20 μL

The kinetic factor (z-value) of the α-amylase was 9.7 ± 0.3 C°, making it ideal to represent the kinetics of bacterial spore and vegetative cell destruction (e.g. *Salmonella* spp., *Listeria* spp. and *Clostridium botulinum*). Most of the processes recommended in the guidelines on Food Pasteurisation Treatments (CCFRA, 1992) can thus be assessed using the α-amylase (see table 2).

Table 2: Selected pasteurisation treatments

Process Type	pH	Time/Temperature Equivalent	T_{ref} (°C)	z-value (C°)
Acid Fruits	3.7 - 4.2	5 minutes at 85.0 °C	85.0	10.0
Tomato Products	4.0 - 4.3	5 minutes at 93.3 °C	93.3	8.3
Tomato Products	> 4.3	10 minutes at 93.3 °C	93.3	8.3
Sous Vide	> 4.5	40 minutes at 70.0 °C	70.0	10.0
Cook-chill	> 4.5	2 minutes at 70.0 °C	70.0	6.7

This paper presents two case studies that used the amylase particles to measure P-values achieved at the centre of fruit particles undergoing a batch heating - continuous cooling process.

Experimental methods

The key development that extended the applications to most pasteurised food products was in modifying the amylase encapsulation method that prevented its contact with the food products during the pasteurisation process. Not only would such contact be undesirable from a food contamination viewpoint but it was likely to affect the reaction kinetics of the amylase. This novel encapsulation method increased the scope of application to a wider range of pasteurisation processes, some of which were difficult to validate with conventional methods (e.g. manufacture of yogfruits in agitated vessels, cooking of poultry products in spiral ovens).

To enclose the amylase, a silicone compound was used because it was transparent, robust, safe to handle, chemically inert and can be moulded into a variety of particle shapes. The physical properties of the silicone have been tested in previous work (McKenna and Tucker, 1991) and shown to be similar to those of food products (e.g. density and thermal characteristics).

Manufacturing the silicone particles to represent the heating characteristics of the critical food particles required several steps. A small quantity (approximately 40 μL) of α-amylase was injected into a silicone enclosed air bubble that could be as small as 3 mm in diameter. The bubble was then inserted directly into the food, for example into a turkey burger, or made into a larger silicone particle that heated at the same rate as the food. After processing, 20 μL of enzyme solution was extracted from the silicone bubble with a hypodermic syringe and the measured activity converted to a pasteurisation value (P-value).

To measure the enzyme activity, 20 μL of α-amylase solution was assayed by adding it to 1 mL of amylase reagent equilibrated at 30 °C, mixing by inversion, and then measuring the rate of increase of absorbance at 405 nm between 1.5 and 2.0 minutes from insertion into a spectrophotometer (Unicam PU8700).

The initial ($A_{initial}$) and residual amylase activities (A_{final}) from each particle were converted to P-values (equation 1) using the value for decimal reduction time at 85 °C (D_{85} = 6.95 minutes).

$$P = D_{85} \times [\log (A_{initial}) - \log (A_{final})] \qquad (1)$$

where A_{final} is the final activity after a specific time-temperature history
$A_{initial}$ is the initial activity

Case studies to validate two yogfruit processes

The first commercial tests for the amylase particles were to validate processes for an intermediate strawberry product and a pineapple & passion fruit product intended for use in yoghurt manufacture. Much of the particle development work arose from the strawberry trial in which some particle losses occurred. Both fruit products used Colflo 67 starch as the principal thickening agent (National Starch). Batch sizes were 400 and 430 kg, with 25 wt% of IQF strawberries and 27 wt% of 10 mm pineapple cubes respectively. The batches were heated to 90 °C in a horizontally agitated vessel (Giusti), held at this temperature for 5 minutes, then cooled to 35-45 °C in a 5-pass 47 mm i.d. Spiraflo tubular heat exchanger (Tetra Pak).

Silicone cubes of 10 mm size were used to represent the strawberry and pineapple particles with the air bubbles positioned precisely at the centre of each silicone cube. This ensured that measured P-values were representative of the pasteurisation achieved at the fruit particles, the critical position. Approximately 40 μL of amylase solution was injected into each air bubble and sealed with fresh silicone compound. Initial amylase activity ($A_{initial}$) was measured for 5 samples using a spectrophotometer.

For the strawberry validation trial, 12 amylase particles were added to the cold batch, processed according to the recipe and retrieved from the 400 kg filled tank after cooling. Subsequent improvements were made to the manufacture of the silicone cubes to improve their strength so that for the pineapple validation trial, 45 amylase particles were used. The amylase particles were laboriously recovered from the fruit batches by spreading the products over a stainless steel sorting table.

The number of amylase particles required to fully define the P-values for the 430 kg batch of pineapple & passion fruit mixture was determined using the sampling plans given in BS 6001: Part 1: 1991. In statistical terms it is impossible to be 100 % sure that no particle has a P-value less than the target of 5 minutes since sampling theory assumes that defects occur and therefore an

acceptable level has to be chosen. However, for process safety, it is unacceptable to have fruit pieces with centre P-values below 5 minutes, and so the silicone cubes were oversized by approximately 10% to take this into account.

The calculation of sample numbers first required a calculation of the number of pots of yoghurt that could be produced from this 430 kg batch. This gave 11,460 pots, for which the sampling plan was designed to ensure that less than 5 % of pots contained a pineapple with a P-value less than 5 minutes. For lot or batch sizes between 3,201 and 10,000 the number of samples to be randomly selected at special inspection level S-3 was 32. To ensure that at least 32 particles were recovered intact and with sufficient amylase for the assays, 45 particles were used.

Results and discussion

Figure 1 shows the heating and holding profile of the strawberry carrier liquid, taken by temperature sampling with a long probe at the batch cold spots. The carrier liquid P-value estimated from these times and temperatures was 52 minutes (T_{ref} = 85 °C, z = 10 C°) which was expected to be much greater than that at the particle (strawberry) centres. The amylase results confirmed this (see table 3), with the target P-value of 5 minutes equivalent at 85 °C achieved by all of the particles. Of the twelve amylase particles introduced to the strawberry batch, only ten were recovered intact.This prompted improvements to be made to the methods used to produce the silicone cubes that increased the chances of them surviving a hostile processing environment intact. The processes in the agitated Giusti vessels represented a severe test for the particles, where the shear forces experienced by the particles were in excess of most other processing systems.

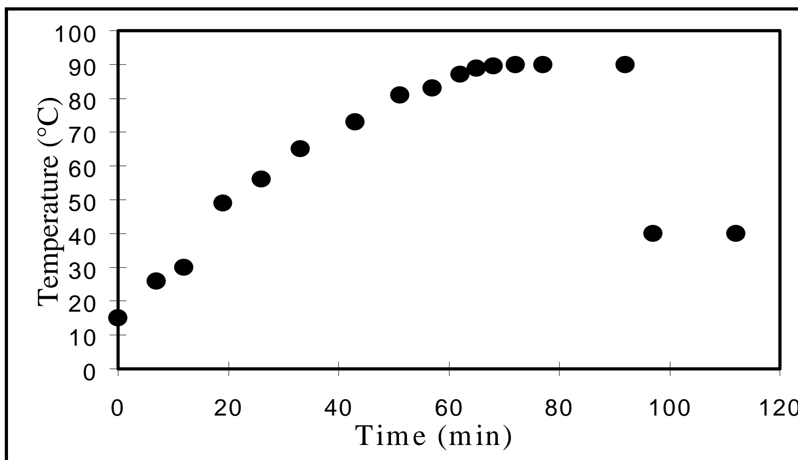

Figure 1: Heating and holding temperature profile of the strawberry carrier liquid

Following improvements to the silicone cube manufacturing method, 45 amylase particles were added to the 430 kg pineapple & passion fruit batch with 44 recovered intact after processing. The increased number of particles was used to build up the distribution of P-values as shown in figure 2. No measured P-values were less than the target of 5 minutes equivalent at 85 °C, with a minimum P-value of 7.0 minutes. This provided the confidence that the processing regime given to this product was safe. Future modelling on the P-value distribution is planned in order to predict the P-values that may be obtained with larger sample sizes, for example for individual pineapple pieces in the 430 kg batches.

Table 3: P-value results for the total process delivered to the strawberry product

Particle number	P-value for $D_{80.7}$ (min)	P-value for $D_{85.0}$ (min)
1	45.9	17.1
2	35.3	13.2
3	35.3	13.2
4	39.0	14.5
5	22.3	8.3
6	22.4	8.4
7	45.9	17.1
8	45.9	17.1
9	49.1	18.3
10	45.9	17.1

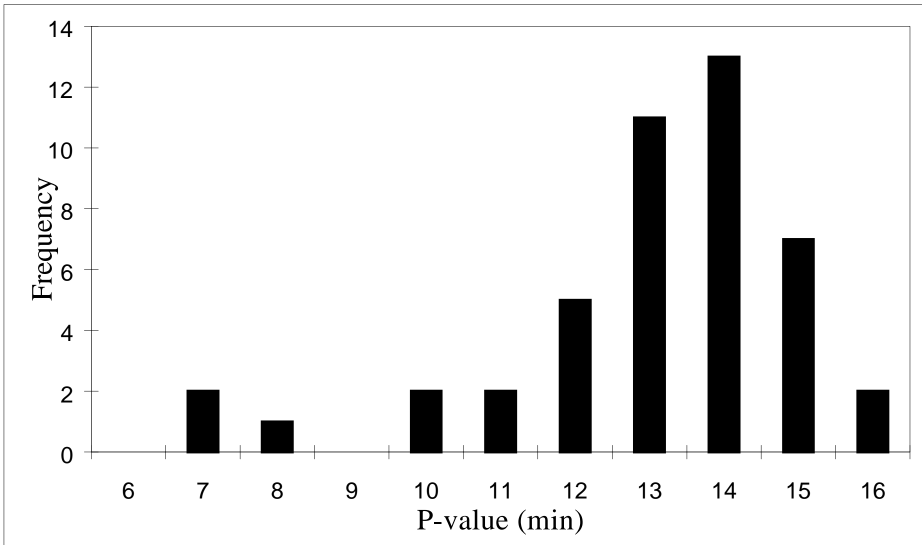

Figure 2: Distribution of the cube centre P-values ($D_{85.0}$) for the pineapple & passion fruit product

Application to other pasteurisation processes

This amylase method has proved to be an important tool for measuring the P-values achieved at the centre of food particles, such as those in the yogfruit examples. Although developed within a research project focusing on continuous processing, it can also be applied to many other pasteurisation technologies where the benefits of no trailing wires can be realised. For example:

- continuous and batch ovens (e.g. chicken nuggets)
- continuous and batch fryers (e.g. doughnuts)
- cook-chill (e.g. recipe dishes)
- baking (e.g. fruit pies)

- hot fill (e.g. cook-in-sauces)
- roasting (e.g. ham joints for slicing)
- heat exchangers (e.g. pie fillings)
- hot smoking (e.g. smoked mackerel)

Many of the above pasteurisation technologies are ones in which the mild thermal process is used in combination with another preservation system, such as reduced water availability or chilled storage. The advantage of utilising such combination technologies is to extend the scope of pasteurisation treatments to many more foods. This ever increasing diversity puts a demand on the validation resources available to food companies, stretching the applicability of traditional trailing wire systems. The amylase encapsulation method described here has an important role to play in providing food companies with a proven system that can be used for validating food pasteurisation treatments where such traditional methods are impractical.

Conclusions

The key benefit of this new method is that no trailing wires are required so the product with its amylase particle inside can travel through a cooking process unhindered. It provides an alternative process validation tool to temperature probe systems and to microbiological methods such as the spores encapsulated in alginate beads (Brown et al., 1984). Its advantages over the alginate spore technique are that each amylase assay requires only a few minutes and perhaps more importantly there is no need to introduce bacterial spores to a factory environment that could present a contamination risk.

Acknowledgement

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Optimal control of microwave combination ovens for food heating

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Abstract

Microwave combination ovens are ovens in which the food is heated by a combination of heat transfer mechanisms, including microwave propagation, convection, steam, radiation and high temperature bottom contact plates. Their aim is to overcome some of the problems with microwave-only ovens such as edge overheating and cold spots, while maintaining the advantage of speed over conventional ovens. However, the combined effect of these heating modes complicates the instructions for and operation of the appliances.

In this contribution an overview is given of the results obtained so far in the framework of project FAIR CT96-1192 which is funded by the European Union. The overall objective of this project is to improve the operation of domestic ovens with multimode heating functions in order to optimize the final food temperature uniformity while minimizing quality losses due to edge overheating. The project team consists of 5 partners covering different areas such as control theory, heat and mass transfer modelling, microwave modelling, microwave oven design and construction, and optimisation theory.

Introduction

Domestic microwave ovens are now being used increasingly to heat and prepare foods as a fast alternative to conventional radiation and/or convection ovens. However, several problems have been identified, including the existence of hot and cold spots, and excessive heating at product edges and corners. This is important from the food safety point of view, as possible contaminating pathogenous micro-organisms may survive in the cold spots. Further, excessive heating of edges and corners may cause dehydratation and undesired discoloration, making the food less attractive. On the other hand, food preparation such as roasting is intended to give a crust and cannot be done in a microwave-only oven.

Appliances combining the microwave heating mode with conventional heating mode such as forced air or grilling are increasingly popular in Europe. These *microwave combination ovens* are intended to overcome some of the problems with microwave-only ovens, while maintaining the advantage of speed over conventional ovens. However, the combined effect of these heating modes complicates the instructions for and operation of the appliances.

During the past decades several model-based procedures have appeared in thermal food processing technology which are aimed at optimizing the quality of foods while assuring the mandatory microbiological requirements. It has been shown that a considerable improvement of the process in terms of decreasing the process time and increasing the surface quality of the treated food can be achieved for the case of thermally sterilized conduction heated foods. It can be readily expected that the application of appropriate optimization and control methods would improve the operation of microwave combination ovens significantly. However, the complexity of the governing models have hampered the application of optimization methods to this process so far.

The overall objective of the proposal is to improve the operation of domestic microwave combination ovens in order to optimize the final food temperature uniformity while minimizing quality losses due to overheating and maintaining microbiological safety. The following subobjectives can be distinguished:

1. To develop models and numerical software to predict the temperature in foods during heating in microwave combination appliances
2. To develop numerical procedures to assess the uncertainty of the temperature distribution inside the heated food as a consequence of the variability of product and process parameters.
3. To optimize the heating process as quantified by a series of performance indicators, while safeguarding microbial safety
4. To develop robust online control procedures that would assure optimal processing in spite of process deviations and product variability
5. To validate the developed procedures using model and real food systems in existing household microwave combination ovens

The partners are: the Katholieke Universiteit Leuven (K.U.Leuven, Belgium, co-ordinator), the Instituto de Investigaciones Marinas, (IIM-CSIC, Spain), the Swedish Institute for Food Research (SIK, Sweden), Whirlpool Sweden AB (Norrköping, Sweden) and the Universidad de Vigo (UVI, Spain). The results described in this paper were extracted from the publications which were generated in the framework of this project and which are included in the reference list at the end of this paper. For further details the reader is referred to the original publications.

Results

The models for heat and mass transfer in the microwave combination oven are a key issue in the project. In the first project year a considerable amount of work has gone into the development of models of increasing complexity and corresponding software implementations. At the lowest level several models which are based on Lambert's law for microwave propagation have been implemented. These models were already described and validated in the literature; however, they are only valid for a particular geometry and are validated only in constant operating conditions. It was clear from the beginning of the project that more complex models, including Maxwell's equations for the propagation of the electromagnetic field and Luikov's equations for coupled heat and moisture transfer are required for the purpose of this project. Software is now available at the different institutes to solve these equations, and qualitative validation experiments have been carried out with satisfactory results for the Maxwell's equations. Validation experiments for the coupled heat and mass transfer are being carried out. Large parts of the code for coupled heat and mass transfer are currently being rewritten in FORTRAN to decrease the execution speed, and to make the code portable so that it can be linked easily to the optimization.

From the results of the optimization studies it can be concluded that a significant improvement of the uniformity of both temperature and cook value can be expected by combining microwave and convection heating in an optimized way. This supports the feasibility of the project objective. The stochastic optimization algorithms appear to be superior to other gradient-based algorithms. It will be very interesting to compare the optimal control profiles with those obtained through the Pontryagin minimum principle. Some theoretical results are expected in the second project year.

Simulations so far indicated that a real time closed loop implementation of such control objectives is feasible. Also, more advanced algorithms such as Internal Model Control indicate a considerably

better performance than the typical on/off algorithms which are used in practice. However, the lack of actuators and sensors complicates the implementation of the controllers in a real microwave combination oven. For example, it is not possible to measure centre temperatures in the food in a cheap way (although laboratory equipment such as fiber-optic probes will be used for model validations). It will be investigated of an estimation of the centre temperature can be made (e.g., using the simplified models developed before) using inferential methods.

Three identical ovens are now available at the K.U.Leuven, IIM and SIK, and the acquisition and control software is operational.

The project is still ongoing, and further results are expected to be published in the literature.

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Determination of the total water content of foodstuffs by variations of the Karl Fischer titration

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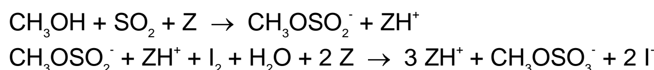
Introduction

The water content is of great significance for technological, microbiological and nutritive properties of foodstuffs as well as having legal and economic ramifications. Its determination is therefore one of the most frequent analyses performed on foodstuffs. Very often drying techniques are applied. They do not, however, yield the water content as result, but a mass loss under the employed conditions, and are therefore variable. All the substances that are volatile under the drying conditions, even those produced by the process itself, contribute to the mass loss and are therefore measured as water, whereas very strongly bound water may escape detection. The Karl Fischer titration has become the most important method to determine the water content in many products. It is also used as reference or calibration method for other techniques that do not really measure the water content, such as drying techniques [1-4] or when the relationship between the measured entity and the water content is only empirical and rather complex such as in NMR and NIR spectroscopy or microwave techniques [5].

The Karl Fischer titration is based on a selective two-step chemical reaction [6]:



$$CH_3OH + SO_2 + Z \rightarrow CH_3OSO_2^- + ZH^+$$
$$CH_3OSO_2^- + ZH^+ + I_2 + H_2O + 2\ Z \rightarrow 3\ ZH^+ + CH_3OSO_3^- + 2\ I^-$$

The titration is carried out in a working medium, which usually consists essentially of methanol. Methanol and sulphur dioxide form the ester methyl sulphite, CH_3OSO_2H, which is then oxidized to methyl sulphate, CH_3OSO_3H, by iodine, the titrating reagent (added in a solution), in a stoichiometric reaction that requires water. In order to achieve a practically complete reaction, a base Z is added, nowadays mostly imidazole, to neutralize the acidic groups of the esters formed. The "historic" pyridine has several disadvantages.

When no more water is left in the titration cell and, therefore, the Karl Fischer reaction can no longer take place, added iodine remains unchanged. This first excess of iodine indicates the end point of the titration. The mostly used detection of the end point is based on an electrochemical effect. Using the bivoltametric variety, two platinum electrodes are polarized by a constant current of about 50 μA. The voltage necessary to maintain this current is monitored. The first excess of iodine and thus the presence of the redox system iodine/iodide renders possible an easy corresponding redox reaction at both electrodes. This makes the voltage drop abruptly. When the voltage remains under a certain value - as final voltage very often 250 mV is chosen - for a certain time, the so-called stop delay, the determination is completed.

The stop delay is very important because of the fact, that water may not be immediately freely disposable. Many samples are not completely soluble in methanol, and consequently water reaches the working medium only with a certain delay by diffusion and extraction. The arrival of delayed water in the working medium makes the voltage rise above the critical value again. The delay time takes this fact into account.

The electrodes may also be polarized by a constant voltage with, consequently, monitoring the current. At the end point this current rises suddenly. When it remains above a certain value during the chosen stop-delay time, the titration is stopped. This variety is the biamperimetric method.

The water to be determined must react with the chemical partners. The ideal sample should therefore be soluble in methanol which usually serves as working medium. Most foodstuffs do not fulfil this condition. This necessitates variations of the standard procedure which aim at a complete liberation of the water and, consequently, a quick contact with the reagents. A selection of such variations is described and some experimental results are given.

Experimental

The titrations were carried out with the KF Titrino 701 from Metrohm, Herisau/Switzerland, using the bivoltametric end-point indication with a polarizing current of 50 μA and a stop voltage of 250 mV. The chemicals were from Riedel-de Haën, Seelze/Germany, particularly the Karl Fischer reagents Hydranal-Composite 5 (one-component system) and Hydranal-Titrant 5/Hydranal-Solvent (two-component system). The homogenizer IKA Ultra-Turrax T25 was from Janke & Kunkel, Staufen im Breisgau/Germany.

Results and discussion

Time- or drift -controlled end point

Some samples need much time to be dissolved. In the case of samples which are not completely soluble the water reaches the working medium only by diffusion and extraction processes from the inner parts of the sample particles into the working medium. In these cases the duration of the titration must be long enough. One measure is a long stop-delay time. If this time, however, is too long, one risks to find no or an incorrect end point, because water intruding from outside may necessitate small reagent additions within the set stop-delay time. It is a better way, therefore, to measure this so-called drift before the start of the analysis and stop the titration when the titration rate has reached the initial value of the drift again. The reagent volume due to the drift with taking the duration of the titration into account should be deducted from the reagent volume. The titrator used allows this procedure automatically.

Extraction of the water

Another possibility is to provide a minimal titration time ("extraction time"). The analysis is not terminated before this time has elapsed, not even if the end-point criteria should be reached before. A pure stirring time before the start of the titration is not so effective, because with continuous titrating during the extraction the gradient of water concentration is steadily kept at a maximum. External extractions with consecutive titration of an aliquot are also possible but contain several additional theoretic and experimental aspects to be considered [7].

Reduction of the particle size

The smaller the particle size, the shorter are the distances the water molecules have to cover to reach the working medium. External milling or a similar operation with consecutive transfer of the sample into the titration cell brings about the risk to loose water that was originally contained in the product. An internal treatment in the titration vessel itself is preferable. This can be carried out by

using a homogenizer. The shaft is introduced through an additional opening in the lid of the titration cell [8]. Table 1 shows results obtained by internal sample homogenization compared to the standard procedure [8]. Titration times for sultanas and cheese are considerably shorter. Cocoa and rye flour have already a particle size below the one the homogenizer can achieve.

Table 1: Comparison of the standard procedure to internal homogenization (5 replicates each)

Product	Standard procedure		1 min Ultra-Turrax	
	Time [min]	Water content [%]	Time [min]	Water content [%]
Emmental	20	32.45 ± 0.08	3	32.4 ± 0.29
Sultanas	30	14.50 ± 0.01	3	14.5 ± 0.06
Cocoa	30	3.78 ± 0.05	same as standard procedure	
Rye flour	30	11.55 ± 0.04	same as standard procedure	

Titration at elevated temperatures

At higher temperatures the migration of water from the core of sample particles into the working medium is faster. Temperatures of up to about 50 °C can be realized in commercial titration cells with thermostatic jackets. A step further is the titration at temperatures up to the boiling point of the working medium. The titration vessel has then to be altered. The usual titration cell consists of a beaker as lower part (possibly with a thermostatic jacket) and a covering lid with holes for the electrodes, the burette tip and other equipment. This normal cell was replaced by a multi-necked round-bottomed flask with ground joints. A reflux condenser with a drying tube at the top was attached to the central neck. The measured drift values proved the tightness of the apparatus. It was even better than that of common titration vessels. It could also be shown that the Karl Fischer reaction follows the same stoichiometry as at lower temperatures [9].

Table 2 lists results for some cereal products [9]. The time gain at higher temperatures is enormous and the determinations are more complete.

Table 2: Karl Fischer titration of cereal products at different temperatures (4 replicates each, but 2 only of wheat flour at 25 °C)

Product	Water content [%]	Temp. [•C]	Titration time [min]
Rye flour (pre-dried)	9.11 ± 0.04	25	30-36
	9.22 ± 0.03	50	9-11
	9.23 ± 0.03	65	4-5
Rusk	3.42 ± 0.03	50	19-21
	3.62 ± 0.01	65	11-13
Pasta	10.38 ± 0.03	50	8-9
	10.43 ± 0.02	65	4-5

Internal homogenization at elevated temperatures

The internal homogenizer can also be used at elevated temperatures. Up to 50 °C the usual cell with thermostatic jacket and the special lid with the additional hole can be used. For analyses at the boiling point a round-bottom flask as for the boiling-point titration but with a further neck has to be used. In this case the shaft of the homogenizer is introduced through the central neck and the reflux condenser is fitted to a side neck [10]. Table 3 lists results obtained at high temperatures with and without use of the homogenizer [10]. A further time gain can be observed.

Table 3: Water content determinations of toothpaste and coffee powder at elevated temperatures with and without use of the internal homogenizer Ultra-Turrax (UT) (10 replicates each)

	Tooth paste		Coffee powder	
	50°C, without UT	50°C, with UT	65°C, without UT	65°C, with UT
Water content [%]	33.8 ± 0.55	34.1 ± 0.44	4.31 ± 0.05	4.33 ± 0.05
Extraction time [min]	5	5 UT + 5	10	5 UT + 5
Titration time [min]	30	5	25	20

Polarity of the working medium

By adding further solvents to the methanol in the working medium the solubility or dispersibility of the sample can be improved. Formamide is recommended for polar substances, chloroform for non-polar samples. It could be shown that in some cases the use of the toxic chloroform can be avoided when the titration is carried out at elevated temperatures and with internal homogenization [10]. Table 4 gives an example [10].

Table 4: Titration of margarine (low fat content) at 50 °C with homogenization by Ultra-Turrax (UT) with and without addition of different volumes of chloroform to the working medium (WM) (10 replicates each)

	WM:$CHCl_3$ = 2:1 without UT	WM:$CHCl_3$ = 5:1 with UT	WM (without $CHCl_3$) with UT
Water content [%]	56.1 ± 0.31	56.0 ± 0.40	56.0 ± 0.19
Extraction time [s]	120	110 UT + 10	110 UT + 10
Titration time [min]	2	2	2

Fat containing products may be titrated in the less polar 1-propanol to avoid the addition of the toxic chloroform. The electric end-point criteria must then be changed considerably because of the different dielectric properties of methanol and 1-propanol [11]. A slight addition of methanol is advantageous to avoid the formation of a precipitate after the first or second titration. Thus several titration without changing the working medium are possible. Table 5 shows examples.

Table 5: Titration of cream and mayonnaise in different working media (10 replicates each); the small addition of methanol to 1-propanol is necessary to prevent the forming of a precipitate

Working medium	Temperature [°C]	Water content [%]
Cream		
12 ml methanol + 6 ml chloroform[a]	20	62.5 ± 0.30
20 ml 1-propanol + 0.5 ml methanol[b]	20	62.6 ± 0.13
Mayonnaise		
12 ml methanol + 6 ml chloroform[a]	50	45.0 ± 0.07
20 ml 1-propanol + 0.5 ml methanol[b]	50	45.1 ± 0.14

[a] polarizing current 50 μA, stop voltage 250 mV
[b] polarizing current 3 μA, stop voltage 100 mV

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Use of cellular systems to characterise plant tissue behaviour

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Abstract

Two cellular systems have been studied to characterise the physiological and physical changes, i.e. cell viability and cellular shrinkage, which take place in plant tissue during osmotic treatments. The behaviour in hypertonic solutions of isolated protoplasts, i.e. single cells without cell wall, from cortex tissue of carrots and cortex tissue of strawberry has been evaluated at several operation conditions. CSLM (Confocal Scanning Laser Microscopy) in fluorescent mode has been used to determine cell volume changes upon osmotic treatment. With a minimum of sample preparation, on-line micro-experiments in which the osmotic treatment of the sample takes place directly on the microscope were carried out. Cellular shrinkage was evaluated for different operation conditions with respect to osmotic pressure which was established through sucrose concentrations ranging from 30 to 60%.

Introduction

By immersing biological materials into aqueous solutions of sufficiently high concentration of solutes as salts or sugars two major and simultaneous counter-current flows are initiated: a release of water from the tissue into the solution and a simultaneous transfer of the solute into the tissue. Although the mass transfer mechanisms involved are not fully understood, many authors have underlined the important role the plant tissue nature (e.g. composition, structure) may play in the mass transport following osmotic treatment. Osmosis seems to be the main phenomenon during the first phases of the process (Marcotte et al. 1991a, 1992; Saurel et al. 1994); because of its properties as a selective barrier, the plasma membrane hence is the most important cellular organ concerned.

Shrinkage has been considered an indicator of the structural changes undergone by the plant tissue and a necessary parameter to model the process. In this way, a relation between total shrinkage and physiological changes as plasmolysis and cellular collapse has been suggested (Yao et al. 1996). Cellular and extracellular shrinkage as a function of macroscopic parameters, such as solute concentration of the osmotic solution, solute gain and mass loss have been calculated to describe the microscopic process (Marcotte et al. 1991b).

Until now several microscopic techniques have been employed to estimate the qualitative effect of osmotic treatment on tissue structure (Saurel et al. 1994; Torreggiani et al. 1997). At present, the new Confocal Scanning Laser Microscopy (CSLM) allows the optical sectioning from bulk specimens with a minimum of sample preparation and to carry out on-line micro-experiments in which the osmotic treatment of the sample takes place directly on the microscope. The confocal microscope is based on the principle that the light is focused on a well-defined depth in the specimen and that information from this focal point is projected onto a pinhole in front of the detector. The light illuminates a very small region of the specimen and the point detector ensures that only light from that small region appears. In this way, the depth resolution in CSLM is much better than in the conventional microscope (Blonk et al. 1993). In CSLM various modes of microscopy can be performed: reflected light, transmitted light and fluorescent incident/emitted light. In biomedical applications and in food microscopy, the latter has been shown to be the most

powerful technique, because the various structural elements do not differ largely in reflective power. In biology CSLM is mainly used in animal physiology while in botany its use has not been so extensive. The application of CSLM to food technology has been restricted to the study of emulsions because of the difficulties to specifically stain plant tissue structures.

Plant single cells and isolated protoplasts, i.e. single cells without cell wall, have been proposed as a model system to evaluate the influence of unit operations or processes (Knorr 1994). These model systems resemble the state of a cell as it is found within the plant tissue and can be reproduced under controlled conditions. Furthermore, the use of plant single cells avoids masking of stress responses to previous handling as cutting or peeling. Protoplasts from plant tissue are obtained upon an enzymic treatment. Enzyme sources and maceration conditions, e.g. cellulase-pectinase ratio, pH, temperature, have to be empirically set up for each plant material. Moreover, small variations in the tissue composition due to physiological changes and age of the raw plant material can drastically modify the isolation process yield.

Other cellular systems, as cortex tissue of strawberry, seem to be particularly suitable to study microstructural changes. On ripening, the connections between the parenchyma cells of the strawberry cortex progressively decrease (Szczesniak et al. 1969). Therefore cells are separated from each other allowing a more efficient labelling of non fixed tissue.

Isolated protoplasts and cortex tissue, both made up of parenchyma cells, are used to study the behaviour of plant material under osmotic stress. These cellular systems allow to study separately the role of the plasma membrane and, on other hand, the effect of the solid matrix (cell wall, intercellular space) on the osmotic process.

The aim of this work is to show the possibilities of CSLM to characterize the physical and physiological changes, i.e. cellular shrinkage and cell viability, which take place during osmotic treatments in two cellular systems: isolated protoplasts from cortex tissue of carrots, and cortex tissue of strawberries.

Material and methods

Carrot protoplasts

Carrots (*Daucus carota L.*) from a local provider were peeled and cut in cylinders. Cortex tissue was separated using a corkborer and cut into approximately 2-mm rings. The segments were transferred to a digestion medium consisting of 0.6 M mannitol (a_w = 0.989), 2% (w/v) cellulase, 0.1 % (w/v) pectinase and 0.02M phosphate buffer (pH 4), all reagents from Merck (Darmstadt, Germany). Four g of tissue were added by 20 ml of digestion medium. The tissue was vacuum infiltrated for 5 min to facilitate penetration of the digestion medium into the cell wall space. Tissue was incubated in enzymic medium for 3 h at 24-26 °C. Protoplasts were separated from partially digested tissue by filtering with polyamide cloth. The filtrate was centrifugated at 250g for 5 min and protoplast pellet suspended in 0.7 M mannitol solution (pH 6.5). The protoplast sediment was suspended in 5 ml of Ficoll 12% (w/v) in 0.7 M mannitol, 0.02 M phosphate buffer (pH 6.5). A discontinuous gradient was formed by successively layering 5 ml of 8%, 5%, and 0% Ficoll in 0.7 M mannitol, 0.02 M phosphate buffer (pH 6.5) over the protoplast suspension. The gradient was centrifugated at 400g for 20 min and protoplasts were collected at the interface between the 0% and 5% Ficoll layers. The protoplasts were diluted with 0.7 M mannitol and centrifugated at 250g for 5 min. The final pellet was

suspended in 0.5 ml of 0.7 M mannitol prior to use in experiments (Gronwald et al. 1982; Langerbartels et al. 1981).

Cytoplasm of isolated protoplasts with enzymic activity was stained with fluorescein diacetate (Sigma Aldrich Chemie). For that, the protoplast suspension was treated with 100mg/ml fluorescein diacetate for 3 min and repeatedly washed with 0.7 M mannitol (250g, 5 min).

Isolated protoplasts were osmotically treated with 30, 40, and 50% sucrose solutions directly on an invert microscope. Experiments using 30 and 40% sucrose solutions were carried out twice using cells from the same isolation process. Four samples, two of them coming from the same protoplast isolation, were treated with 50% sucrose.

Strawberry tissue

Fresh strawberries (*Fragaria ananassa,* var. Elsanta) from a local provider were cut in halves. A 2-mm slice of each half was longitudinally cut and pith and epidermis tissue were removed with a razor. Cortex tissue cuts were held in isotonic solution, 0.3 M (a_w = 0.995) mannitol, for 1 hour (Flowers et al 1992). After that cytoplasm and cell wall were labelled. For this, cytoplasm was stained with fluorescein diacetate; cell walls were labelled with congo red (Sigma Aldrich Chemie) because of their affinity to cellulose and β-glucanes. Cortex tissue cuts were incubated in a 100 μg/ml solution of congo red for 1 hour at room temperature, then the sample was treated with fluorescein diacetate, 100 μg/ml for 60 minutes; finally it was gently washed.

As for carrot protoplast, the osmotic treatment was carried out directly on the microscope. Strawberry tissue was treated with 40 and 60% sucrose solutions. Each osmotic treatment was repeated eight times.

Microscopy

The micro-experiment took place in a cylindrical sample chamber placed over the microscope lenses. The chamber was made up of two cylindrical pieces to hold the coverslip. The coverslip was coated with poly-L-lysine solution, an effective compound in promoting adhesion of sections (Sigma Aldrich Chemie), in order to attach the plant specimen (carrot protoplasts or strawberry tissue) to the coverslip's surface. After the sample had been placed into the 5 ml volume chamber, osmotic sucrose solution was added by pumping at 20°C ± 2°C.

A Zeiss LSM 410, Anxiovert 135 invert microscope and a Zeiss 20x objective (NA 0.5) was used. An argon laser generates two excitation beams of 488 and 543 nm. Three dichroic mirrors and a set of filters for blue, green, red and far-red fluorescence were employed to split the different emission signals.

During osmotic processing images of the same group of cells were stored in a digital format for further processing.

Image analysis

OPTIMAS 6.2 was the image analysis software employed to measure the protoplast surface in different phases of the process.

Results and discussion

Dynamic microscopic studies of cell plants have been found to be an efficient tool not only to describe qualitative structural changes but also to estimate some parameters related to the plant structure such as cellular shrinkage. The laborious sample preparation required in classical light microscopy and also in some electronic microscopies has restricted their use to qualitative studies of structural modifications caused by osmotic treatments. CSLM allows to overcome this limitation as no chemical fixation is necessary to prepare the sample.

Protoplast suspensions are biological model systems in which a good fluorescent labelling can be obtained as the diffusional problems of on-block staining of plant tissue are negligible. Cell wall and cytoplasm of strawberry cortex tissue could be effectively stained because the rather loose connection between parenchyma cells allowed a good diffusion of the fluorescent probe. The use of fluorescent vital stains, on the other hand, provides information about the physiological state of the plant material, as the fluorescence of fluorescein diacetate, a reagent which diffuses into the cells and is colourless until its ester bonds are hydrolyzed in the cytoplasm, is related to cell viability. So the absence of a fluorescence signal indicates a loss of membrane integrity and, hence, cellular death.

Changes undergone by a sample of carrot protoplasts during the first 20 minutes of osmotic treatment with 40% sucrose solution are shown in Fig.1. During the first minute, a quick cellular shrinkage can be observed, while no important cellular volume decreasing has been found from 15 minutes of treatment. Both a breakdown of the plasma membrane and an out-of-focus image because of protoplast detachment can be the reasons of a vanishing of the signal from some protoplasts along the process.

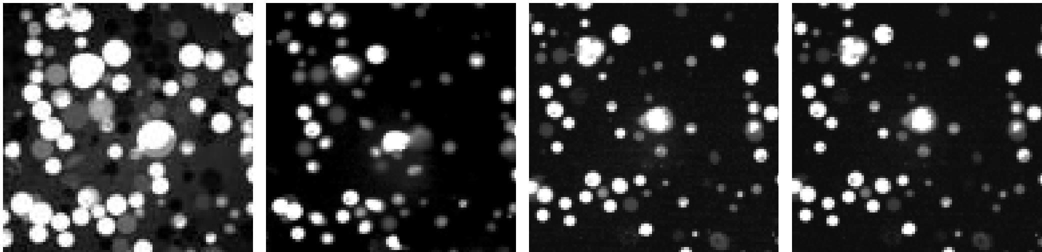

Figure 1: Carrot protoplasts from cortex tissue after 0, 1, 15 and 20 min of treatment with 40% sucrose solution at room temperature (20x magnification)

Cell wall and plasma membrane of strawberry cortex tissue are shown at different processing times in Fig. 2. It should be pointed out that, at the original colour images, protoplasts and cell wall emitted green and red fluorescence, respectively, allowing a better structure visualization. Different stages of protoplast separation from the cell wall (plasmolysis) due to the osmotic pressure of the sucrose solution can be clearly observed.

To show the possibilities of CSLM as a tool to evaluate cellular changes, protoplast shrinkage was calculated. Assuming that changes in volume are proportional to surface changes in the xy plane, cell shrinkage was calculated for every cell from the ratio of the surface at any time to the initial surface (S/S_0).

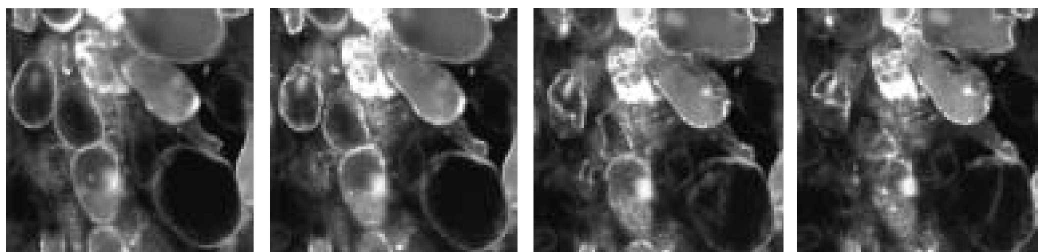

Figure 2: Strawberry cortex tissue processed with 40% sucrose solution at room temperature after 0, 15, and 30 min of treatment (20x magnification)

For carrot protoplasts the surface of approximately 100 cells corresponding to two experiments was measured for treatments with 30% and 40% sucrose solutions. Every cell could be identified and the cellular shrinkage was calculated as the average of the single cellular shrinkage of n cells ($S/S_0=\Sigma(S_i/S_{0i})$). In the case of experiments with 50% sucrose, a very fast cellular response gave rise to a high detachment rate, which hindered the identification of every protoplast along the process. Therefore cellular shrinkage was calculated from the ratio of the average surface at any time to the average initial surface ($S/S_0=\Sigma S_i/\ \Sigma S_{0i}$).

A value of 0.87±0.25 was obtained for the shrinkage of carrot protoplasts after 15 minutes of 30% sucrose solution treatment; for the same processing time with 40% sucrose solution, a value of 0.56±0.15 was obtained. After 15 minutes of treatment with 50% sucrose solution 0.37±0.20 and 0.26±0.20 were the values obtained for the shrinkage of 2 different protoplast populations. The good agreement between these experimental values allows to validate the experimental procedure as an efficient tool to estimate changes at the cellular level.

Fig. 3 shows the average cellular shrinkage as a function of the processing time. For all of the operation conditions used, the cellular response was very fast and an apparent swelling in the first 5 minutes could be noticed. It could be as a consequence of a subestimation of the cell surface at the beginning of the treatment. When the cells shrink so quickly, depending on the direction of the shrinkage, the image can be out-of-focus until the operator refound the right plane. Another possible explanation is that under osmotic stress the permeability of the membrane is modified and a small gain of sucrose solution can take place (Yamaki et al. 1988).

Shrinkage of protoplasts of strawberry cortex tissue was calculated as $S/S_0=\Sigma(S_i/S_{0i})$ because cells could be easily identified during the process. Fig. 4 shows S/S_0 versus processing time of tissue treated with 40 and 60% sucrose solutions. For a high osmotic pressure (60% sucrose) the shrinkage is faster, that is, S/S_0 decreases in the first 20 minutes but with longer processing times it remains almost constant (S/S_0=0.69±0.2). On the other hand, after 25 minutes of 40% treatment S/S_0=0.82±0.2, but a more extended shrinkage could be expected because of the negative slope of the curve and a still high ratio of living cells (n/n_0=0.83). The proportion of number of living cells at any time to the initial number of living cells has been defined as living cell ratio (Fig. 5). In the first 10 minutes of 60% sucrose treatment the ratio of cells with a functional plasma membrane is still higher than 80%, but with longer processing times it decreases dramatically down to 26%.

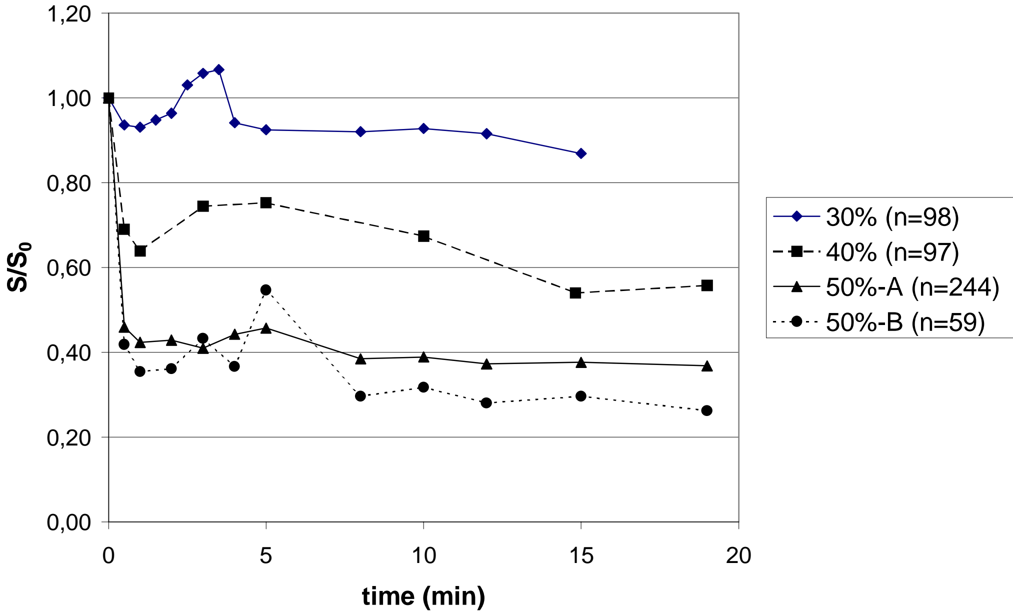

Figure 3: Shrinkage of protoplasts treated with 30, 40 and 50% sucrose solutions versus processing time

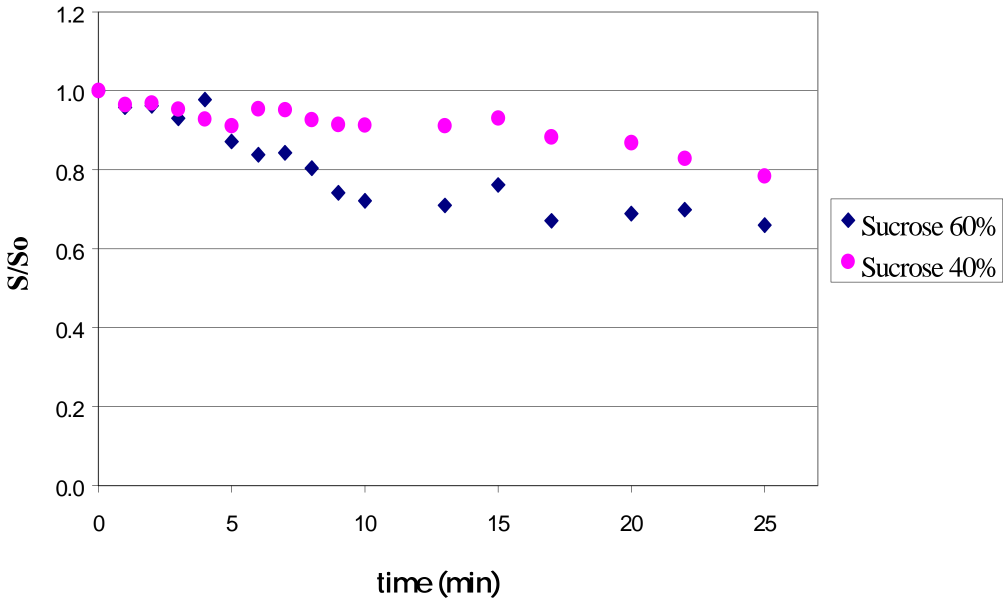

Figure 4: Cellular shrinkage (S/S_0) versus processing time of strawberry cortex tissue treated with 40% and 60% sucrose solution at room temperature

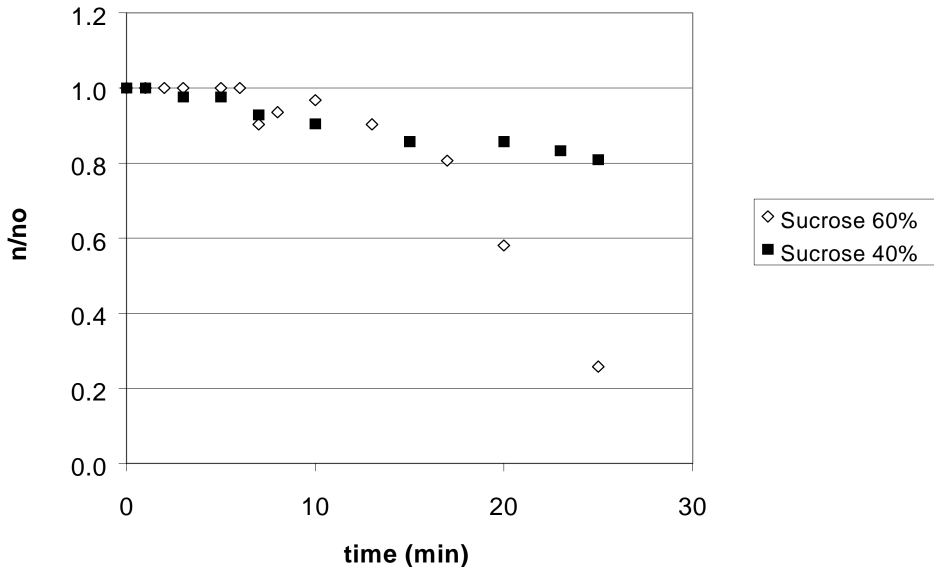

Figure 5: Living cell ratio (n/n_0) versus processing time of strawberry tissue treated with 40% and 60% sucrose solution at room temperature

In Fig.6, the protoplast shrinkage of carrot protoplasts suspension is compared with the protoplast shrinkage of strawberry cortex tissue both treated with 40% sucrose solution. A much faster cellular response is observed for protoplasts, which after 20 min of treatment seem not to shrink any more. Protoplast shrinkage is much slower in strawberry tissue, indicating that longer processing times are necessary to determine maximal protoplast shrinkage (or critical volumen) in tissue.

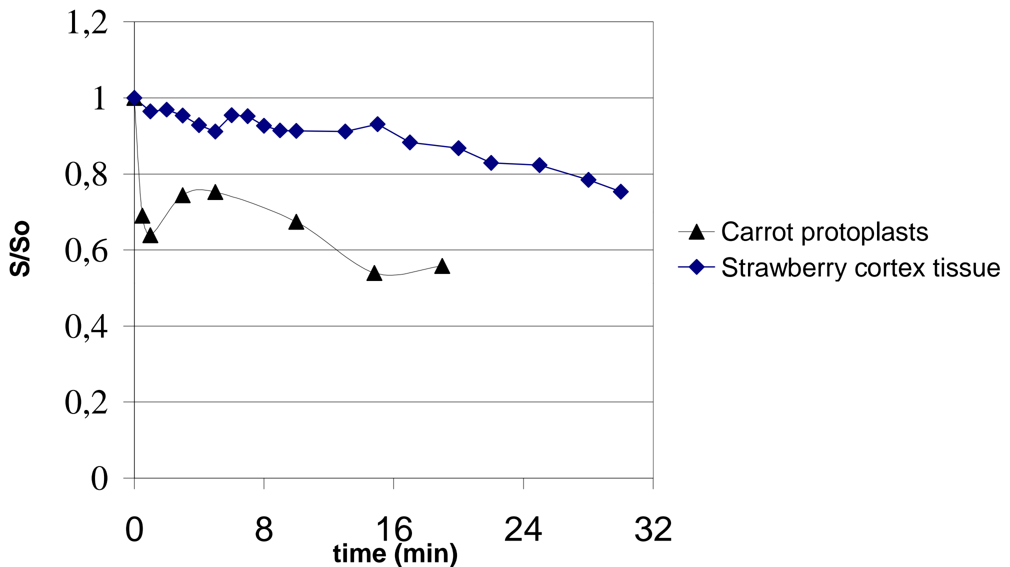

Figure 6: Cellular shrinkage (S/S0) of carrot protoplasts and strawberry cortex tissue treated with 40% sucrose solution

Although two different plant materials are used for the study, it has been shown that the combined use of isolated protoplast and some (simple) tissue structures can give information about the effect of the main cellular structures concerned - plasma membrane, cell wall, middle lamella - on the mass transfer processes.

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Improvement of overall food quality by application of osmotic treatments in conventional and new processes

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Abstract

Research into the application of osmotic treatments in food processes is going on throughout Europe. A Concerted Action was organised to promote advancement in this field by co-ordinated research with integrated objectives and efficient exchange of the knowledge. The final goal is to provide the necessary scientific and technological tools for industrial application of osmotic treatments. Main tasks and work are describe.

Introduction

The principle of osmosis is used to remove water of solid food material in various traditional preserving methods, e. g. salting of meat and fish, and candying of fruit. The technique is simple: plant or animal tissue is immersed, whole or in pieces, into concentrated aqueous solution with an osmotic pressure higher than the tissue pressure. Difference of concentration between food and solution give rise to a migration of water from the tissue into the solution and an impregnation of the tissue by the environmental solutes.

Currently, renewed attention is paid to this technique as a pre-step to further processing to improve the nutritional and sensory quality of food. Osmotic treatment - also known as osmotic dehydration or dewatering impregnation soaking (DIS) - has a wide potential of application: fruit, vegetables, meat and fish can be osmotically treated previous to freezing, chilling, convective drying, microwave or high pressure processing, and other conventional and new processes; combined processes can be designed to obtain food products and food ingredients.

The recent increase in interest in osmotic treatments arises primarily from the need for quality improvement and from economic factors. Quality improvement is related to water removal without thermal stress and the stabilising effect of impregnated solutes. With the correct choice of solutes, and a controlled and equilibrated ratio of water removal and impregnation, it is possible to enhance natural flavour and colour retention in fruit products, hence to avoid additives, obtain softer textures in partially dehydrated products and improve technological properties of food materials.

Potential energy saving are an essential advantage of this processing technique. In classical drying processes water is generally removed by evaporation or by sublimation, i.e. high energy consuming methods, whereas by osmotic treatment up to 60% of the initial water can be removed from the solid food at moderate temperatures and without phase change. The reconstitution of the diluted osmotic solution can be done by membrane processes or with cheap-waste energy if it is at all necessary. Under regular industrial production conditions, an energy cost reduction of 40% to 50% can be expected. The savings can even be higher if the process is applied to partial water removal of products to be frozen in a next processing step, in this case freezing loads and transport volumes are reduced in addition. Little work has been done so far on this aspect of osmotic treatments, most of the work has been focused on energy needs during drying of osmotically treated materials in comparison with fresh materials.

Most applications have been developed for plant material, mainly focused on intermediate moisture- and dried fruit products. More recently, osmotic treatments have been used for the production of fruit based food ingredients. Examples of these ingredients currently in trade are: soft frozen fruit pieces, such as raspberries, strawberries, cranberries or tropical fruit, for ice creams, bakery products and refrigerated dairy products; banana slices infused with a high fructose corn syrup solution for cream pies, gelatine desserts, cakes, cookies; dehydrated apple pieces; infused peaches, cherries, carrots.

There is already much practical experience on the osmotic treatment itself available. To fulfil consumer, industrial and environmental expectations, however, some problems remain to be solved. Osmotic treatments have been frequently applied as a low cost processing method neglecting process optimisation; but the current interest in this technique and the development of industrial applications on a large scale demand a controlled process. At present it is recognised that attention must be focused on the following: (a) developing of predictive models; (b) optimisation of combined processes, osmotic treatment and further processing; (c) management of osmotic solutions under economic and environmental aspects.

Adequate predictive models are needed to realise the necessary process control and progress in the design of industrial equipment. Consumers are interested in a wide range of safe products of excellent sensory and nutritional characteristics. By osmotic treatments improvement of the overall quality of existing products and development of new ones is possible; however, optimisation of the combined processing, osmotic treatment and following up process, is still necessary. The management of osmotic solutions remains to be one of the critical points on an industrial scale.

To support such work a Concerted Action has been organised within the frame of the fourth EU-Framework Program.

The Concerted Action

In this Action 15 Research Centres and Universities of 11 European countries, Israel and Canada participate. The project funded by the Directorate General XII of the Commission of European Communities under research grant FAIR-CT96-1118.

Main task is to create and improve the links among the different groups working in this field to acquire the necessary knowledge to optimise overall food product quality by application of osmotic (pre-)treatments and to control the operation from the engineering point of view. Moreover to improve the scientific knowledge for the evaluation and control of modifications of food processed by osmotic treatment by: collection and evaluation of data on chemical, physico-chemical and physical properties of fresh and processed food materials (plant and animal) and the correlation with processing conditions; evaluation and description of mass transport mechanisms in biological tissues during osmotic treatment both at the microscopic and macroscopic level.

The final goal of the Action is to provide the necessary scientific and technological tools for industrial application of osmotic treatments.

The aims are achieved by working in three main areas with specific objectives (Fig. 1) and disseminating the knowledge gain throughout different channels.

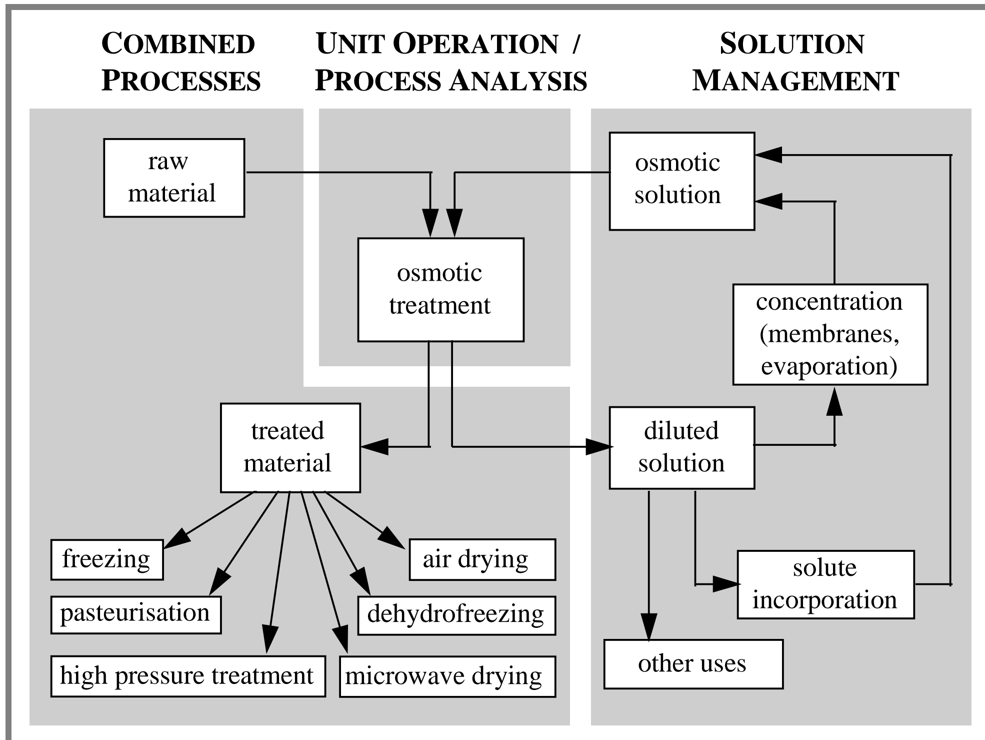

Fig. 1: Working areas

Unit operation / Process analysis

The final task is to achieve an adequate description of the system and of the mass transfer mechanisms inside the food material that allow the development of predictive mathematical models of the operation. The specific task are:

- Study the internal equilibrium of the samples (model food and real food) at different conditions of water and solute content, in terms of chemical potential, as well as the different contributions to the matrix (activities, cell turgor, system pressure and matrix potential).
- Analysis of the mass transfer mechanisms inside the food, at cellular level. Evaluation of the structural changes of the food material (shrinkage, surface cells and pore collapse) and their effect on the mass transfer characteristics in the solids.
- Prediction of the kinetics of the different mass transfer mechanisms by means of the adequate equations.
- Development of predictive mathematical models of the operation.

Adequate predictive models are needed for process control and equipment design and the lack of them is an obstacle to industrial applications. Poor understanding of the fundamentals of mass transfer in biological cellular structures - a problem common also to other areas of food processing dealing with transport phenomena- is the main hindrance of advance in this field.

Osmotic dehydration may be defined as a solid-liquid operation where a solid food material is immersed in a concentrated aqueous solution in order to remove water from the material. Differences in chemical potentials of water and solutes in the system result in fluxes of several components of the material and solution, being the water outflow from the food into the solution and the solute uptake by the food the two main flows taking place during osmotic treatment.

As the name of the process indicates, osmosis is the mechanism responsible for high water losses with reduced solute uptake, at least as long the tissue membranes are intact. However, depending on the tissue and the operating conditions such as temperature and pressure, diffusion, convection and flux interaction may occur at the same time and contribute to the complexity of the process. Furthermore, modifications in composition and structural changes (shrinkage, porosity reduction, cell collapse) taking place in the food material during osmotic treatment modify the heat and mass transfer behaviour in the tissue and must be considered.

Most of the existing models start from the macroscopic approach, which assumes the tissue to be homogeneous, and are based on concepts of diffusion and irreversible thermodynamics. These models try to describe mass transport in mathematical terms ignoring the mechanisms which take place at the cellular level. These semi-empirical models are generally useful in the individual case. Today it is recognised, however, that mass transfer mechanisms inside solid food should be analysed at a cellular level.

The microscopic approach takes the heterogeneous properties of the tissue into account and has been developed for plant material on the basis of plant physiology studies on the effect of osmosis on water balance and transport in growing plants. Many tissue parameters (e.g. membrane permeability, porosity, cell size) are required for the development of models regarding all the mechanisms acting on the various components (intercellular and extracellular spaces, vacuole, etc.). For most tissues subjected to osmotic treatment, lack of the data required for this modelling approach represents a hindrance to progress.

The importance of the mechanisms taking place at the cellular level and the need of understanding them before mass transfer in biological tissues is described in a proper way are recognised. Research into the microscopy of the process started at some laboratories, and their results are will be of great value not only for osmotic processing, but for all processes involving mass transfer in cellular materials.

Combined processes

The objective is to integrate osmotic treatments in traditional and new food processes to obtain food products and ingredients with defined functional properties, designed for specific complex food systems. The specific tasks are:

- Chemical, physical and physicochemical characterisation of fresh plant and animal material and osmotically treated materials.
- Evaluation of modifications on the food materials. Establish correlation between the modifications observed on the chemical composition, physical and functional properties of the food materials and the osmotic treatment operating conditions.

- Consecutive processing. Analyse the behaviour of the modified material during the further processing by conventional and new food processes and establish the optimal operating conditions for the combined processes.
- Evaluation of nutritional and sensory quality parameters after processing and storage.
- Microbiological assay. Validation of the hygienic status of the osmotically treated and finished products.
- Formulation of food products and food ingredients.

Optimising combined process includes the selection of: solutes and processing temperature, pressure and time for the osmotic treatment; processing parameters for subsequent treatment; storage conditions for the endproduct. This requires knowledge of the chemical and physical properties of the raw material, of the osmotically treated material and of the endproducts; of the relationship among osmotic process variables and the changes achieved in the material; and of the behaviour of the treated material during further processing and storage.

Different plant and animal food material have been subjected to osmotic treatments in hypertonic solutions. The product obtained was consequently processed by convective drying, freezing, pasteurisation, microwaves. The effect binary and multicomponent solutions of different osmo-active solutes in the overall composition changes and the stability of products was evaluated. The effect of the different processing parameters (temperature, pressure, solution composition and concentration, sample size and shape) on the solid - liquid exchanges and on the characteristics of the osmotically treated products was also evaluated. Raw materials, osmotically treated and final products have been characterised with respect to chemical compounds, physicochemical and physical properties. The relationships between the modification of the composition of the raw material achieved by the osmotic treatments and the protective or stabilising characteristics achieved in the final products have been investigated. The final products obtained by the different combined methods have been characterised with respect to the nutritional and sensory quality and the storage stability of products was evaluated.

Studies have been done in: fruit (strawberry, apricot, apple); vegetables (carrots, potato); mushrooms (champignons); fish and sea food (Black cod, Salmo salar, squid); meat (venison).

Some research into tissue parameters related to the behaviour during processing and storage has been done, but much work still remains to be done.

Experiences in the application of osmotic treatments make it possible to design an endproduct of better colour stability or better texture, but the mechanisms responsible for the improvement achieved by water removal and impregnation are not always fully understood. Research into the mechanisms stabilising the material is important for process optimisation.

The protective effect of different sugars on plant pigments is known, but not so the mechanism responsible for stabilisation. It is postulated that trehalose can stabilise membranes during dehydration by occupying the place of the water removed; mannitol, which is synthesised in plants under oxidative stress, may prevent oxidative damage by protecting different enzymes. By direct formulation during osmotic treatment, i.e. water removal and incorporation of polysaccharides, elevation of the Tg of the material and consequent enhanced storage stability of frozen products is expected. The protective effect of impregnated sucrose, sorbitol and maltose on colour stability of

plant pigments during frozen storage has been described; however, modification of the Tg could not fully explain the results obtained; viscosity has been suggested as a controlling parameter for stabilisation.

Solution management

The objective is to give an hygienic, economical and environmental based answer to the solution problematic. Possible ways of treating the diluted osmotic solutions so as to reincorporate it in the osmotic operation or to use it for other food processes will be evaluated. The specific tasks are:

- Microbiological evolution of the solution. Study the microbiological status of the diluted solutions in terms of the major food-borne pathogens, such as *Listeria*, *Salmonella*, *Staphylococcus aureus* and *Vibrio*.
- Characterisation of osmotic solutions. Experimental determination of chemical, physical and physicochemical parameters of the concentrated and of the diluted solutions. Evaluate changes suffered throughout processing.
- Recycling methods. Evaluate different methods for recovering the diluted solution, (such as reconcentration, incorporation of solutes) in order to be reused in the osmotic process. Evaluate the effect of pasteurisation on microbial and chemical characteristics, specially in relation to non enzymatic browning.
- Use of diluted solutions in other processes. Evaluate the use of the diluted solutions for purposes other than the osmotic process.

Research towards osmotic treatments has been focused on the effect of different parameters on the mass exchange and the evaluation of quality improvement of endproducts. Little work has been done towards the solution recycling and this has come to represent a major hindrance to industrial development of the process.

Composition of the solution (kind of solutes and concentration) and the weight ratio of solution to food are key points of an efficient osmotic process. During the process the composition changes, mainly due to the outflow of water from the food material, but also due to the uptake of solutes and leaching out from the tissue. High weight ratios of solution to food promote the efficiency of the process, but this requires handling of large volumes of solution and increases the cost of the process.

One of the main requirements of a low-cost and energy saving osmotic process is an optimised management of the solution, which involves minimisation of the solution volume, control of composition during the process and solution recycling. Furthermore, the hygienic quality of the process depends on the correct management of the solution. Implementation of HACCP is necessary to assure hygienic quality of the process.

The weight ratio of solution to food is determined by the kind of food processed and the kind of solutes. When using low ratios of solution to food, control and readjustment of the solution composition are important to prevent a loss of efficiency by the dilution effect. In-line sensors for prediction of soluble and total solids in continuous processing of fruit juices have been evaluated and could be easily instrumented in the osmotic process for binary solutions.

For recycling, the solution must eventually be filtrated to exclude particles (seeds, tissue pieces, etc.), thermally treated to reduce microbial contamination (e.g. pasteurisation) and concentrated (evaporation or addition of solutes).

The use of evaporators for solution reconcentration is the most energy consuming choice, increasing processing costs; concentration restoration by addition of solutes is limited by the consequent volume increase of solution; use of membranes for concentration has to be studied, including evaluation of implementation costs. Processing costs and hygienic quality of products are directly influenced by solution recycling.

The solution can be recycled several times; recycling number of times is limited by the efficiency of the recycling method selected, by the sensory characteristics of the recycled solution, e.g. excess of extracted plant pigments or flavours, or the caramelised colour of thermally treated syrups. When the solution cannot be recycled anymore, it may be used in another processing line or must be discarded.

Several uses have been suggested for sugar solutions of osmotic processes; they include production of beverages (mixing with fruit juices, diluting with water and adding carbon dioxide), jams, infusion of extracted fruits, fruit candying or animal feed. Discarded multicomponent solutions containing salt and sugars present more problems.

Recycling and discard of osmotic solutions are still obstacles for industrial application on a large scale and require an economical and environmental based answer. Evaluation of process costs using different recycling methods is required.

Results dissemination

Practical guides for the industry

⇒ Compilation of possible applications of osmotic treatments with respect to products and processes.

⇒ Recommendations for the processing and handling of agricultural raw material by osmotic treatment to semifinished and finished products.

⇒ Recommendations for the handling of osmotic solutions, utilisation and discharge.

The first document is based on the collection and evaluation of published material and the other two on the basis of the knowledge base of the group. The material will be presented in a proper way to give SME short and clear information on practical matters.

Industry workshops

One day industrial seminars or workshops are organised with the main task of giving the experience of the group directly to the industry sector. The seminar proceedings are edited by the seminar organiser in each case and published.

The first Seminar took place in Porto, Portugal (October 1997)and the second at Bertinoro, Italy (April 1998). The third Seminar will take place in Valencia, Spain (March 1999).

Osmotic treatment newsletter

This is a quarterly publication created in the framework of the Action. With the newsletter it is intended to:

⇒ disseminate the advances in the field of the application of this technique throughout academia and industry; not limited to the background of the Action, but seeking a Worldwide cover;

⇒ widen the links among research groups and industry working on or interested in the technique.

http://www.dainet.de/osmotic/

The Action has a homepage in the Web. The information is continuously actualised and covers the following points: Partners (work of each group on osmotic treatments and links to the institution's homepage); Project description (objectives; methodology; expected results and applications); Dissemination activities (plenary meetings; industry workshops / seminars; publications); General information (publications, etc.).

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Osmodehydrofreezing to improve frozen kiwifruit quality: The influence of raw fruit texture

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Introduction

Kiwifruit is considered an important commercial fruit with an increased processing potential. There are various ways of preserving kiwifruits, but the most suitable has been the freezing of slices and pulp, because sensory attributes, such as colour and flavour, and the nutritional value are retained (Dalla Rosa et al., 1980; Venning et al., 1989; Crivelli et al., 1990; Papadopoulou and Monolopoulou, 1997). Freezing, however, has some negative consequences on the texture of the fruit, because of turgor loss and cell breakage (Brown, 1979). Furthermore the frozen fruit and vegetable industry uses much energy in order to freeze the large quantity of water present in the fresh products. As pointed out by Huxoll (1982), a reduction in moisture content of the material reduces refrigeration load during freezing. Other advantages of partially concentrating fruits and vegetables prior to freezing include savings in packaging and distribution costs but, above all, achieving higher product quality because of the marked reduction of structural collapse and juice dripping at thawing (Lazar, 1978).

The products obtained are termed "dehydrofrozen" and the concentration step is generally realized through conventional air drying. Osmotic dehydration, better defined as "Dewatering-Impregnation-Soaking" (DIS) in concentrated solutions could be used instead of air drying mainly to obtain a quality improvement especially for fruit and vegetable sensitive to air drying (Dalla Rosa et al., 1980; Forni et al., 1990; Vial et al., 1991; Torreggiani, 1995; Bressa et al., 1997). Air dehydration to about 50% weight reduction of kiwifruit, even at 45-50°C, causes considerable colour and texture defects; a "yellowing" of the fruit is observed together with a "woody" texture (Torreggiani et al., 1987). A 2 hrs osmotic treatment at room temperature gives high quality dehydrofrozen kiwifruit in terms of natural colour and flavour. The sugar uptaken during osmosis modified the low-temperature phase transitions of kiwifruit slices and significantly improved colour and chlorophyll pigments stability during storage at -10°C up to 6 months (Torreggiani et al., 1994). Osmotic dehydration, applied before freezing, could also improve texture of thawed fruit, as shown for strawberry slices (Maestrelli et al., 1997).

Since there is a strong correlation between texture characteristics and pectin composition of fruits, the structural modifications of the cell walls have to be taken into account. Among the different forms of pectins, which are linked to their different solubility, molecular weight, etc., the protopectin is considered the most important pectin fraction to determine the fruit texture (Shewfelt et al., 1971; Shewfelt and Smit, 1972; Souty and Jacquemin, 1976).

In a previous work on osmodehydrofreezing of kiwifruit slices at different ripening stages, and thus different texture levels, no relationships were found between texture and pectic composition changes during osmotic dehydration (Torreggiani et al.,1998). The different protopectin contents of the three groups of kiwifruits (firm, medium and soft), were directly correlated with texture values. As the soft kiwifruit showed the highest texture modification during osmodehydration, the

protopectin/soluble pectin ratio (protopectin index) (Forni et al., 1986) could be used to identify cultivars and maturity stages of fruit suitable for osmotic processing.

The purpose of the present work is to investigate how freezing and frozen storage at -20°C affects texture characteristics of osmodehydrated kiwifruit slices processed at three different texture levels, as well as studying the correlation between texture and pectic composition modifications.

Material and methods

Raw material

Kiwifruits, cultivar Hayward, of the same lot, were purchased just after harvesting in the P.A.F. cooperative (Faenza, Italy). The fruits were then divided in 3 groups and were differently stored in order to obtain the 3 texture levels of : 4-5 kg (firm), 1.8-2.5 kg (medium) and 0.8-1.5 kg (soft). The 3 groups storage conditions were as follows: (firm) 30 days at 0°C + 8 days at room temperature, (medium) 30 days at 10°C and (soft) 10 days at 0°C + 20 days at room temperature.

Flesh texture of the fruits was determined with an Instron Universal Testing Machine model 4301 (Bucks, U.K.) by penetration test. The fruits were held against a hard static surface and the maximum penetration force (kg) of 8 mm plunger penetrating 8 mm into the fruit was measured. Two readings were taken on opposite sides of each fruit after removing a small area of fruit skin of 1-2 mm thickness with a sharp blade. The reported data are the means of 50 determinations obtained with a crosshead speed of 20 cm/min.

Osmotic dehydration

The kiwifruits of the three different texture groups were hand peeled, mechanically cut into 10 mm thick slices (Dicer Bertuzzi, Milan, Italy) and osmodehydrated for 2 hours (Forni et al., 1990) at 25°C in a glass tank filled with 70% (wt/wt) sucrose syrup continuously recirculated through a peristaltic pump. The slices were held submerged by a plastic screen and the fruit - syrup ratio was 1/3 (wt/wt). The process was repeated three times.

Osmotic dehydration parameters

Solid gain (SG), water loss (WL) and material balance were calculated according to Giangiacomo et al. (1987), are expressed as g/100 g of initial fresh fruit and are reported in a previous paper (Torreggiani et al., 1998).

Freezing

Osmodehydrated kiwifruit slices were frozen in a pilot air-blast tunnel operating at - 48°C air temperature and 4 m/sec air velocity. For all the samples the freezing time was about 15 min. The frozen products were packaged in sealed polyethylene bags, then in cardbord boxes and stored at -20°C.

Analyses

Chemical and physical analyses were performed on osmodehydrated and thawed osmodehydrofrozen kiwifruit slices. Thawing was performed at room temperature for 30 min. Chemical data are the means of four determinations.

Sugars (glucose, fructose and sucrose) were quantified by HPLC on a Polyspher CHCA (Merck, Darmstad, Germany) column (0.65 x 30 cm) at 90°C, eluting with deionised water, with a Jasco (Tokyo, Japan) 830 refractometric detector, according to the method of Forni et al. (1992).

Extraction of AIS (alcohol-insoluble substances) and fractionation of the pectic substances (W=water soluble, O=oxalate soluble and R=residual protopectin) was performed according to Forni et al. (1986). The determination of the pectic substances was effected by the analysis of the galacturonic acid in the fractions W, O and R. Galacturonic acid content was determined by HPLC on enzymically depolymerized pectins (Forni et al., 1987).

Texture was measured on the kiwifruit slices using an Instron Universal Testing Machine model 4301 (Bucks, U.K.) measuring the maximum force (kg) with a standard Kramer Shear Press single blade (3 mm). Two readings were taken on opposite sides of the flesh of each slice, 5-8 mm from the core (columella). The reported data are the average of 40 determinations obtained with a crosshead speed of 10 cm/min.

Analysis of variance and Tuckey multiple range tests were used to determine the statistically significant differences ($P \leq 0.05$).

Sensory evaluation

Sensory evaluations were carried out through a preference test (Larmond, 1977), by using a nine-point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely) and taking into account texture and flavour of thawed osmodehydrofrozen kiwifruit slices. The panel was composed of 15 trained judges and each tasting was repeated three times on subsequent days. Analysis of variance and Tuckey multiple range tests were used to determine the statistically significant differences ($P \leq 0.05$).

Results and discussion

Figure 1 gives the texture values (kg) of osmodehydrated kiwifruit slices before and after freezing, and during frozen storage at - 20°C. A significative decrease of texture was observed, due to the freezing process. Firm, medium and soft kiwifruit showed percentage texture decreases of 42%, 44% and 62%, respectively, indicating a very low suitability to freezing of the soft fruits. During the first 4 months of storage texture of firm and medium fruits showed a further significative decrease, while texture values of soft fruits were stable. At the end of the 12 months of storage the texture of the firm fruits was still significantly higher than that of the medium and soft ones, even if the frozen storage had partly reduced the texture differences observed in the fresh fruits.

Firm osmodehydrated kiwifruit had the highest content of AIS and pectic substances (Figure 2). The AIS content of medium kiwifruits was significantly greater than that of the soft fruits, while pectin contents were similar. After freezing both AIS and pectin content increased while during frozen storage not significative modifications were observed, except for the decrease of total pectin of the firm kiwifruit.

As shown in Figure 3, freezing caused a significative increase of the water soluble (W) fraction content whatever the texture levels of the raw fruits. During the frozen storage the W fraction content was stable in the medium fruits, while constantly decreased in firm and soft fruits, indicating a probable further degradation of pectin substances. The oxalate soluble (O) fraction content did not

significantly change both during freezing and subsequent frozen storage (Figure 4). The protopectin (R) content of the firm and medium osmodehydrated fruits showed, during freezing, a marked decrease, which carried on progressively during the frozen storage only in the firm fruits (Figure 5). The R fraction content directly correlate with texture (R^2 = 0.95), pointing out the major role of protopectin in tissue firmness even during processing and storage. Protopectin content in soft kiwifruits did not significantly change both after freezing and during frozen storage.

The protopectin/soluble pectin ratio (protopectin index) modifications were directly correlated with texture changes (R^2 = 0.90) (Figure 6).

After processing and storage the percentage distribution of the differently soluble pectin fractions in the fruit at different texture levels is like that observed in the raw fruit. So while in the firm fruits the protopectin (R) is the predominant fraction, in medium fruits the W fraction content, and in soft fruits both W and O fractions contents are higher than that of the R.

Sugars content was not significantly modified by the freezing process and frozen storage (Table 1).

The changes in the sensory attributes of texture and flavour of the firm, medium and soft osmodehydrofrozen kiwifruit just after freezing and after 12 months of frozen storage are reported in Figure 7. Texture of the firm and medium fruits was judged agreeable all through the storage period, while that of the soft fruits was considered unacceptable from the beginning of storage. The same behaviour was observed for the sensory acceptance of flavour. The sugar uptake , due to the osmotic pre-treatment (Torreggiani et al., 1998), could probably balance the higher acidity of the firmer, thus less ripe, kiwifruits, making them fully acceptable all through the storage period.

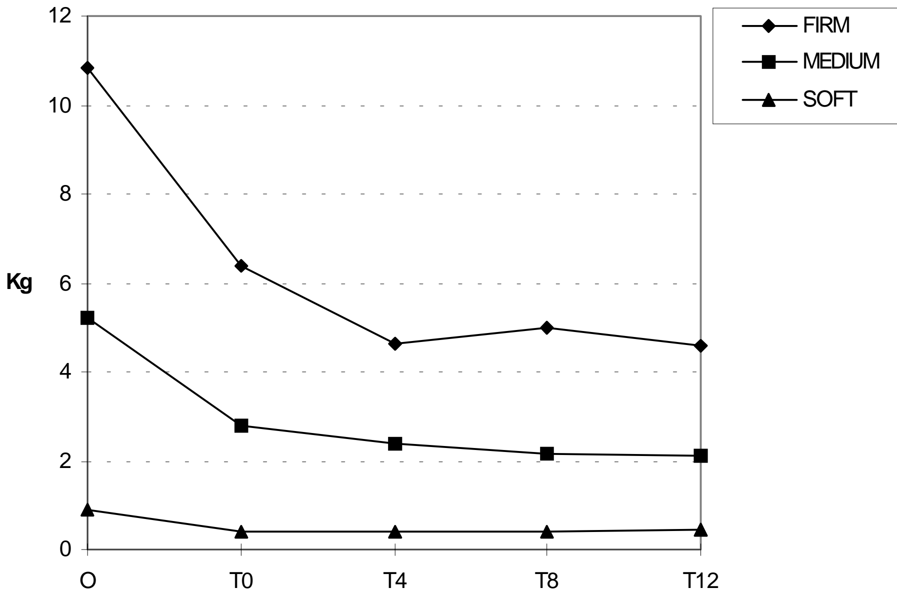

Figure 1: Texture values of kiwi fruit slices before (O) and after freezing (T0), and after 4 (T4), 8 (T8) and 12 (T12) months of storage at -20°C.

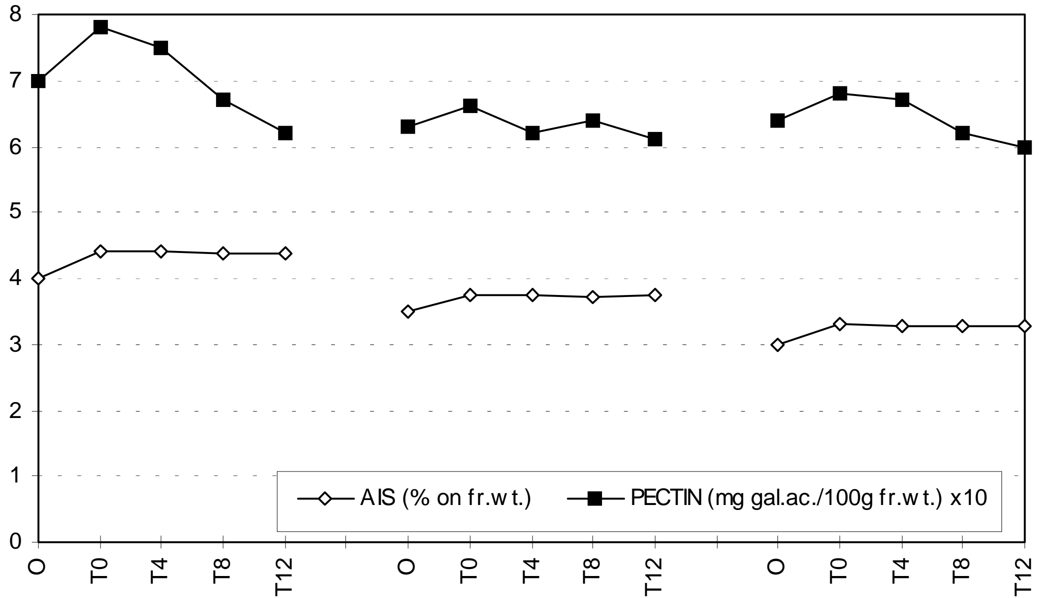

Figure 2: AIS and total pectin content of kiwi fruit slices before (O) and after freezing (T0) and after 4 (T4), 8 (T8) and 12 (T12) months of storage at -20°C.

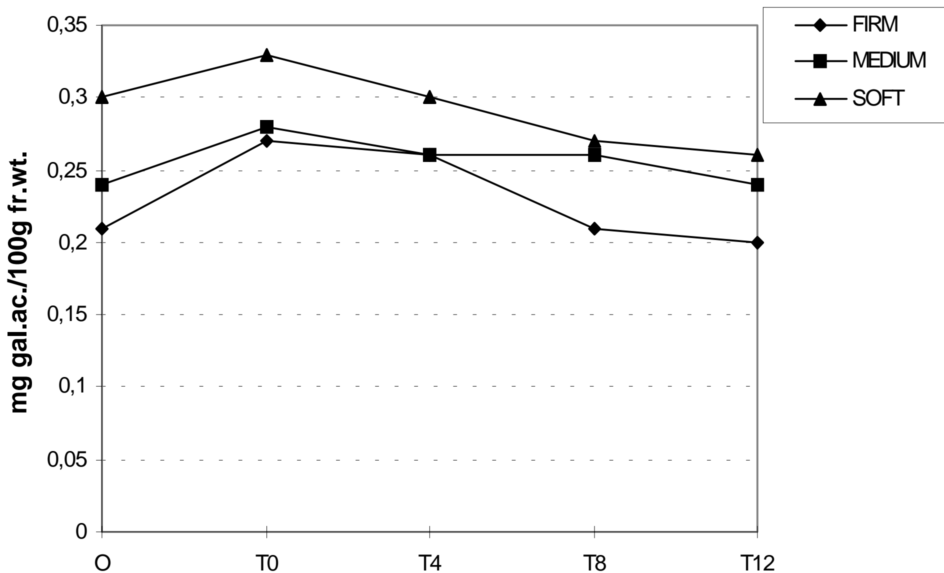

Figure 3: Pectin fraction W of kiwi fruit slices before (O) and after freezing (T0) and after 4 (T4), 8 (T8) and 12 (T12) months of storage at –20°C

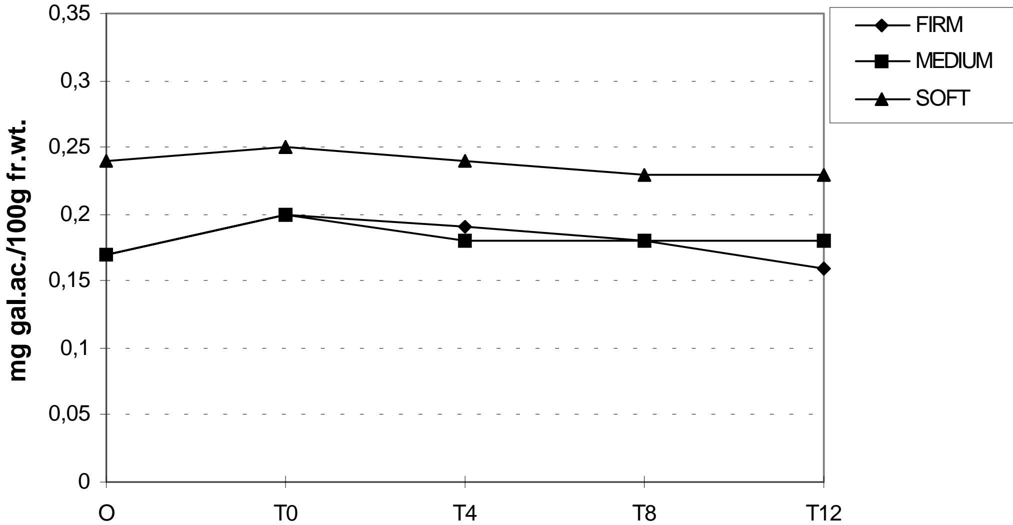

Figure 4: Pectin fraction O of kiwi fruit slices before (O) and after freezing (T0) and after 4 (T4), 8 (T8) and 12 (T12) months of storage at –20°C

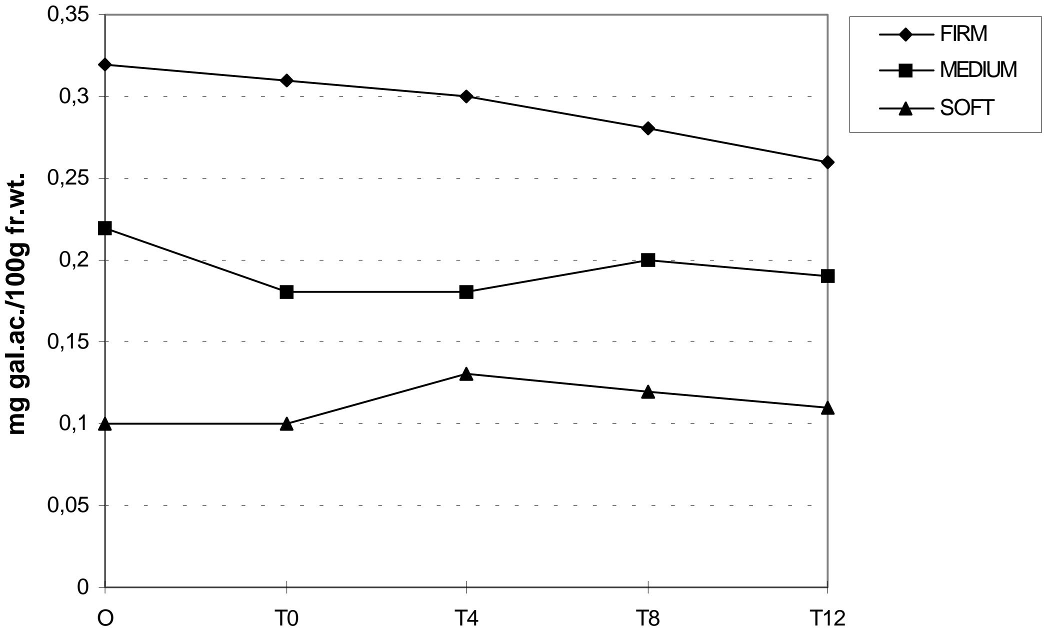

Figure 5 : Pectin fraction R of kiwi fruit slices before (O) and after freezing (T0) and after 4 (T4), 8 (T8) and 12 (T12) months of storage at –20°C

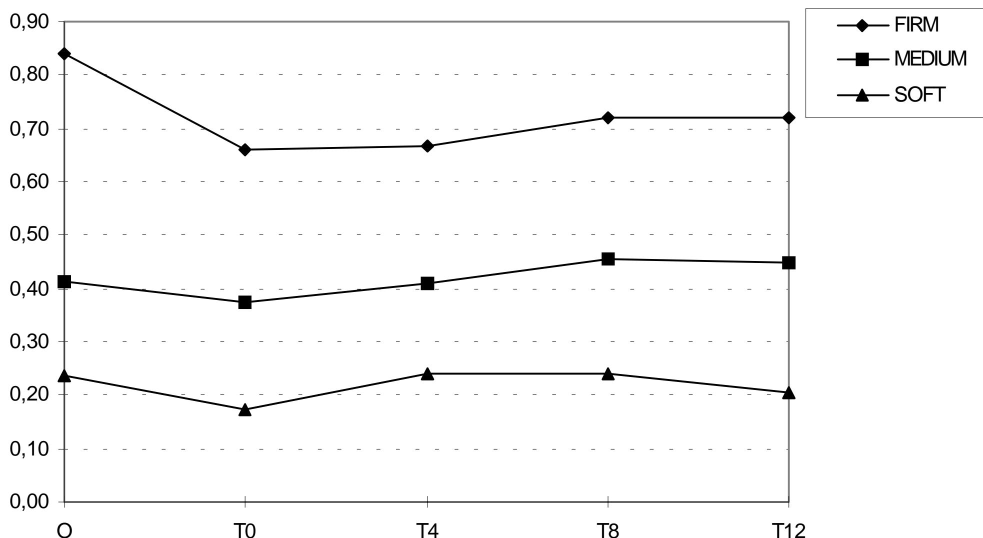

Figure 6: Protopectin index (R/W+O) of kiwi fruit slices before (O) and after freezing (T0) and after 4 (T4), 8 (T8) and 12 (T12) months of storage at –20°C

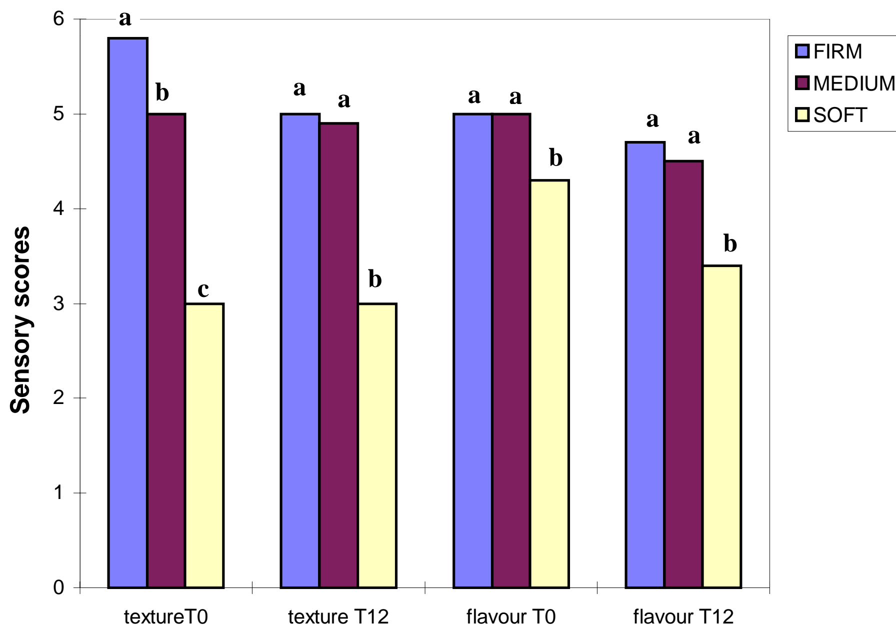

Figure 7: Texture and flavour acceptance of osmodehydrofrozen kiwifruit slices after freezing (T0) and 12 (T12) months of storage at - 20°C.
* different letters indicate significative differences (P<0.05)

Table 1: Sugar content (%on fr.wt.) of kiwi fruit slices before (0) and after freezing (T0) and after 4 (T4), 8 (T8) and 12 (T12) months of storage at - 20°C

	GLU	FRU	SAC	TOT
FIRM				
O	5,1	5,33	5,1	15,53
T0	5,1	5,3	5,12	15,52
T4	5,05	5,25	4,9	15,2
T8	4,55	4,9	4,92	14,37
T12	4,5	4,7	5	14,2
MEDIUM				
O	4,35	5,15	5,7	15,2
T0	4,4	5,16	5,72	15,28
T4	4,4	5,03	6,08	15,51
T8	4,33	4,7	5,82	14,85
T12	4,29	4,6	5,8	14,69
SOFT				
O	4,15	5,05	4,71	13,91
T0	4,17	5	4,65	13,82
T4	4,2	5	4,58	13,78
T8	4,15	4,65	4,61	13,41
T12	4,1	4,5	4,6	13,2

Conclusions

The analysis of the pectin composition performed on osmodehydrated kiwifruits at different texture levels after freezing and frozen storage confirmed the major role of the protopectin in tissue firmness. In firm and medium kiwifruit the texture decrease, caused by freezing and frozen storage was directly correlated to pectin substances degradation, which was demonstrated by the decrease of the insoluble fraction R and at the same time the increase of the water soluble fraction W. So pectin degradation could be regarded as one of the causes of the structural collapse observed in freeze-thawed kiwifruits.

As soft kiwifruit showed the highest texture decrease after freezing, the protopectin/soluble pectin ratio (protopectin index) might be taken as an index of the suitability of kiwifruit to osmodehydrofreezing and could be used to identify suitable cultivar and maturity stages.

A direct correlation ($R^2 = 0.92$) was found between texture of the raw kiwifruit and texture acceptance of the processed fruit, so indicating the ripening stage of the raw fruit as a key point in the production of high quality osmodehydrofrozen kiwifruit.

In order to further understand the changes undergone by the osmodehydrated fruit during the freezing process, special attention has to be paid to analyse at microscopic level the structural transformations during ripening. In this way the effect of these changes both on solid liquid exchanges during osmotic dehydration and structural stability during freezing could be established, so improving the osmodehydrofreezing process.

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Case hardening in osmotic dehydration of fruits. Effect of vacuum impregnation

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Abstract

Osmotic dehydration of fruits and vegetables implies important changes in composition and structure. When very high osmotic solution concentrations are used, solute gains are seen to decrease. This behaviour is attributed to a case hardening effect related with a fast collapse of the surface cells. In this paper the case hardening phenomenon has been analyzed in osmotic dehydration of apple tissue, with and without applying Vacuum Impregnation. A less intense case hardening effect was observed in Vacuum Impregnated samples. Differences could be explained in terms of structural changes promoted by Vacuum Impregnation.

Introduction

Osmotic dehydration of fruits and vegetables implies important changes not only in composition but also in structure and liquid retention capability. In general, the higher the osmotic solution concentrations and/or work temperature, the greater the solute gain and water loss (Beristain et al., 1990). Nevertheless, when very high osmotic solution concentrations, and relatively low temperatures ($T \leq 50$ºC) were used, solute gains were observed to decrease as compared with those obtained at low osmotic solution concentration (Lenart and Flink, 1984, Lerici et al., 1988). This behaviour was attributed in previous works to a case hardening effect related with a fast collapse of the surface external cells in the osmosed tissue.

The objective of this paper was to analyze the case hardening phenomenon through the development of water and solutes fluxes throughout the process time in osmotic dehydration of apple tissue. The effect of Vacuum Impregnation (VI) on the case hardening phenomenon was also studied.

Materials and methods

The osmotic dehydration experiments were performed with apple slices and sucrose solutions in a pilot plant (Mata, 1993) at controlled temperature, pressure and flow rate of solution. Atmospheric pressure was maintained in OD, and a pulse of vacuum pressure (180 mbar) during the first 5 minutes was applied in the PVOD experiments, carrying on afterwards at atmospheric pressure. Working temperatures were 30, 40 and 50ºC and sucrose solution concentrations were 55 and 65 Brix. The solution-fruit ratio was great enough (50:1) to avoid significant changes in the solution concentration during the process. The solution flow rate was adjusted to ensure the internal control of the mass transfer rate. To prevent evaporation the fruit-osmotic solution system was hermetically closed.

Apples (Granny Smith) were peeled, the core taken out, and sliced perpendicular to the apex-base direction. Slices 10 mm thick, having 64 mm and 20 mm external and internal diameter respectively were cut. Three slices were obtained from each apple; one for the OD experiment, another for the

PVOD and the third for fresh apple analysis. The apple slice dimensions (Ed, Id and H) and weights were measured at the beginning and at the end of the experiment. Apple samples were placed in trays and processed for 15, 30, 45, 60, 120 and 420 minutes. At the end of the osmotic treatment the apple slices were removed from the solution, their surface gently blotted with tissue paper, and their moisture content and the soluble solid content in the fruit liquid phase determined. Each experiment series was carried out in triplicate. Moisture content was determined gravimetrically (AOAC standard methods. 20.013, 1980) and sugar content with a refractometer.

A biexponential equation (eq. 1) was fitted to water and solutes net gains throughout the process time.

This equation (eq. 1) was derived with time in order to obtain the mass rate. The instantaneous mass fluxes at the solid-liquid interface were obtained using equation 2.

$$\Delta \dot{M}_t = a_1(1-e^{-k_1 t}) + a_2(1-e^{-k_2 t}) \quad \text{eq. 1}$$

$$J^j_t = \frac{M^o_0}{A_t} \cdot \frac{d(\Delta M^j_t)}{dt} \quad \text{eq. 2}$$

Results and discussion

In fig. 1 and 2 water and solute fluxes calculated with the experimental data for the OD experiments can be seen. This kind of plotting is quite useful in order to observe the case hardening phenomenon (Salvatori et al., 1998).

It can be observed that case hardening mainly affects solute transport (fig. 2). Solute fluxes for OD experiments at 65 Brix OS concentration are practically the same for all three temperatures. For 55 Brix OS the temperature effect and solute fluxes are greater as regards to OS of 65 Brix (fig. 2), even when the concentration driving force is lower. One explanation to this phenomenon would be the big differences in viscosity between OS of 55 and 65 Brix (fig. 3). Although it is evident that the differences in viscosity affect mass transport, and mainly solute transport due to its bigger molecular size, this can’t be the only explanation to this fact. The equality in solute flux values for 65 Brix OS for all three temperatures supports this idea.

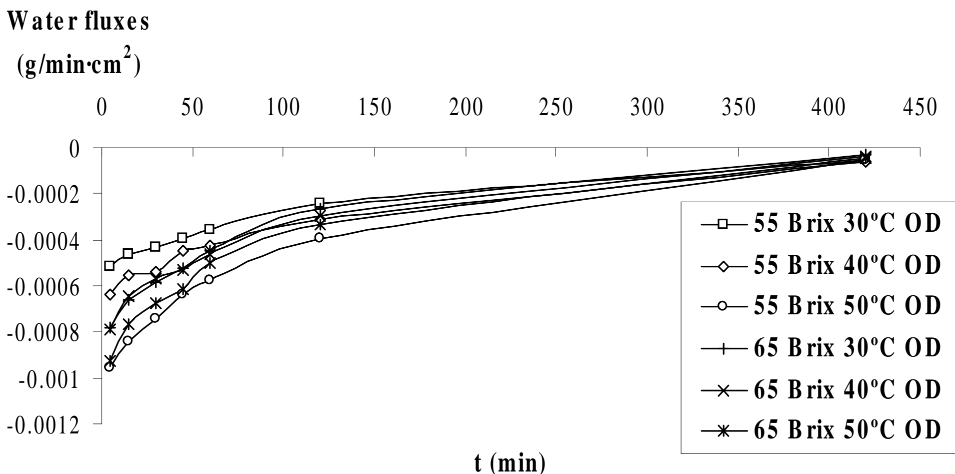

Fig. 1: Water fluxes for OD experiments.

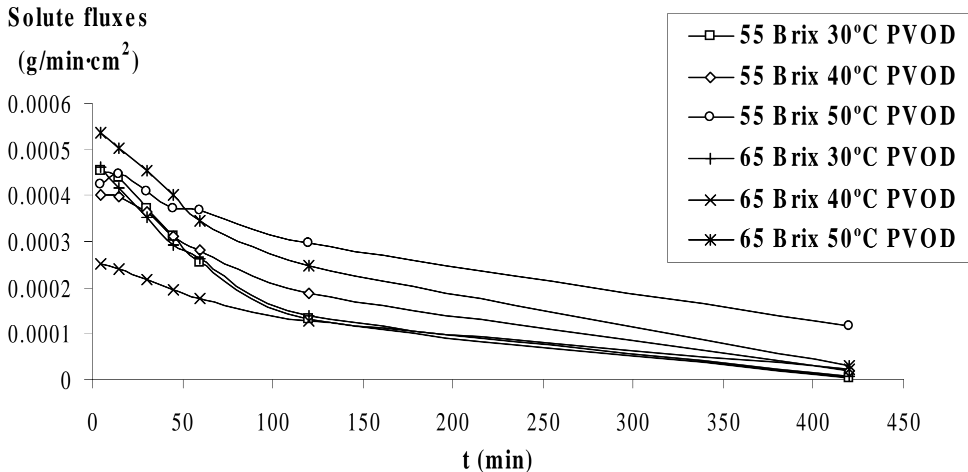

Fig. 2: Solute fluxes for OD experiments.

In fact the idea that surface cell collapse provokes the case hardening would be explained by the increase in viscosity, because the free spaces remaining in the cell wall structure are big enough to permit solute and water flux, affecting only the viscosity of the solution. Maximum values for the volume changes can be observed in table 1. As can be seen, although the maximum values are different, practically the same solute fluxes were observed. This would support the idea that differing viscosity is not the only factor that provokes case hardening.

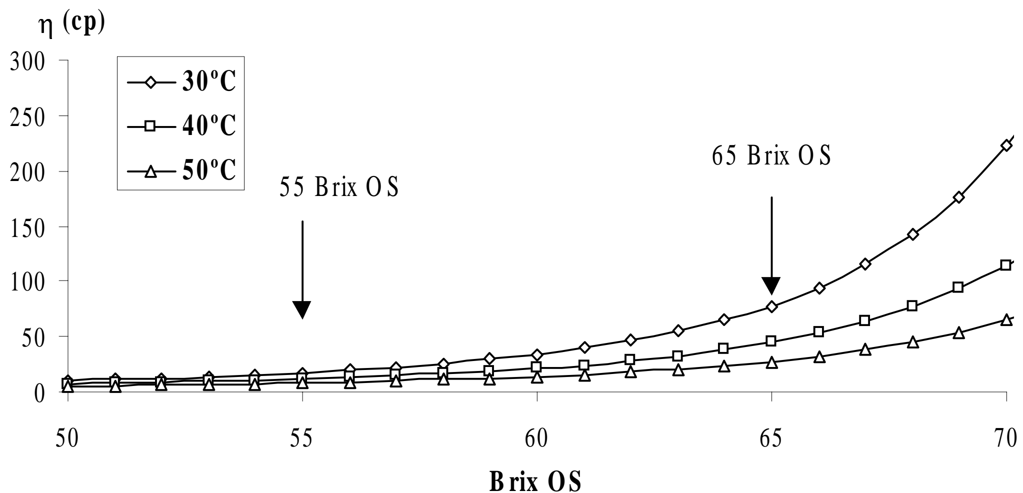

Fig. 3: Sucrose OS viscosity at different temperatures

Table 1: Maximum volume changes ($(V^o_t - V^o_0)/V^o_0$) for each experimental condition

Temperature	55 Brix OS. OD	65 Brix OS. OD	55 Brix OS. PVOD	65 Brix OS. PVOD
30°C	-0.347	-0.395	-0.297	-0.38
40°C	-0.375	-0.466	-0.34	-0.416
50°C	-0.457	-0.539	-0.273	-0.397

When VI is applied at the beginning of the experiments (PVOD), important differences in water and solute fluxes are observed at 55 and 65 Brix OS (fig. 4 and 5). A possible explanation is that gas phase is replaced by osmotic solution during the VI process. Although this fact clearly affects the VI samples` behaviour as opposed to those not vacuum impregnated, this can't be the only reason that explains these differences. The maximum differences that can be explained by the impregnation of pores would be similar to the apple porosity (23%, Salvatori, 1997). Nevertheless in figures 4 and 5, it can be observed that the differences in fluxes are bigger than 23% for the PVOD samples.

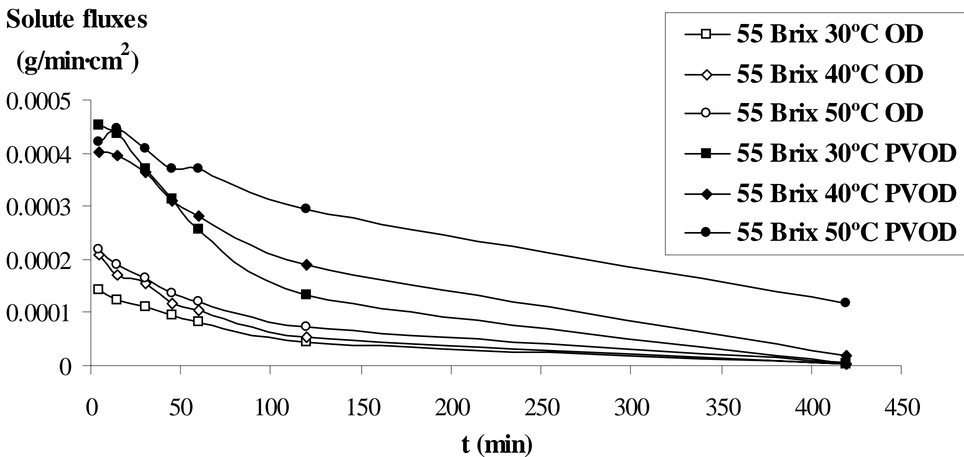

Fig. 4: Solute fluxes for OD and PVOD experiments at 55 Brix OS.

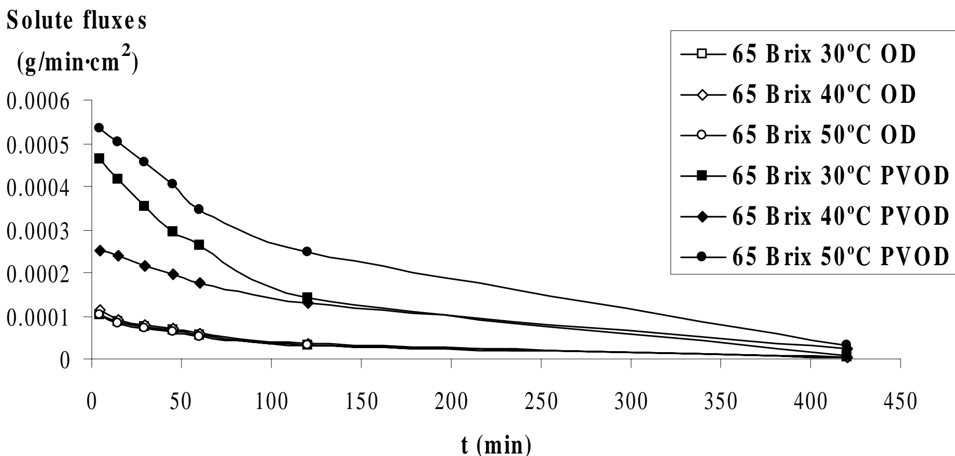

Fig. 5: Solute fluxes for OD and PVOD experiments at 65 Brix OS.

In figure 6, it can be noticed that a lower case hardening effect due to change in OS concentration (from 55 to 65 Brix) exists as regards to OD. In the PVOD experiments the maximum volume changes are much bigger for 65 Brix OS than for 55 Brix OS, which, together with the bigger viscosity, could explain this different behaviour. The lower fluxes at 65 Brix and 40ºC for PVOD could be partially explained in this case by the bigger volume reduction as regards to PVOD at 65 Brix at 30 and 50ºC (table 1).

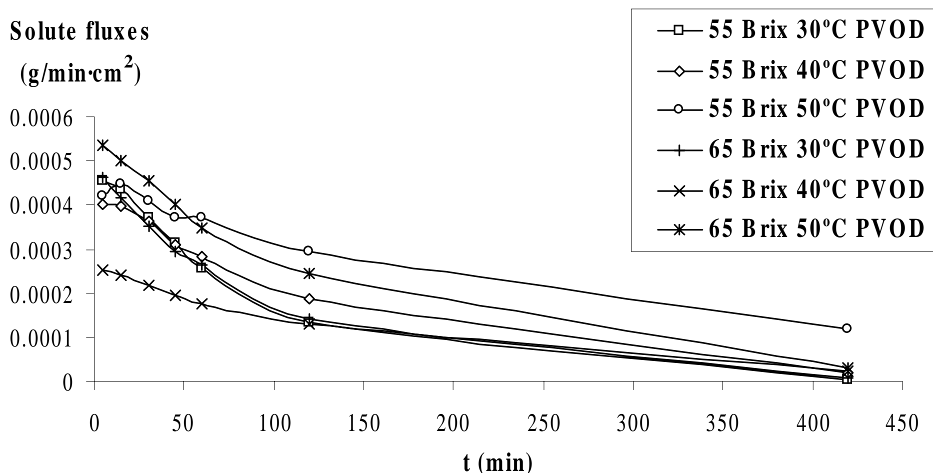

Fig. 6: Solute fluxes for PVOD experiments at 55 and 65 Brix OS.

Another explanation for the decrease in solute fluxes when working at 65 Brix, as compared to 55 Brix OS, would be that the increase in the driving force for 65 Brix and the viscosity would be favourable to the water flow, which would interact with the solute uptake, thereby decreasing its flow.

But even taking into account all the factors explained before, the differences in solute fluxes observed between OD and PVOD treated samples are not explained. These differences could be explained considering that when VI is applied and cells deform, the cell membrane separates from cell wall due to differences in mechanical forces (Barat et al., 1998). This phenomenon has been observed with Cryo-SEM observation (Martínez-Monzó et al., 1998). On the other hand when working at OD, when the tissue shrinks, the cell membrane is joined to cell wall. Thus, in both cases a cell membrane barrier appears in the collapsed surface cell layer. This barrier would be denser when a more concentrated osmotic solution were used, due to the bigger shrinkage. As cell membranes are permeable to water but not to solutes, this would be the reason why case hardening mainly affects the solute transport. When applying Vacuum Impregnation prior to the osmotic dehydration, cell membranes separate from the cell walls during shrinkage. This leads to a collapsed structure more permeable to solutes. This different behaviour is explained in figure 7. The presence of this cell membrane barrier would disappear when working at very high temperatures, which could be one of the explanations to the increase in solute gains under such conditions (Lerici et al., 1988).

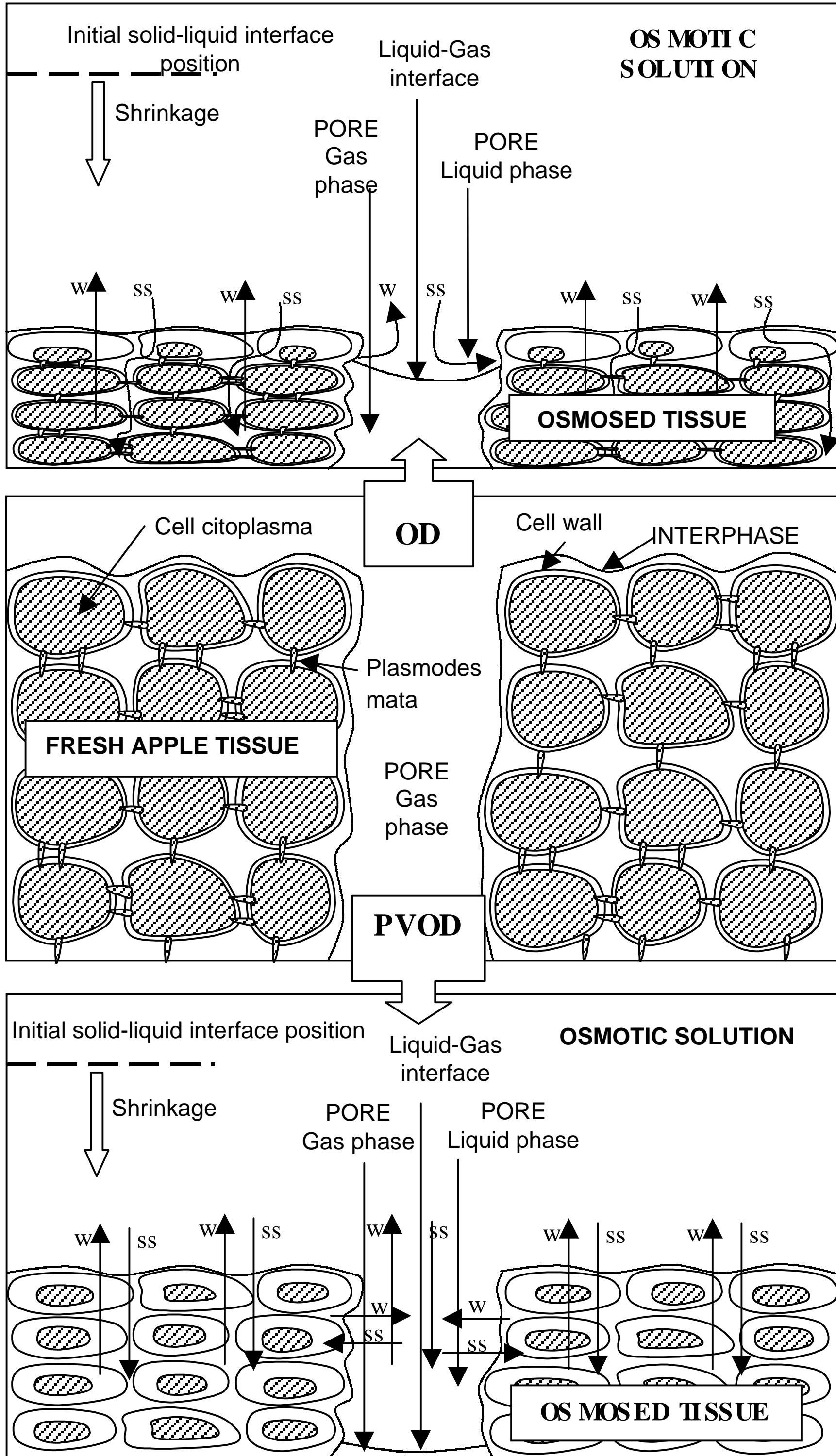

Fig. 7: Scheme of differences in structural behaviour in OD and PVOD samples.

Conclusions

The case hardening phenomenon in osmotic dehydration appears when working with very high concentrated osmotic solutions. Solute transport is the main effect in the case of the case hardening.

The explanation to case hardening, would not only be the collapse of the surface cell layer and an increase in viscosity, but also the formation of a barrier made up of the deformed cell membranes bonded to the cell wall. This barrier would be denser when a more concentrated osmotic solution is used, due to the bigger shrinkage. As cell membranes are permeable to water but not to solutes, this would be the reason why case hardening mainly affects the solute transport.

When applying Vacuum Impregnation prior to the osmotic dehydration, cell membranes separate from the cell walls during shrinkage. This leads to a collapse structure more permeable to solutes. Additionally, pores become full of osmotic solution, which increases the solute and water transport area without any barrier. The phenomenon would explain why Vacuum Impregnation reduces case hardening when applied.

Nomenclature

A: Surface area (cm^2)

a_1, a_2, k_1, k_2: Biexponential model constants

E_d: External diameter (cm)

H: Height (cm)

I_d: Internal diameter (cm)

J: Mass flux (g/min·cm^2)

M: Weight (g)

OD: Osmotic Dehydration at P_{atm}

OS: Osmotic Solution

P: Pressure

PVOD: Pulsed Vacuum Osmotic Dehydration

t : Time

VI: Vacuum Impregnation

$\Delta M_t^o = \frac{M_t^o - M_0^o}{M_0^o}$: Weight change at time t (g./g.)

Superscripts:

j = ss: Soluble solids

j = w : Water

o : Total mass or volume

Subscripts:

0 : Initial values (t=0)

t : Values at time t

atm: atmospheric

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Vacuum impregnation: A useful tool in candied fruit/vegetables processing

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Abstract

Osmotic dehydration is the main phenomena occurred in candied fruit processing. The osmotic dehydration process step lasts a very long time. In this study the analysis of the long-term osmotic dehydration process has been carried out and successive pseudo-equilibrium steps have been analyzed and defined. This analysis enables us to explain the different changes undergone by samples throughout the process. It has been seen that the fruit cellular structure plays an important role in the product development. The Vacuum Impregnation of the fruit with the external solution at the beginning of the treatment also greatly affects the product development.

Introduction

Osmotic dehydration is the main phenomena occurred in candied fruit processes. In some cases a subsequent surface air-drying is carried out in order to obtain a more commercial product (Lloría, 1997). The osmotic dehydration process step lasts a very long time (depending on the fruit size), as opposed to the usual osmotic processes in fruits (1 to 4 hours approximately). So, throughout the long time treatment not only is the sample compositional equilibrium with the external solution reached, but also the penetration of the external solution inside the fruit occurs, while the fruit degasifies and gains transparency. All these phenomena reflect the response of the fruit on the way to the equilibrium with the external solution.

In figure 1, the Flow-Chart of a candying process can be observed. Candied products are obtained by means of an osmotic dehydration process in which the main objective is to reach a product rich in soluble solids. Commonly, an osmotic solution composed of sucrose and glucose is used and its concentration is increased by evaporation throughout the time working at the boiling point temperature. In some cases vacuum pressures are applied in order to decrease the boiling point temperature and subsequently reduce the temperature damage of the product. Even in those cases quite high temperatures are applied to the product (minimum 60-70 °C when working at vacuum pressure). These high temperature conditions imply a serious damage in the organoleptic properties of the fresh fruit. The candy process is usually traditional and its bases are not very well known.

The objective of this study is the analysis of the equilibrium in the osmotic dehydration process in order to understand the main mechanisms that are involved in the candying process. The effect of vacuum impregnation on the behaviour of the samples is also studied. The third objective is to study the feasibility of use of vacuum impregnation as described by Fito (1994), in the candy fruit processing in combination with low temperatures, looking for a higher yield and less degraded product.

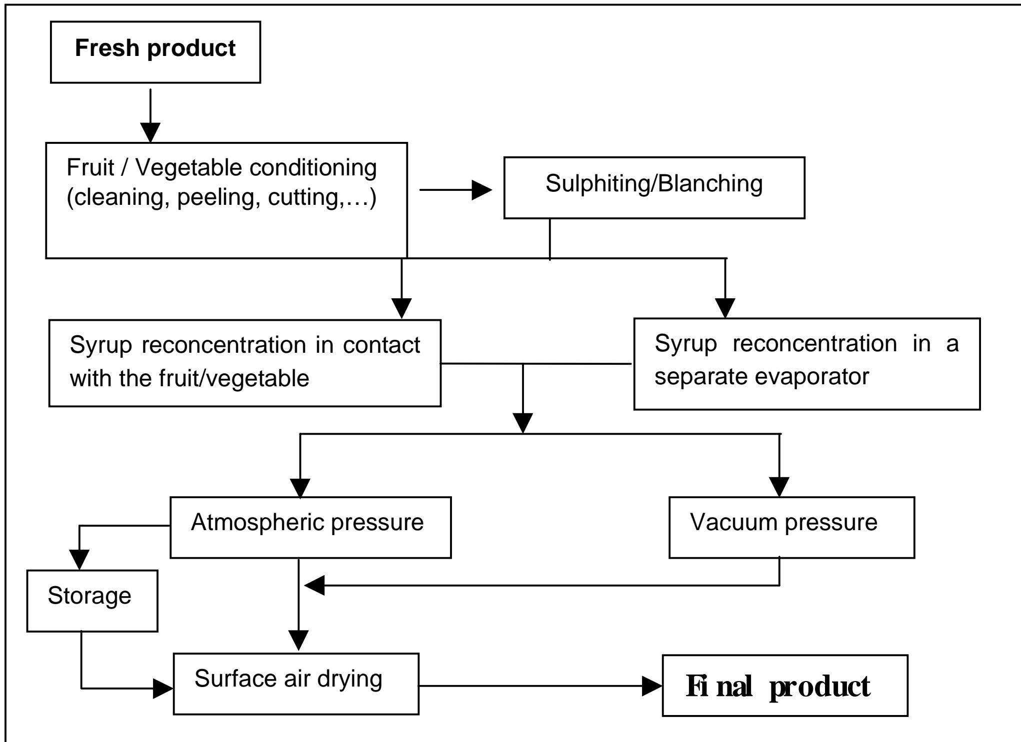

Figure 1: Flow-Chart of fruit / vegetable candying process.

Materials and methods

Apples (Granny Smith) were peeled, and cylindrical samples (2 cm length and 2 cm diameter) were cut with a borer, with the longitudinal axes in the fruit apex-base direction. Initial weight, moisture, soluble solids and volume of samples were determined. Fruit samples from the same fruit piece were placed into a flask, which contained a non-stirred sucrose osmotic solution (45 and 65 Brix). The solution-fruit ratio was higher than 20 in all cases. Temperature of the system was maintained at 30 and 40°C by placing the flask into an oven. Potassium sorbate (2000 ppm) was added to the osmotic solutions in order to stabilize the samples microbiologically. For each treatment the weight and volume of three of the samples were controlled throughout the equilibration time. For the PVOD series, samples were impregnated with OS applying an absolute pressure of 180 mbar during the first 5 minutes of the treatment. After that, atmospheric absolute pressure was restored and the increase in sample weight determined. After vacuum impregnation the PVOD samples were placed into the oven and the experiment continues as in the OD case.

At the end, an apple candying attempt was carried out using the combination of low temperatures (30 and 40°C), OS concentration (45 and 65 brix) and VI, compared with the atmospheric process. Four apple pieces were obtained after peeling each apple in this case.

Analytical determinations

Sample volume was determined with a solid pycnometer, using in each case the respective osmotic solution as reference liquid. Moisture content of samples was determined by placing the samples in a vacuum oven until constant weight was reached (AOAC 20013). Soluble solids were analysed by measuring the refraction index in a refractometer (Atago, NAR T3, Japan) at 20 °C. In order to obtain a clear refractometric measure; samples were homogenised with distilled water using an ultraturrax mixer. Refractometric measures were affected by the dilution factor to obtain the original sample concentration of soluble solids in the food liquid phase (FLP).

Bulk density was calculated applying Eq. (1) (Barat et al., 1998a). This one was empirically obtained (Barat, 1998) by correlating the experimental data: mass fraction of soluble solids of the liquid phase of osmotically dehydrated apple samples (z_s) against the density of these samples after their homogenisation and air evacuation.

$$\rho^{FLP} = 0.48 z^{SS} + 0.9997 \qquad (r^2=0.996) \qquad (1)$$

Results and discussion

Study of the candy fruit/vegetable processes in the range of low temperatures

Changes in weight and volume of apple cylinders were measured throughout the osmotic dehydration process working at 30°C-45 Brix and 40°C-65 Brix, using Vacuum Impregnation or not (fig. 1 and 2). These experimental conditions were chosen because of the low temperatures and the use of an intermediate and highly concentrated osmotic solution. It is supposed that the observed sample behaviour is similar to that seen at high temperatures.

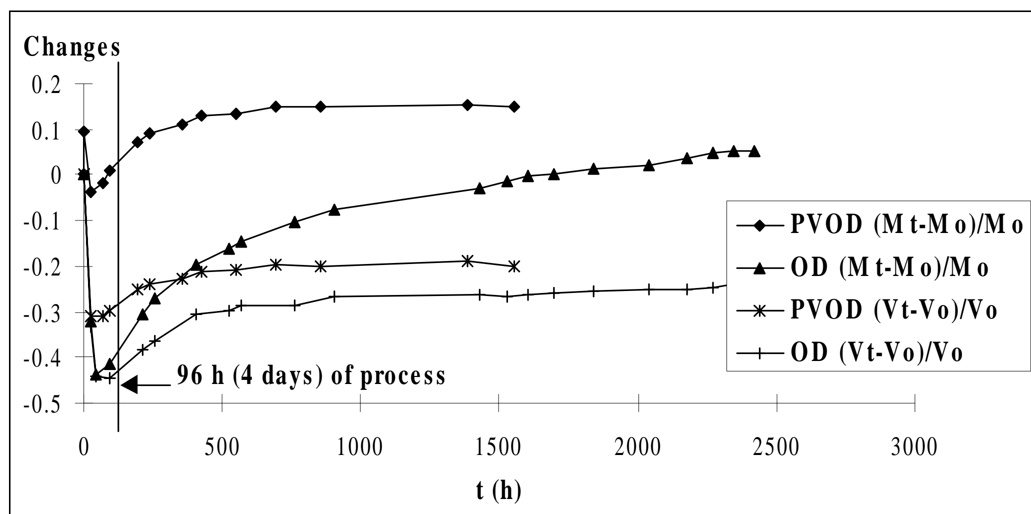

Figure 1: Changes in weight and volume in osmotically dehydrated apple cylinders at 30°C and 45 Brix OS concentration.

The behaviour of osmosed apple on the way to equilibrium is such that a minimum in weight and volume is reached during the first two days. This point corresponds to the equality in concentrations between the OS and the FLP. After that, volume and weight begin to increase. The recovery in weight and volume, due to the structural stress generated during shrinkage, is faster and in a

greater extent when lower OS concentration are used, as can be seen in the comparison of fig. 1 and 2. This would explain that in the industrial procedure, an increasing OS concentration is used, probably to reduce the irreversible changes and big weight and volume losses that occur when a very high concentration gradient exists between the OS and the FLP (Barat et al., 1998a). It can be seen that samples osmotically dehydrated with a 65 Brix OS deform to a greater extent and that weight and volume recovery is more irreversible.

On the other hand, when comparing the behaviour of vacuum impregnated samples with those dehydrated under atmospheric conditions, it can be observed that those vacuum impregnated (PVOD) have a bigger weight and volume than OD samples. This is closely related with the yield of the process.

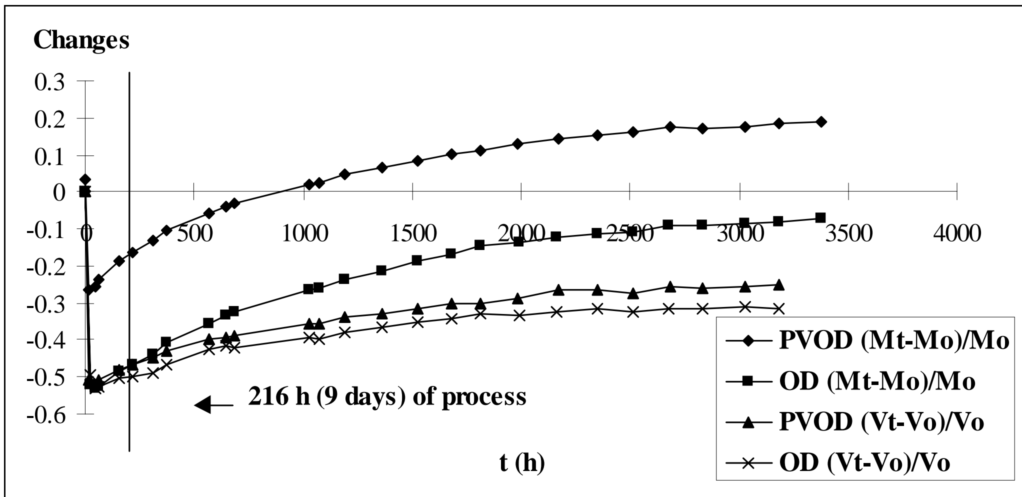

Figure 2: Changes in weight and volume in osmotically dehydrated apple cylinders at 40°C and 65 Brix OS concentration.

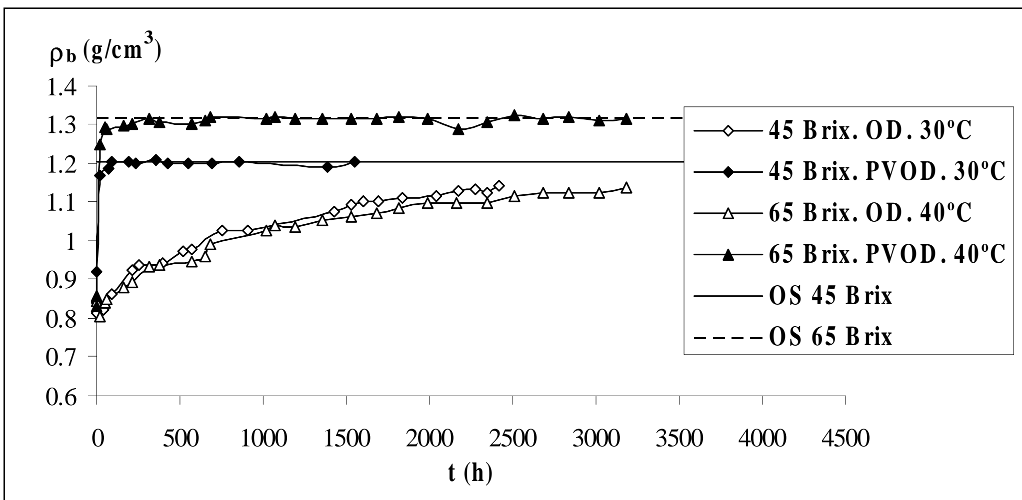

Figure 3: Changes in bulk density of apple cylinders throughout the process.

Bulk density evolution throughout the time (fig. 3) shows that VI samples reach equality with the OS faster than those processed at atmospheric pressure. This implies that VI samples have the characteristic glassy aspect of candied fruits and vegetables in very short time, but not those processed at atmospheric pressure due to the remaining gas. Industrial process implies high temperatures favourable to the flow out of gas.

Use of vacuum impregnation in the candying fruit processing. Apple candying attempt

Taking into account the experimental results obtained with apple cylinders, the candying of apple pieces was carried out with a sucrose osmotic solution of variable concentration as can be observed in fig. 4. It can be noticed that vacuum impregnated samples lose weight to a lesser extent than those not impregnated. The time needed to reach the minimum in weight is longer than in the case of cylinders due to the bigger size of the apple pieces. This new procedure to obtain candied fruit or vegetable has been patented (Barat et al., 1998b).

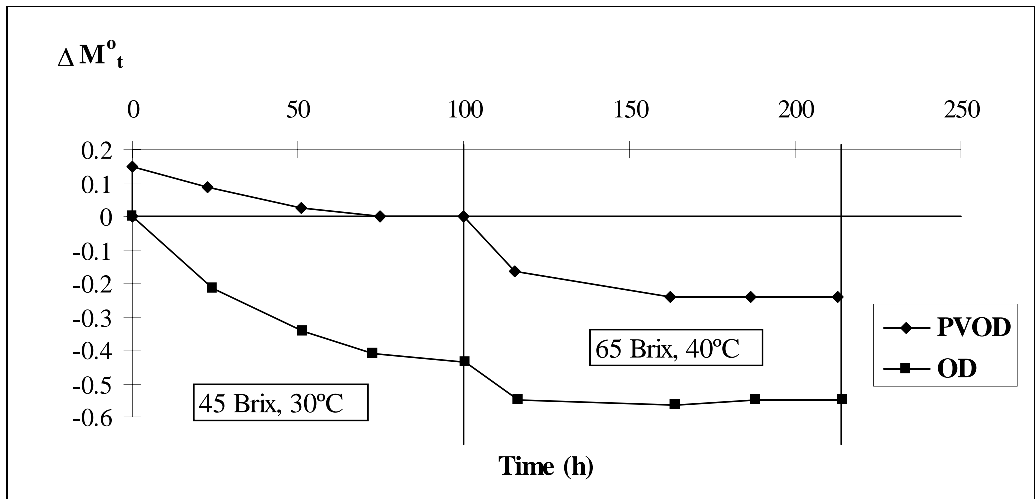

Figure 4: Changes in weight of apple pieces throughout a candying experiment.

In the first period (45 Brix, 30°C) the minimum weight is reached at 74 hours and in the second (65 Brix, 40°C) at 163 h, at the end of both periods equality in concentration of the OS and FLP is accomplished as can be seen in table 1.

Table 1: Concentration values of candied apple pieces and OS at different moments.

	OD			PVOD		
	y^{ss}	z^{ss}	x^{w}	y^{ss}	z^{ss}	x^{w}
T= 0	0.45	0.13	0.85	0.45	0.13	0.85
t= 4 days	0.41	0.39	0.58	0.41	0.40	0.59
t= 9 days	0.65	0.67	0.32	0.65	0.64	0.34

The yield when working using Vacuum Impregnation (PVOD) is much bigger than for the product processed at atmospheric pressure (OD).

Conclusions

The use of an increasing concentration of the osmotic solution in order to achieve an important yield in the process and avoid irreversible deformations and weight losses is essential. High temperatures are applied to the process to reconcentrate the OS, decrease the time needed to reach the equilibrium concentration, increase cell membrane permeability and so promote the uptake of soluble solids. The replacing of gas in the porous structure with liquid phase is also favoured with the use of high temperatures. The role of the structure is very important in this kind of processes in which long term processes are used and a recovery in volume appears due to structural stress relaxation. It has been seen that vacuum impregnation also greatly affects the product development and enables us to obtain a candy fruit with a very high yield and by using quite low temperatures. The application of vacuum impregnation in the candied fruit manufacturing gives a better process yield (lower net mass losses), while allowing us to work at low temperature, thereby better maintaining the fruit's organoleptic properties. The behaviour of fruit and vegetables during the candy process is also understood.

Nomenclature

FLP: Food Liquid Fraction M : Sample mass (g) OD: Osmotic Dehydration at P_{atm} OS: Osmotic Solution P : Pressure (N/m^2) PVOD: Pulsed Vacuum Osmotic Dehydration t : Time (h) V: Volume (cm^3) VI: Vacuum Impregnation x^j : Mass fraction of j in food (kg. j / total kg) y^j . Mass fraction of j in osmotic solution (kg. j / total kg) z^j : Mass fraction of j in food liquid phase (kg. j / total kg)	ρ_b : Bulk density (g/cm^3) ρ^{FLP} : FLP density (g/cm^3) $\Delta V_t^o = \frac{V_t^o - V_0^o}{V_0^o}$: Volume change at time t (g./g.) $\Delta M_t^o = \frac{M_t^o - M_0^o}{M_0^o}$: Weight change at time t (g./g.) Superscripts: j = ss: Soluble solids j = w : Water o : Total mass or volume Subscripts: 0 : Initial values (t=0) atm: Atmospheric t: Values at time t

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BARO-Thermal processing to improve feeding value of faba bean – rapeseed concentrate

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Introduction

High costs of feeds based on imported components is major concern of poultry producers in Central Europe and many other countries. Therefore, considerable effort has been directed to obtain protein for poultry diets from sources which are less expensive, and moreover, can be grown locally, which improves the import-export balance. In countries not able to afford costly imported feedstuffs, the search for lower-cost alternatives is frequently an economical necessity.

Since several years, the feed industry has been interested in the nutritional potential of faba beans and rapeseed, both easily produced in Central Europe, as a source of protein in poultry diets. Both faba beans and rapeseed, because of their high protein content, are attractive feedstuffs for chickens, however, the nutritional value of these feeds, especially when applied as a sole source of protein, has been questioned by several feed specialists. The main concern addressed includes the presence of toxic substances, nutrient antagonists, and low nutritional value [4, 5, 12, 16, 17, 19, 23, 25, 27, 29].

The low nutrition value could be improved by feeding mixtures of faba beans with rapeseed, which is relatively rich in sulphur amino acids [23]. Further improvement may be achieved by extrusion cooking of the mixture, as various antinutritional agents may be destroyed, at least partially, in this way [19].

Despite the experimental evidence attesting the nutritional potential of faba beans and rapeseed, there exists considerable reservation regarding the suitability of both ingredients as a feedstuff. Consequently, their routine application is not widely spread. The study reported here was undertaken to evaluate the suitability of FB-RS concentrate as an energy and protein source for broiler chick diets.

Material and methods

The following raw materials were used for the baro-thermal processing: dehulled faba beans and full-fat „00” rapeseed (41.44 % of oil in dry matter), and rapeseed extracted meal (1.00 % oil in dry matter). Beans were ground in a hammer mill before the mixing with rapeseed. More details are given in the previous papers [19, 20].

The mixtures processed in the twin-screw extrusion cooker, type 2S-9/5, manufactured by Z.M.Ch. Metalchem (Gliwice, Poland). The extrusion-cooking parameters differed in the initial water content: (a) lower water content (12 %); (b) higher water content (18 %). Another variable was the maximum temperature of the temperature profile (i.e. temperatures in the five consecutive segments of the extruder): (a) lower temperature: 100-120-150-180-120°C; (b) higher temperature: 120-140-170-200-140°C.

For the evaluation of faba bean-rapeseed concentrate as a dietary protein and energy supplement, 96 (1 day old) broiler chickens were used. The following experimental diets were compared: C = soybean meal-based control diet, RO = soybean meal-based diet supplemented with rapeseed oil, T = soybean meal-based diet supplemented with beef tallow and FB-RS = faba bean-rapeseed based extruded concentrate.

Feed and water were available *ad libitum*. Birds were fed the starter diets from 0 to 3 weeks, and the finisher diets from 4 through 7 weeks. The feed intake and body weights were monitored at weekly intervals. The measurements were performed on the same day of the week in each case.

Table 1: The composition and analysed nutrient content (%) of starter diets (0-3 weeks)

Component	C	FB-RS	T	RO
Wheat	63.9	39.5	51.5	51.5
Soybean extracted meal	26.1	6.5	29.6	29.6
Powdered milk	3.0	3.0	3.0	3.0
Fish meal	3.0	3.0	3.0	3.0
Beef tallow	-	-	8.9	-
Rapeseed oil	-	-	-	8.9
Calcium diphosphate	1.4	1.4	1.4	1.4
Limestone	1.3	1.3	1.3	1.3
Sodium chloride	0.3	0.3	0.3	0.3
FB-RS concentrate *	-	44.0	-	-
Vitamin-mineral premix**	1.0	1.0	1.0	1.0
Analysed nutrient content:				
Dry matter (DM)	91.5	92.1	92.7	92.8
Crude protein (CP)	21.9	22.2	22.4	22.1
Crude fat (CF)	2.3	11.4	11.3	11.1
N-free extract (NFE)	57.2	49.9	49.0	49.5
Fibre	3.6	2.5	3.4	3.4
Ash	6.5	6.1	6.7	6.7
Calcium (Ca)	1.2	1.2	1.3	1.3
Total phosphorus (TP)	0.8	0.8	0.7	0.7
Magnesium (Mg)	0.12	0.10	0.09	0.09
Metabolizable energy *** MJ/kg	11.73	13.74	13.58	13.58

C = soybean meal-base control diet; RO = soybean meal, supplemented with 8.9 % refined rapeseed oil; T = soybean meal, supplemented with 8.9 % beef tallow; FB-RS = extrusion cooked dehulled-faba bean full-fat rapeseed based concentrate

* The concentrate was a mixture of dehulled faba beans and rapeseeds (1:) and contained (5); 92.7 % DM, 26.6 % CP, 20.9 % CF, 3.1 % fibre, 3,6 % ash, and 38.5 % NFE

** Vitamin-mineral premix provided per kg diet: 8 000 IU vitamin A, 1 200 IU vitamin D3, 10 IU vitamin E, 2 mg vitamin K, 4 mg riboflavine, 0.4 mg pyridoxine, 0.015 mg cobalamin, 12 mg nicotinic acid, 8 mg Ca pantothenate, 20 mg folic acid, 150 mg choline chloride, 50 mg Mn, 0.3 mg I, 30 mg Zn, 10 mg Se, 40 mg Co

*** Calculated as described by Janssen (1986)

The digestibility study (using the conventional balance method) of dry matter (DM), crude protein (CP), crude fat (CF), nitrogen-free extract (NFE), and organic matter (OM) was undertaken at the end of the 3rd and th weeks of the experiment.

At the end of experiment, 4 birds (2 males and 2 females), having body weights approximating the mean of each group, were used to evaluate the carcass quality, the yield, and the fat content.

Analyses of diets and faeces were performed after the conventional standard methods [8, 9, 11, 16, 17, 18].

The statistical analysis was carried out using GLM ANOVA software. The statistical significance was evaluated on the probability level of 0.05.

Table 2: The composition and analysed nutrient content (%) of the finisher diets

Component	C	FB-RS	T	RO
Wheat	73.9	54.4	64.4	64.4
Soybean extracted meal	19.1	2.6	21.6	21.6
Powdered milk	-	-	-	-
Fish meal	3.0	3.0	3.0	3.0
Beef tallow	-	-	7.0	-
Rapeseed oil	-	-	-	8.9
Calcium diphosphate	1.4	1.4	1.4	1.4
Limestone	1.3	1.3	1.3	1.3
Sodium chloride	0.3	0.3	0.3	0.3
FB-RS concentrate *	-	36.0	-	-
Vitamin-mineral premix**	1.0	1.0	1.0	1.0
Analysed nutrient content:				
Dry matter (DM)	90.3	91.4	90.6	90.8
Crude protein (CP)	18.6	18.7	19.3	18.9
Crude fat (CF)	2.7	9.4	9.6	9.0
N-free extract (NFE)	57.2	49.9	49.0	49.5
Fibre	3.6	2.5	3.4	3.4
Ash	6.5	6.1	6.6	6.7
Calcium (Ca)	1.2	1.2	1.2	1.3
Total phosphorus (TP)	0.8	0.8	0.7	0.7
Magnesium (Mg)	0.12	0.10	0.08	0.09
Metabolizable energy *** MJ/kg	11.88	13.44	13.16	13.27

C = soybean meal-base control diet; RO = soybean meal, supplemented with 7.0 % refined rapeseed oil; T = soybean meal, supplemented with 7.0 % beef tallow; FB-RS = extrusion cooked dehulled-faba bean full-fat rapeseed based concentrate

** Vitamin-mineral premix provided per kg diet: 7 000 IU vitamin A, 10 000 IU vitamin D3, 0.01 mg vitamin E, 1.5 mg vitamin K, 4 mg riboflavine, 0.3 mg pyridoxine, 0.01 mg cobalamin, 10 mg nicotinic acid, 3 mg Ca pantothenate, 0.2 mg folic acid, 200 mg choline chloride, 50 mg Mn, 0.2 mg I, 30 mg Zn, 0.2 mg Se, 0.3 mg Co

*** Calculated as described by Janssen (1986)

Results

The nutrient retention values are shown in Tab. 3. Higher digestibilities ($P< 0.05$) of CF in birds fed the RB-RS and RO supplement diets is noteworthy. The addition of fat (the diets FB-RS, RO and T) resulted in better overall performance of chicken (Table 4). The birds fed of the T diet tended to have higher body weights in comparison to those fed the three remaining diets. The utilisation of the feed was better during the first three weeks (Table 4). The diets RO and T resulted in the feed conversion factor lower ($P< 0.05$) than in the birds fed the C diet. However, among the birds fed the FB-RS diet, the feed conversion factor was lower ($P< 0.05$) than in any other experimental group. The utilisation of protein unit of growth differed ($P< 0.05$) among dietary treatments being in the order: FB-RS T< RO < C.

The carcass quality, yield and fat content were not affected by dietary treatments. However, the carcasses from the birds fed diets supplemented with the FB-RS concentrate had slight pigment tinge, when compared with the carcasses of the birds fed remaining three diets.

Table 3: Nutrient retention by chicks fed the four experimental starter and finisher diets

Diet	Dry matter	Crude protein	Crude fat	N-free extract	Organic matter
Starter diets:					
C	70.1 ab	87.0 ab	77.3 a	71.7 bc	73.4 ab
FB-RS	74.2 c	87.2 ab	92.7 c	73.3 c	77.2 c
T	69.2 a	87.0 b	76.5 a	69.5 a	72.5 a
RO	71.1 b	85.4 a	91.1 b	70.2 ab	74.5 b
(SE)	0.34	0.55	0.62	0.52	0.33
Finisher diets:					
C	76.5 a	83.5 a	80.3 a	82.2 b	80.3 a
FB-RS	76.7 a	82.7 a	90.2 b	81.2 ab	80.3 a
T	75.9 a	83.7 a	79.3 a	81.9 ab	79.8 a
RO	76.0 a	82.3 a	90.8 b	80.5 a	79.6 a
(SE)	0.43	0.32	0.74	0.47	0.43

C = soybean meal-based control diet; RO = soybean meal, supplemented with 8.9 % (starter) or 7 % (finisher) refined rapeseed oil; T = soybean meal, supplemented with 8.9 % (starter) or 7 % (finisher) beef tallow; FB-RS = extrusion cooked dehulled-faba bean full-fat rapeseed based concentrate

a, b, c - means followed by different letter are significantly different ($P< 0.05$); SE = standard error of the mean

Table 4: Performance of broiler chicks fed the four experimental diets of starter and finisher types

Parameter	C	FB-RS	T	RO	(SE)
Body weight 3 wk (kg)	0.556 ab	0.546 b	0.600 a	0.564 a	0.030
Body weight 7 wk (kg)	1.749 b	0.815 b	1.980 a	1.850 ab	0.121
Feed utilization per kg body weight gain (kg)					
3 wk	1.629 a	1.391 c	1.493 b	1.495 b	0.016
7 wk	2.356 a	2.063 c	2.138 bc	2.180 b	0.029
Overall	2.132 a	1.870 c	1.952 b	1.981 b	0.021
Protein utilization per kg body weight gain (kg):					
3 wk	0.356 a	0.309 c	0.334 b	0.340 b	0.0035
7 wk	0.438 a	0.386 c	0.413 b	0.406 b	0.056
Overall	0.414 a	0.363 c	0.390 b	0.387 b	0.0041

Conclusions

1. In the present study, the addition of FB-RS concentrate to broiler chicken diets resulted in performance parameters, which were similar or better than those obtained from soybean meal-based diets.
2. Slightly higher digestibilities of DM, CF, NFE and OM were reported in birds fed the FB-RS diet (Tab.3), and also, the presence of faba beans in the diet did not affect the retention in the most efficient ($P < 0.05$) utilization of protein per unit of growth (Tab. 4).
3. The sound performance of chicks fed the FB-RS diet can likely be attributed to the alleviation of the antinutrient activities from both the faba beans and rapeseed as a result of dehulling, and the improved biological value of the FB-RS diet's proteins by supplementation with methionine. It is noteworthy that the energy from rapeseed was as efficient as rapeseed oil, since the digestibility of CF in the FB-RS diet was comparable with that of the RO supplemented diet.
4. The present study has shown that both the protein component and the energy component from the FB-RS concentrate were utilized very efficiently by chickens. The 1 : 1 ratio (W/W) used in the formulation of our concentrate allowed complete substitution of soybean protein, and provided an appropriate energy supplement in the diet.
5. The improved performance of faba-bean-rapeseed mixture may be attributed to relatively high contents of sulphur amino acids (cysteine and methionine) in rapeseed protein, compared with legumes. Another advantage is the extrusion treatment of the feed mixture as some toxic and antinutritional components are decomposed, at least partially, during the extrusion-cooking.
6. The carcass evaluation showed that the respective diets did not affect the quality and yield. However, it has to be emphasized that the carcasses from birds fed diets supplemented with the FB-RS concentrate had slightly different tinge of pigment from that of the birds from the remaining dietary groups. This phenomenon can be, apparently, attributed to higher contents of carotenoid and chlorophyll pigments in rapeseed.
7. Faba bean-rape seed concentrate can be used in broiler chicken diets as the replacement of soybeans. The protein value of the concentrate, if supplemented with methionine, appears to be comparable with soybeans. In addition, the nutritional value of energy from rapeseed included in such a concentrate is comparable to that obtained from refined rapeseed oil.

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Combined microwave and air drying of apple (var. Granny Smith)

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Abstract

A drying pilot plant with combined action of microwaves and conventional hot air was designed and built to study the drying kinetics of some foods. Apple var. Granny Smith was used in this work. To develop an optimised process of drying, different air temperature, microwave incident power and constant air rate were tested. The effect of vacuum impregnation of the fruit with isotonic solution was also analyzed. Empirical equations were used to model the observed drying kinetics.

Introduction

Microwave drying has significant advantages over conventional hot air drying: higher drying rate without case hardening, minimal overheating of locations with less water, and generally better product quality as less oxidation of pigments and vitamins (Decareau, 1985). The proper use of combined microwave and hot air drying improves the efficiency keeping the costs of drying down. Note that drying with microwaves alone can be very expensive in terms of both equipment and operating costs (Schiffmann,1997) .

Drying of food materials is a complex process involving simultaneous, coupled heat and mass transfer phenomena occurring in the material being dried (Rovedo et al., 1994). In hot air drying, the outer layer of the product is heated by convection while conduction heats the remainder of the sample. Nevertheless, microwave drying generates a volumetric heating by means of dipole rotation and ionic polarization (Bouraoui et al., 1994).

In this work the drying rate has been determined through the water fluxes which take place in the apple slice interface and the results obtained for the different experimental conditions were compared.

Materials and methods

Photographs describe the experimental pilot plant dryer. A modified household microwave oven with a mode stirrer was used (Fig.1). Two paralell lines were conected, one for hot air source and other for microwave generator . The air was heated by six electric resistances (500 W each one) and the desired temperature was controlled. The sample was suspended into the oven cavity by a nylon filament from a top mounted balance (Electronic Analytical Mettler Balance) for continuous monitoring changes of weight (Fig.2).

During each drying experience, the air rate, temperature out and inside the cavity, ambient relative humidity and the incident/reflected microwave power, were also measured by means of an higrometer, an anemometer and temperature sensors (all Systemat models). All these values were continously recorded through computer data acquisition.

The sample internal and surface temperatures were mesured and registered during microwave drying by means of three Luxtron Fluoroptic temperature probes (Fig.3).

The apples used for the drying experiments were purchased locally. The samples where sliced and the corresponding thicknesses measured with a dial micrometer. In all cases, the thickness (aprox. 7 mm.), was much smaller than diameter. In this condition drying took place mainly from the two major faces of the slice, thus simplifying the mathematical treatment of the experimental results. The core of the fruit was eliminated with a cylindrical 2.58 cm. diameter core borer.

Experiments were done on fresh and vacuum impregnated samples (5 min at P=50 mbar + 10 min at P=1013 mbar) using apple juice as isitonic impregnation solution. For the experiments, air rate was 2 m/s, heating temperatures 40 and 50°C, and microwave powers 20 and 40 W.

The weight development, m(t), was fitted by an empirical function with a sixth grade polynomic form and the surface water flux was obtained from:

$$J_{wx} = \frac{1}{A} \cdot \frac{dm}{dt} \quad (1)$$

where:

J_{wx} (g.water/s.m^2): surface water flux in x direction (through the sample),

dm/dt (g.water/s): drying rate

A_t(m^2): interchanging surface (a function of time)

The values can be determined using the following equations:

$$A_i = 2 \cdot (\frac{\pi \cdot D_{out}}{4} - \frac{\pi \cdot D_{in}}{4}) \quad (2.a)$$

$$A_t = \frac{(0{,}3 \cdot A_i)/t}{\Delta t} \quad (2.b)$$

where A_i is the initial evaporating area (m^2), D_{out} is the outer diameter of the slice apple (m), D_{in} is the inner diameter of the slice apple (0.0258 m) and Δt is the time increase (s).

Results and discussion

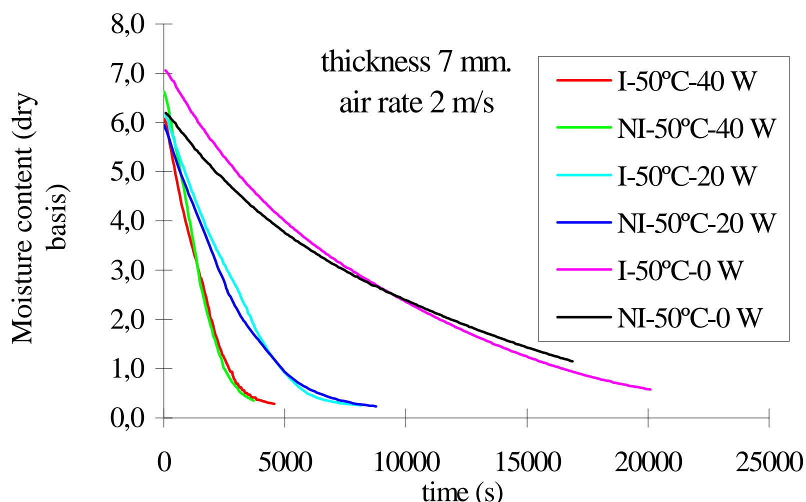

Fig. 1: shows moisture content development vs. time for impregnated and fresh samples, dried at 50°C only with hot air or combinated with microwaves (20 and 40 W).

Table 1: shows the experimental data for sample characteristics and process parameters

	Slice Apple data		**Air parameters**			**Microwave Power(W)**		
	Xi(dry basis)	**Outer Diameter (m)**	**inlet Tª(°C)**	**inlet RH%**	**drying RH%**	**Incident**	**Reflected**	**Absorbed**
NI-40°C-0 W	6,628	8,12	17,56	36,34	9,78	0	0	0
NI-50°C-0 W	6,189	7,29	23,88	38,63	9,78	0	0	0
NI-40°C-20 W	6,252	7,42	28,64	59,56	31,54	21	9	12
NI-50°C-20 W	5,954	7,52	29,31	63,61	20,86	21	9	12
NI-40°C-40 W	6,052	7,64	27,2	58,1	29,4	40	13	27
NI-50°C-40 W	6,628	7,89	28,5	64,64	20,25	40	13	27
I-40°C-0 W	5,708	7,59	27,43	57,58	28,31	0	0	0
I-50°C-0 W	5,99	7,63	27,9	53	15,88	0	0	0
I-40°C-20 W	6,59	7,51	29,3	58	32	20	7	13
I-50°C-20 W	6,16	7,51	27,42	69,12	20,3	21	10	11
I-40°C-40 W	5,82	7,7	30,22	59,57	20,63	40	14	26
I-50°C-40 W	6,077	8,11	28.5	58,31	21.2	40	13	27

Table 2: shows the coefficients values of the polynomic functions m(t) fitted for each experiment.

$$m(t) = a{\cdot}t^6 + b{\cdot}t^5 + c{\cdot}t^4 + d{\cdot}t^3 + e{\cdot}t^2 + f{\cdot}t + g \qquad (3)$$

	a	b	c	d	e	f	g
NI-40°C-0 W	2,78117E-25	-2,96035E-20	1,21137E-15	-2,44727E-11	2,88361E-07	-2,72631E-03	2,18345E+01
NI-50°C-0 W	-6,90995E-25	4,40202E-20	-9,18774E-16	4,24699E-12	1,13930E-07	-2,50003E-03	2,77990E+01
NI-40°C-20 W	-1,12604E-22	3,67348E-18	-4,84095E-14	2,99644E-10	-4,61061E-07	-4,20802E-03	2,15762E+01
NI-50°C-20 W	-8,83637E-23	3,11976E-18	-4,47741E-14	3,02160E-10	-5,14523E-07	-4,89982E-03	2,66928E+01
NI-40°C-40 W	-9,22753E-21	1,52902E-16	-9,95798E-13	3,10150E-09	-3,42786E-06	-6,21353E-03	2,18849E+01
NI-50°C-40 W	5,28608E-20	-5,96565E-16	2,32280E-12	-3,12364E-09	5,70651E-07	-7,37402E-03	2,48517E+01
I-40°C-0 W	3,24686E-28	-9,40341E-23	1,20110E-17	-9,29053E-13	4,74549E-08	-1,46362E-03	2,46198E+01
I-50°C-0 W	1,59766E-25	-1,89817E-20	8,79150E-16	-2,05925E-11	2,89007E-07	-3,15296E-03	2,79519E+01
I-40°C-20 W	3,42687E-23	-1,92515E-18	3,78541E-14	-3,36043E-10	1,60585E-06	-7,20869E-03	3,32833E+01
I-50°C-20 W	4,34563E-23	-4,81618E-18	8,55882E-14	-5,67905E-10	1,94623E-06	-7,98967E-03	3,13567E+01
I-40°C-40 W	5,80161E-21	-1,08999E-16	7,18703E-13	-1,99202E-09	3,06628E-06	-9,59509E-03	2,58976E+01
I-50°C-40 W	9,06987E-21	-1,70825E-16	1,12353E-12	-3,16976E-09	4,88783E-06	-1,21174E-02	2,87028E+01

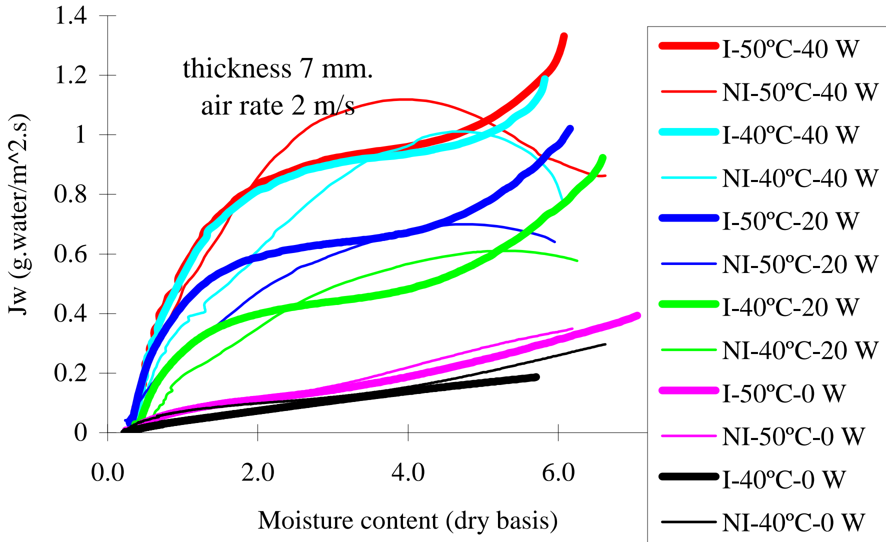

Fig. 2: shows calculated Jw vs. Xw (db) in all the studied samples.

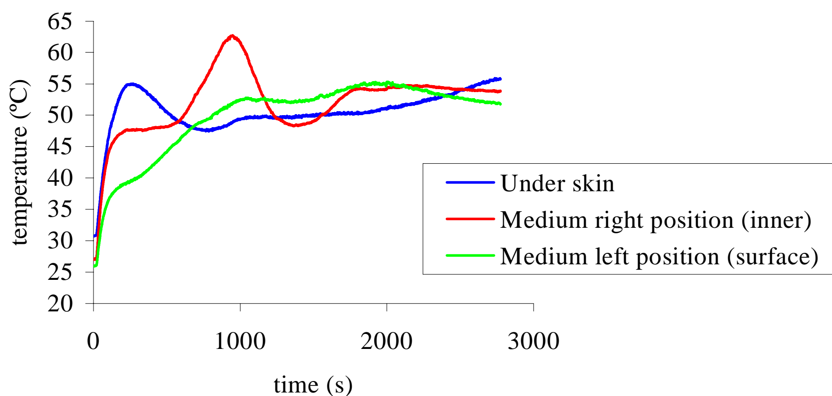

Fig. 3: shows the temperature development along the time of different points in the apple slice

Conclusions

Water fluxes are much lower for hot air dried samples.

Fresh samples showed also greater fluxes than those treated only with hot air, althoughits maximun flux is lower than the corresponding for impregnated.

As expected, combinated use of air and microwaves in drying decreases significantly the process time, also for fresh as for impregnated samples.

When microwaves are used, vacuum impregnation of fruit with isotonic solution increase the drying rate (maximum flux) while microwave effect dominate during the process (at high moisture contents).

The temperatures measured in different sample points during the microwave drying increased rapidly to above 65°C and then stabilized around the drying air temperature, although on the surface the increase is much lower probably due to the evaporated cooling.

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Modification of technological and nutritional properties of pea by germination and microwave heating

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Key words: pea seeds, germination, carbohydrates, microwave heating

Summary

Effect of germination and microwave heating is the resulting way of the improve of nutritional quality of legume products - the significant reduction of galactooligosaccharides (raffinose family oligosaccharides - RFO) and trypsin-inhibitor activity. Influence of germination on the carbohydrates composition of pea seeds was studied. After 4 days of germination the content of RFO in pea was decreased to 17.5 % of the original value and ratio sucrose/RFO in cotyledon increased from 0.5 to 4.5. Evaluation of drying curves for microwave drying of treated products were performed.

Introduction

The problem of flatulence causing oligosaccharides (RFO) in legumes is one of the general phenomena which have influence on the consumption and use of legumes in human and animal nutrition. Germination is one of the way of processing, which allows reduction of antinutritional factors, such as α-galactosides and trypsin-inhibitor activity (TIA) and affects the nutritive value of legumes. The significant reduction of TIA is possible to reach by heating - by hot air, infra-red or microwave. TIA decreases utilisation of protein content in grain legumes and therefore reduces of grain legumes use in nutrition. Combined effect of germination and the following microwave heating process was proposed for improving of nutritional quality of legume products. For microwave heating of pea and germinated pea was proposed as the main criteria ratio sucrose-to-RFO and trypsin inhibitor activity (TIA).

Material and methods

Plant material

Samples of pea (*Pisum sativum, ssp. sativum* L.), five cultivars (Merkur, Profi, Grana, Lantra, Primus), each cultivar from three various breeding farms in Czech Republic (Zatec, Caslav, Jaromerice nad Rokytnou), harvest 1997.

Germination tests

Seeds were incubated in covered Petri dishes on moist filter paper at 20 °C. Paper was moistened by distilled water, pH 7.0. Time of germination were: 24 h, 48 h, 72 h, 96 h. After 24 h and 48 h of germination seeds had germs only 1-2 mm length. These germinated seeds were analysed whole. After 72 h and 96 h of germination germs (1 to 3 cm length), cotyledons and testa were analysed separately.

Microwave heating

Sample were heated in microwave oven Whirlpool MT 243 / UKM 347 (frequency 2450 MHz, microwave power 90, 160 W, cavity volume 25.4 l, position of load in cavity - near of periphery on the turning glass plate)

Determination of dry matter content

Dry matter content (%) was determined after drying at 100 °C to constant weight on HA 300 Moisture Balance (Precisa, Switzerland), reproducibility 0.02 %.

Determination of drying curve

Approximately 20 g of sample in open Petri dish (diameter 6.7 cm) was placed into microwave oven and heated. Each minute were measured surface temperature of sample and weight of sample. Time of heating: up to 20 - 30 min.

Measurement of surface temperature

Surface temperature of sample was measured by means of the contactless infrared thermometer Ahlborn, type AMiR 7811, range: -20 to + 500 °C, resolution: 1 °C, accuracy: +/- 2 °C, Repeatability: +/- 1 % of reading value, response time: 500 ms.

Extraction and assay of soluble carbohydrates

Approximately 2 g of ground sample was homogenised in 20 ml of ethanol:water (80:20, v/v), refluxed (boiled) for 60 min. After boiling extract was diluted by demineralized water, filtered through a membrane filter 0.45 μm pore size and analysed by HPLC.

HPLC determination

The identification and quantification of monosaccharides, sucrose and galactooligosaccharides (raffinose family oligosaccharides - RFO - raffinose, stachyose, verbascose) contents were carried out using HPLC chromatography as described by Kvasnicka et al. (3). Detection limits for individual saccharides were the following (in mg/100 ml): glucose - 1.39, fructose - 1.79, galactose - 1.39, sucrose - 0.60, raffinose - 0.79, stachyose - 0.78, verbascose - 0,78.

Results

Average values of concentration of soluble carbohydrates and dry substances in pea seeds (cotyledons) during germination are shown in Table 1. Concentration of soluble carbohydrates in all samples of testa were zero always. Composition of soluble carbohydrates in germs is in Table 2. Germinated pea seeds were dried by microwave heating, surface temperature of microwave treated germinated pea was maximum 66 °C for power 90 W and 90 °C for power 160 W.

Table 1: Composition of soluble carbohydrates (g/100 g dry matter) in pea during germination (average values)

Time of germination	Dry mass (%)	Monosaccharides	Sucrose	RFO	Decrease RFO (%)	Ratio sucrose/RFO (1)
0 h	97.09	0.43	2.89	5.44	0.00	0.53
24 h	80.15	0.44	2.30	3.29	39.27	0.70
48 h	74.35	0.36	2.90	2.21	59.21	1.36
72 h	72.34	0.25	3.39	1.00	81.78	3.78
96 h	62.96	0.27	4.02	0.95	82.51	4.56

Table 2: Composition of soluble carbohydrates (g/100 g dry matter) in germs during pea germination (average values)

Time of germination	Dry mass (%)	Glucose	Fructose	Galactose	Sucrose	RFO
72 h	13.01	7.39	1.01	1.59	5.12	0
96 h	19.46	4.83	0.33	2.39	2.70	0

Conclusions

In agreement with papers concerning the effect of germination on the composition of various grain legumes, chiefly lentils and lupin seeds (1), (2), (4), our results show that germination treatment decreases the content of RFO in pea to 17.5 % of the original value and ratio sucrose/RFO increases from 0.5 to 4.5. Germination and the following microwave heating, where surface temperature of heated product is up to 75 °C, can be recommended to make-up the good quality pea meals.

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Pasteurisation and sterilisation with pulsed electric fields

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Abstract

NIZO food research and the University of Technology Eindhoven started a partnership to develop a novel treatment chamber for applying pulsed electric fields (PEF) on liquid food. In this paper some preliminary results are presented. With the PEF treatment chamber bacteria and spores are inactivated at temperatures below 40 °C. The energy-input is 30% of values reported in literature and patents with comparable inactivation levels of bacteria.

Introduction

The use of high-intensity pulsed electric fields (PEF) in food applications such as fruit juices and milk is gaining popularity, since it is claimed to represent one of the most promising non-thermal alternatives to conventional pasteurisation methods. The results with PEF published in literature show large differences in lethal effects and levels of energy [1,2] under apparently similar conditions. Figure 1 shows schematically the supposed mechanism of inactivation of bacteria cells with PEF. NIZO food research has started a research program to develop a well-defined treatment chamber in which both bacteria and spores are efficiently inactivated.

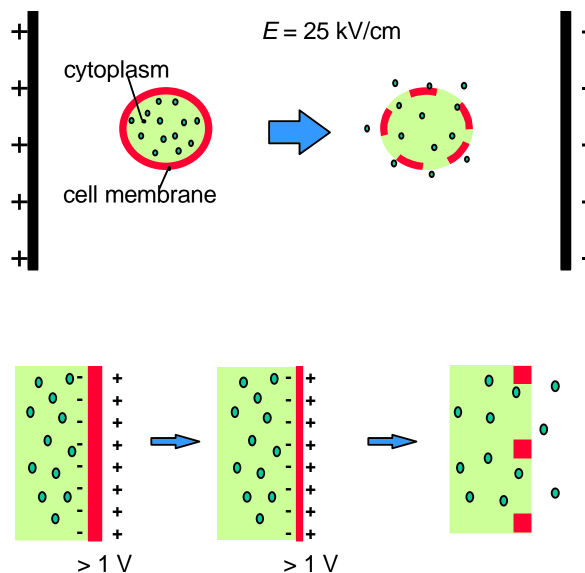

Figure 6: Mechanism of inactivation of bacteria cells with PEF (hypothesis)

In literature a number of bacteria and yeasts have been subjected to PEF. Varied are the field strength (5-70 kV/cm), the pulse length (2-40μs) and the number of pulses (2-50). The largest effect is obtained with *E. coli* (9 log reductions). The general feeling of the authors is that spores cannot be inactivated by PEF alone.

However, the hardly reproducible results in literature indicate that the design of the PEF treatment chamber is still not optimal:

- non homogeneous field,
- relatively high energy input (> 500 kJ/L),
- many batch systems.

NIZO prototype of treatment chamber

In co-operation with the University of Technology Eindhoven, NIZO has developed a new continuous treatment chamber for PEF applied to liquid foods. Characteristics:

- Low-energy consumption (<30% of conventional equipment)
- Homogeneous electric field
- Flexible (pulse length 0.05-5μs)
- High capacity is possible (> 1000 L/h)

Preliminary results

The first experimental results with the new treatment chamber are promising. At a temperature of 32 °C both *Pseudomonas fluorescens* and *B. cereus* spores were inactivated, resp. >3 and 1 log reduction (see figure 2). The energy-input was less than 200 kJ/L.

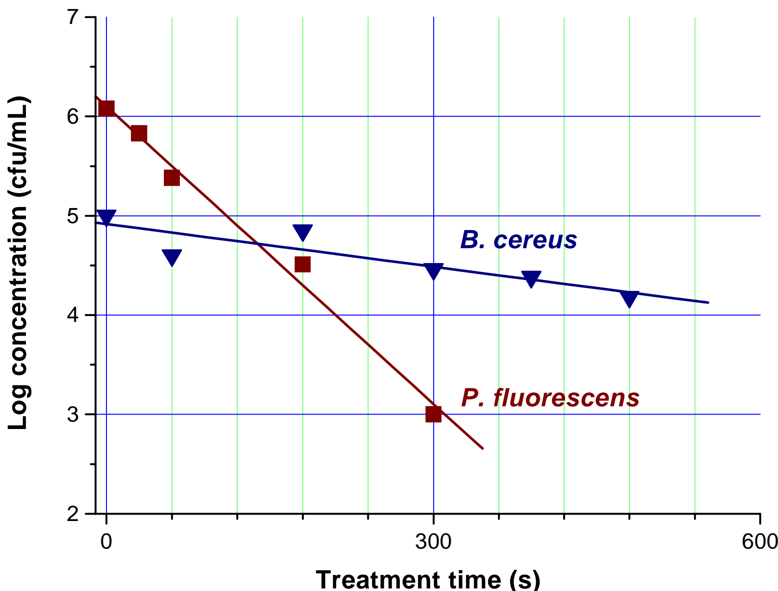

Figure 7: Inactivation of bacteria and spores with the new treatment chamber

Discussion and conclusions

In principal with PEF it is possible to produce 'fresh' liquid foods with a high quality. Examples of applications:

- ULT milk (ultra low temperature)
- fruit juices
- whey protein concentrates
- fermented drinks, etc.

Other advantages may be reduced deposition of bacteria, proteins and minerals in equipment and decrease of growth rate of bacteria.

The results published in this paper are the basis for the further investigations. The research will be focused on (further) inactivation of bacteria and spores. For the future NIZO will optimise the prototype treatment chamber and develop an aseptic system for application research in the food industry.

In conclusion, with the new PEF treatment chamber of NIZO food research and the University of Technology Eindhoven bacteria and spores are inactivated at temperatures below 40 °C. The energy-input is 30% of values reported in literature and patents with comparable inactivation levels of bacteria.

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Use of computational models to study inhomogenities in electrical field processing of foods

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Summary

Ohmic heating uses the inherent electrical resistance of a material to generate heat when a current flows through it. It can be used effectively to sterilise solid-liquid food mixtures. To produce safe, high quality, food, it is important to identify any inhomogenities that may occur.

Both modelling and experimental work have been carried out to study and explain the effects due to local electrical conductivity change. A solid of significantly low electrical conductivity can cause a cold shadow within the liquid. As a result, local changes in heating rate occur. These have been successfully modelled for an (a) electrically insulating and (b) electrically conducting solid caught in this shadow.

Introduction

Direct electrical ('ohmic') heating is an effective method for the rapid heating of solid-liquid mixtures. For foods, this makes it possible to speed up the heating of mixtures, which can give a higher quality product than conventional heating. With ohmic heating, the levels of electrical conductivity of a solid and surrounding fluid are critical in determining the heating rate[1]. The presence of a single inclusion of significantly different electrical conductivity will have an effect[2]: high conductivity (such as metal) will 'suck in' the local electric field and low conductivity inclusions (such as wood or bone) will divert it. In both cases, regions of low field strength will result which will tend to heat less rapidly - a *cold shadow region*. This produces problems for a process designer. No work has been done previously to study how particle heating is affected by the shadow region, and to see what variation in physical properties is acceptable.

Objective

The aim of work here is to see over what scale thermal problems are caused: we have combined a computational and an experimental approach, to study the extent to which these shadows might cause processing problems in a real industrial situation. We also wish to identify the extent to which computational models can be used to study foods.

Experimental study

Apparatus

A static electric heating cell was used to provide a simple geometry for the experiments. The cell comprises a 184x80x77 mm polypropylene box with 75 mm square plate electrodes at each end, a voltage regulator, and thermocouples, plus instrumentation to measure temperatures, voltage and current.

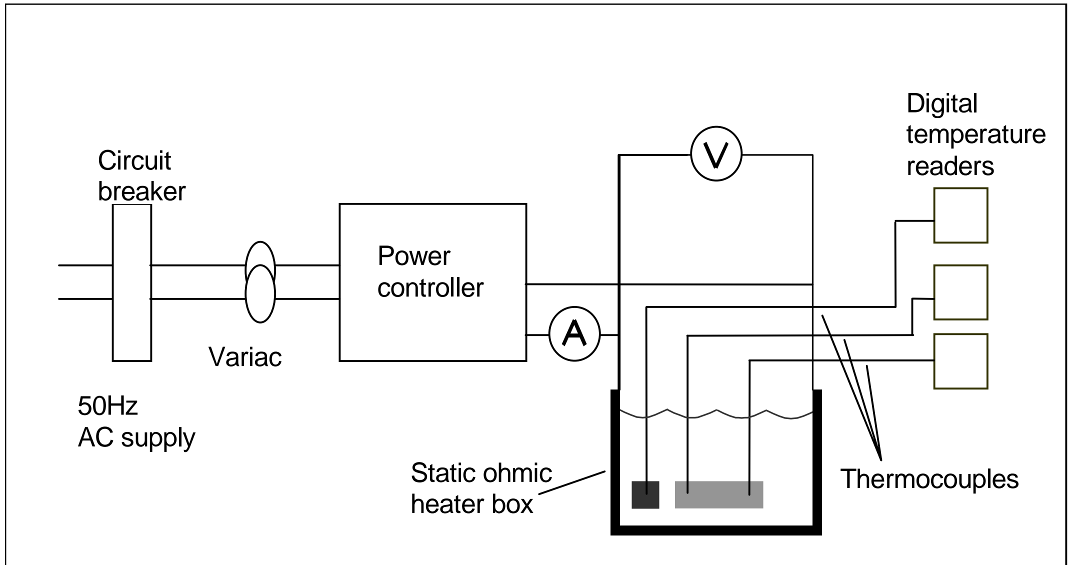

Figure 1: Schematic diagram of the static electric heating apparatus[1] (modified).

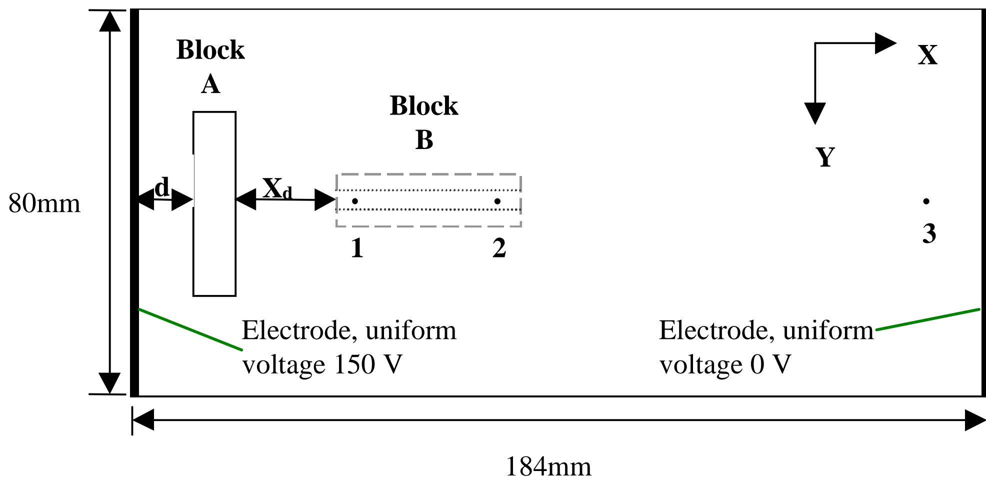

Figure 2: Plan view of the experimental system in the static electric heater cell. The walls of the cell are thermally and electrically insulated.

Materials in the heating cell and experimental method

In the experimental system shown in Figure 2, block A, of area 10x50 mm, was made of perspex - an electrically insulating material. This block casts a 'shadow' in the electric field, which will affect local heating rates. Experiments were carried out where block B was:

(a) an electrical insulator (dotted lines in Figure 2) of area 3x50 mm, made from perspex. This thinner block was such that thermal conduction occurred over the timescale of the experiment.

(b) a model food particle (dashed lines in Figure 2) of area 10x50 mm, made from egg albumen, with electrical conductivity twice that of the surrounding solution.

The system is essentially two dimensional: solids were 35 mm high, and carrier solutions surrounding the solid blocks were of depth 35 mm. Solutions were solidified agar gel in case (a), and salt water in case (b). The blocks were aligned along the vertical centre line of the vessel. Distance **d** was fixed at 10 mm, and distance $\mathbf{x_d}$ between the blocks was varied between 0 mm and 50 mm. A voltage of 150 V was used, and temperature readings were recorded at points **1**, **2**, and **3**.

Computational study

Two-dimensional simulations were conducted, corresponding to the geometries and conditions of the ohmic heating experiments illustrated in Figure 2. The required material properties are given the Table 1. Perspex properties were taken from Kaye & Laby[3], electrical conductivities of salt water, agar gel, and egg albumen were determined experimentally, and all other properties were approximated by those of water, the main constituent. The modelling was carried out on a Silicon Graphics Indigo workstation using FIDAP (Fluid Dynamics Analysis Package), a commercial Computational Fluid Dynamics software package, which uses the Finite Element Method and solves energy and potential field equations.

Table 1: Material properties for simulations

Material Property	Agar	Perspex	Water	Albumen
Density ρ (kg m^{-3})	1000	1170	1000	1000
Specific Heat c_p (J kg^{-1} K^{-1})	4810	1500	4810	4180
Thermal Conductivity λ (W m^{-1} K^{-1})	0.6	0.17	4*	0.6
Electrical Conductivity σ (S m^{-1})	0.0412T(°C) + 0.9479	10^{-12}	0.0412T(°C) + 0.9479	0.0952T(°C) + 2.1595

Enhanced thermal conductivity, λ, used for water, to take account of convection effects.

Governing equations of the computational model

Thermal field: $$\frac{\partial}{\partial t}\left(\rho c_p T\right) = \nabla\left(\lambda \nabla T\right) + Q \quad (1)$$

Heat source: $$Q = \sigma \left|\nabla V\right|^2 \quad (2)$$

Electric field: $$\nabla.\left(\sigma \nabla V\right) = 0 \quad (3)$$

where the electrical conductivity is linear in temperature, $\sigma = \sigma_0 + mT$ with σ_0 and m depending on the properties of the material.

c_p	Specific heat [J kg^{-1} K^{-1}]	λ	Thermal conductivity [W m^{-1} K^{-1}]
m	Electric conductivity coefficient [S m^{-1} K^{-1}]	ρ	Density [kg m^{-3}]
Q	Volumetric heating rate [W m^{-3}]	σ	Electric conductivity [S m^{-1}]
T	Temperature [°C]	σ_0	Reference value for σ, [S m^{-1}]
V	Electric potential [V]		

Motivation for the static model

When there is little convective heating, then the temperature differences between different regions of a food mixture will be more pronounced. With no fluid motion, the model is much simpler: we do not solve the Navier-Stokes equation, and there is no convective term, $\rho c_p v.\nabla T$, in eqn (1). However, we can compensate to some extent for convection effects by defining an enhanced thermal conductivity[2,4], λ^*, in eqn (1).

Results and discussion

Shape of the shadow region in systems of high viscosity

Figure 3 shows the heating rate at point **1** within the perspex block B for various $\mathbf{x}_d$. This is compared with the heating rate at point **2**, when $\mathbf{x}_d$ = 50 mm; i.e. where block B is affected minimally by the thermal shadow region. The carrier fluid was agar gel, to minimise convective mixing. The point at which uniformity in the heating rate in block B is reached can be considered to be the boundary of the thermal shadow region. Even when $\mathbf{x}_d$ = 50 mm, the heating rate at point **1** is lower than at point **2**. For all cases, model and experiment agree that the region extends a distance greater than 50 mm.

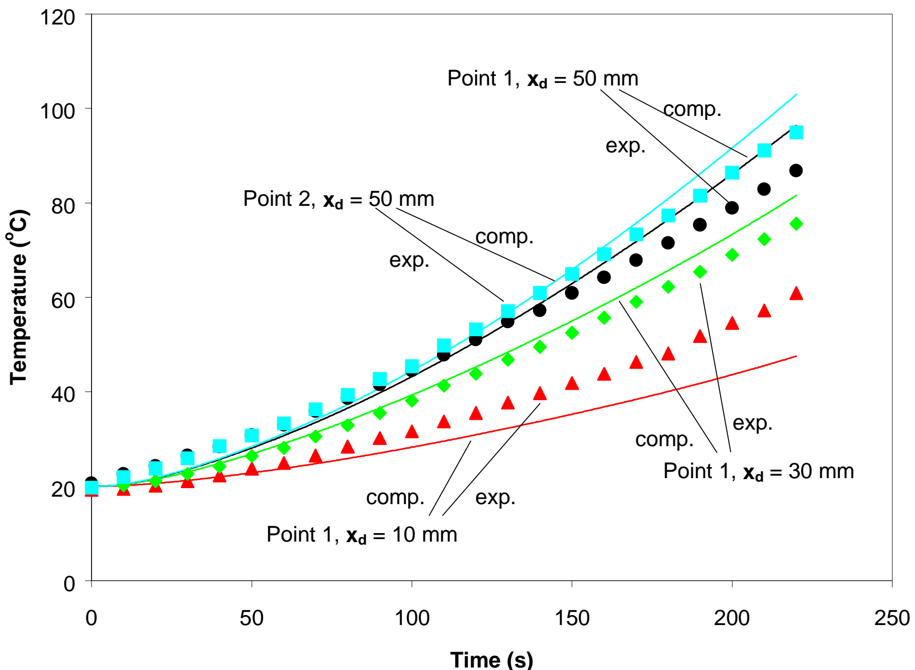

Figure 3: Heating rate at point **1** within block B (perspex) for distances $\mathbf{x}_d$ = 10 mm, 30 mm, 50 mm. Points are experimental data, lines are simulation results.

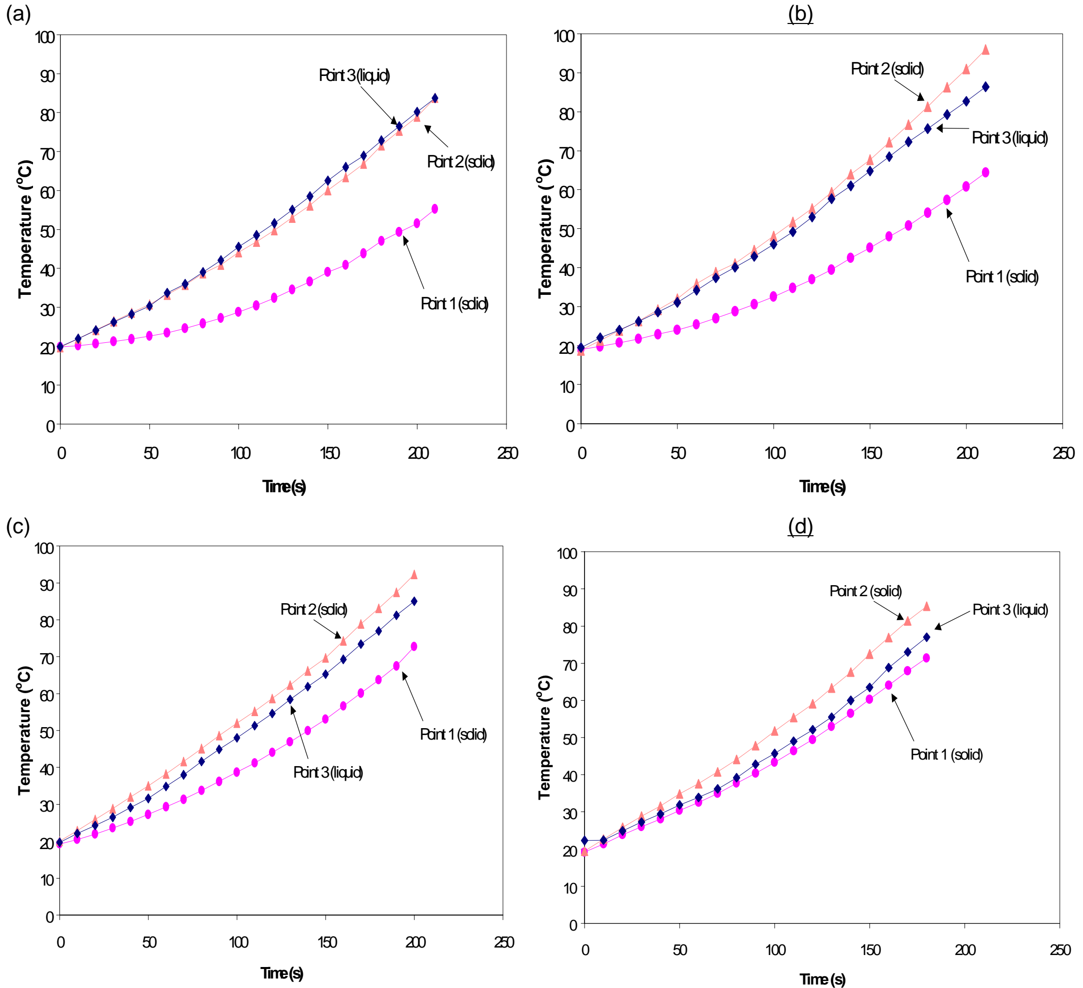

Figure 4: Effect of distance $\mathbf{x_d}$ between blocks A and B on the heating rate at three points (numbers as in Figure 2), where block B is egg albumen. Distances tested are: (a) 0 mm, (b) 5 mm, (c) 10 mm, and (d) 20 mm.

Effect on electrically conducting food solids

When block B is a 10x50 mm egg albumen particle, its internal temperature results primarily from electrical resistance heating; thermal conduction is slow. In isolation, egg albumen will overheat the liquid. However, when block B is in the thermal shadow, then significant temperature differences arise both between solid and liquid and within the solid itself.

Figure 4 plots the temperatures at points **1** and **2**, and that of the liquid - point **3** - well away from the shadow region. As $\mathbf{x_d}$ is increased from 0 to 20 mm, one half of the block heats *faster* and the other half heats *slower* than the liquid - seen by comparing the experimental data for points **1** and **2** with that for point **3** in Figure 4 (b) - (d). The point at which uniformity in the heating rate is reached can be considered the boundary of the thermal shadow region. Since point **2** is 5 mm from the right end of block B, and its heating rate increases with $\mathbf{x_d}$, these results indicate that the thermal shadow region extends a distance greater than 65mm: the effects on inhomogenity can be widespread.

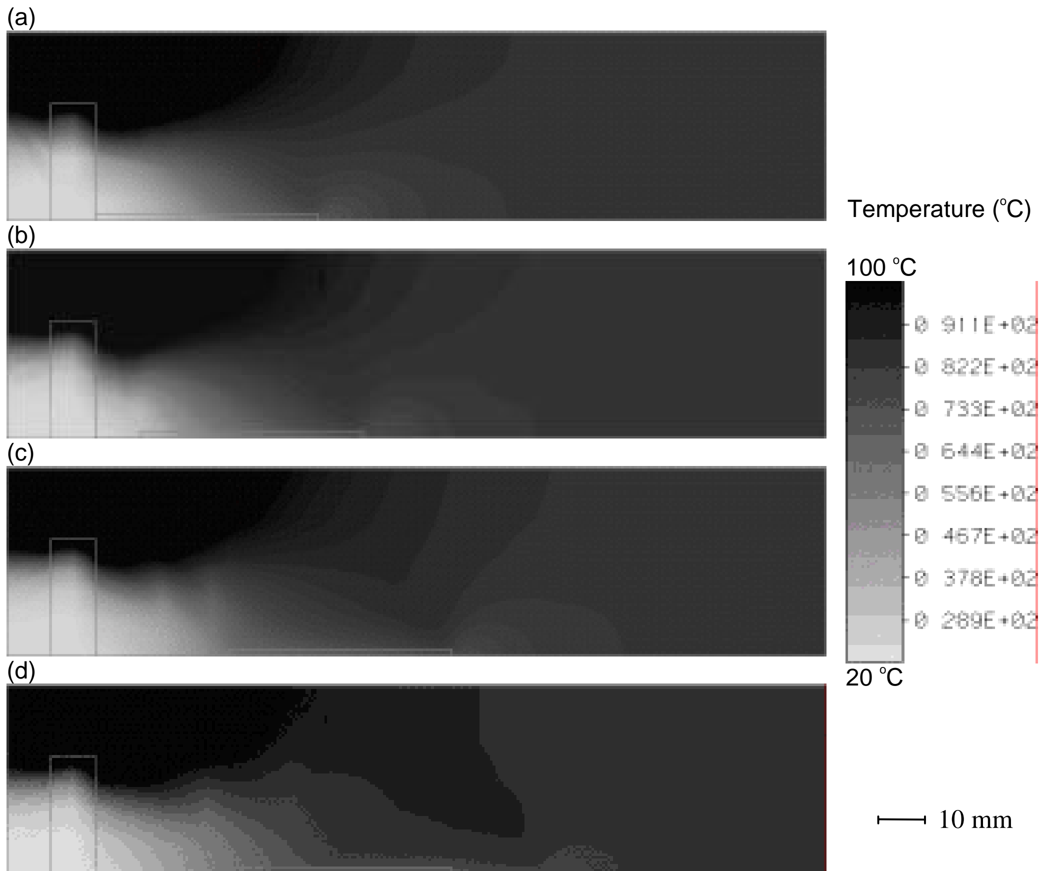

Figure 5: Modelled temperature distribution after 170 seconds of electrical heating, when block B is perspex (upper half of cell shown only). Distances between blocks are (a) 0 mm, (b) 10 mm, (c) 30 mm, (d) 50 mm.

Computational results: Shape of the shadow region

FIDAP has been used to show the thermal field across the experimental domain. Temperatures after 170 seconds are given in Figures 5 and 6 for different block positions. The effect of the perspex block A is to divert the current around it, creating hot regions in the liquid along its parallel edges to the current flow, and cold regions within the perspex itself and along its perpendicular edges to the current flow.

Figure 5 simulates the conditions of Figure 3, and shows that the position of the perspex block B does not significantly change the extent of the low temperature region. Thermal uniformity is established approximately 75mm away.

For the electrically heated (egg albumen) block, temperature distributions are more complicated, as indicated by the experimental results of Figure 4. The region in the albumen which overheats is seen clearly in Figure 6 (c and d).

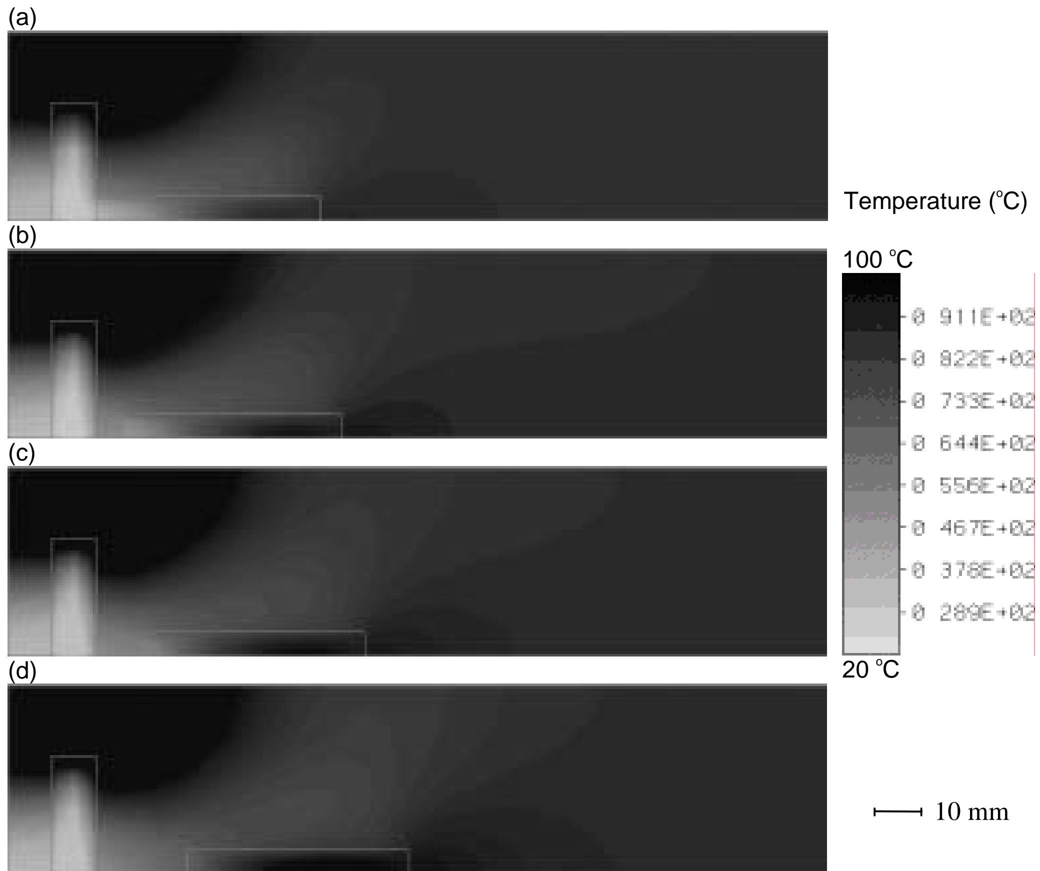

Figure 6: Modelled temperature distribution after 170 seconds of electrical heating, when block B is egg albumen (only upper half of cell shown). Distances between blocks are (a) 0 mm, (b) 0.5 mm, (c) 10 mm, (d) 20 mm.

The model also predicts the situation where the albumen both underheats and overheats, seen most clearly in Figure 6 (b and c). A "hot shadow" cast by the albumen is also seen: high current flow through the albumen leads to a region of high temperature immediately behind it. These distributions are complex, but show that computational models reproduce the effects seen in practice.

Conclusions and current work

Homogeneity in the heating rate is crucial to ensure uniformity of local heating rates under electrical heating of solid-liquid mixtures. The cold shadow effect of an electrical insulator on the thermal field was shown by using perspex. When a second particle is sufficiently electrically conductive to undergo resistive heating, it is possible that portions of that particle can overheat and underheat with respect to the surrounding liquid - a result of the difference in local electric field strengths.

These results show that centimetre-scale inclusions can create inhomogenities which cannot be removed by thermal conduction over the timescales typical of ohmic processes; care must be taken with the preparation of formulations for this type of process.

Work is underway to study the relationship between heating uniformity and the distribution of physical properties, and to identify how FIDAP can be used to model electrical heating.

Acknowledgements

This work has been supported by BBSRC and Unilever.

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Effect of low-dose gamma irradiation on *Listeria monocytogenes* inoculated onto pre-cut packaged white cabbage and radish

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Summary

Shredded white cabbage and sliced radish were inoculated with *Listeria monocytogenes,* then packaged aerobically in low-density polyethylene pouches. The effect of low-dose (1 kGy gamma rays) irradiation on the viable cell counts of the test organism and the naturally contaminating bacterial flora was followed as compared to the unirradiated samples at storage temperatures of 5, 10 and 15 °C. Irradiation reduced drastically the viable load of spoilage bacteria, improving thereby the microbiological shelf-life and extending the sensorial keeping quality. In case of irradiated samples this later was not limited by bacterial growth but by desiccation and discolouration and/or physiological deterioration. Losses of the vitamin-C content as an effect of irradiation was not higher than those occurred in the unirradiated samples during their shelf-life. Irradiation reduced the viable count of *Listeria monocytogenes* by several log cycles, and the surviving population showed to be more susceptible to low temperature than its unirradiated population. Considering its regularly found low contamination level on fresh produce, this low-dose treatment may, under conditions of good manufacturing practices, practically eliminate this most enduring environmental contaminant non-sporeforming pathogen from prepared vegetables.

Introduction

There is an increasing consumer trend for fresh-like, less severely processed ready-to-eat foods with reduced levels of chemical preservatives. The catering industry is also strongly interested in such minimally processed non-frozen meals and meal components. Among these foods are the minimally processed chilled vegetables usually carrying Pseudomonads, Enterobacteria and lactic acid bacteria as natural microflora. The high moisture and numerous cut surfaces provide excellent conditions for the growth of microorganisms.

Modified atmosphere packaging (MAP) by producing elevated concentrations of CO_2 or N_2 can suppress the growth of the spoilage bacteria, especially *Pseudomonas* species. However, these extended shelf-life chilled produce, potentially, involve a microbiological hazard due to the growth of psychrotrophic pathogenic bacteria, particularly, because there is always a risk of temperature abuse (BEUCHAT, 1995). Food poisoning outbreaks related to fresh produce have been increasingly reported in the last two decades (SCHECH *et al.,*1994; ARCHER, 1996).

Considering that sanitation with chemical means such as chlorine treatment is not very reliable whereas modified atmosphere packaging, although suppressing the aerobic spoilage flora, may even favour the growth of microaerophilic, or anaerobic pathogens, there is a need for a procedure that can reduce the incidence of pathogenic microorganisms associated with fresh produce, without changing their minimally processed character. Ionising radiation offers a physical means for pasteurisation without changing the fresh state of these commodities. Thus, the aim of our studies was to investigate the effect of low-dose irradiation on selected vegetables inoculated with *L.*

monocytogenes which has the highest radiation resistance and environmental stress tolerance among the non-sporeforming pathogens mentioned above (MONK *et al.,*1995). Our results with gamma irradiated pre-cut bell pepper and carrot cubes stored in polyethylene pouches at various temperatures within the range of 1 to 16 ^{o}C have been already reported elsewhere (FARKAS *et al.,* 1997). In the present paper, further studies on the effect of gamma irradiation on the fate of the native bacterial flora and a high inoculum level of *L. monocytogenes* on shredded white cabbage and sliced radish are reported. On the basis of preliminary sensory panels, a dose of 1 kGy has been selected again for radiation treatment, which did not change the sensory properties of the test materials.

Materials and methods

White summer-cabbage and white autumn-radish were purchased from the local market. Outer leaves of cabbage were discharged, and inner leaves were mechanically shredded into narrow strips, the radishes were diced into approx. 5 mm thick slices.

The *Listeria monocytogenes* strain 4ab No.10 was the same test organism as used in our former studies with bell pepper and carrots (FARKAS *et al.,* 1997). The stock culture of this strain was activated by culturing in brain heart infusion broth at 30 °C for 18 h and this culture was diluted with tap water to yield a suspension of approx. 10^5 CFU/ml. Two batches of six kg each of pre-cut cabbage or radish were mixed in 20 litre volumes of suspensions of *L. monocytogenes* for one minute, allowing an initial contamination level of 10^4 - 10^5 CFU/g, then thoroughly drained and approx. 100 g portions of pre-cut inoculated vegetables were placed in low-density polyethylene pouches and sealed under air atmosphere. The oxygen transmission rate at 25 °C of this type of 25 µm thick foil is approx. 7800 cm^3m^{-2} per 24 h, the nitrogen transmission rate approx. 2800 cm^3m^{-2} per 24 h, the CO_2 transmission rate approx. 42000 cm^3m^{-2} per 24 h while the water vapour transmission rate at 38 °C is 18 $g.m^{-2}$ per 24 h at 90 % R.H.

Half numbers of inoculated samples were irradiated at a dose rate of 0.5 kGy/h at room temperature by a panoramic Co-60 irradiator. Untreated and irradiated batches were stored at 5, 10 and 15 °C, respectively, for 10 days. Directly after irradiation and periodically during the storage, duplicate samples from both untreated and irradiated batches were analysed at each sampling time.

Besides total aerobic plate counts (APC), selective estimations of *L. monocytogenes,* counts of presumptive lactic acid bacteria, the Enterobacteriaceae counts as well as HPLC-analyses of the ascorbic acid and dehydro-ascorbic acid content of the vegetable samples were performed as described in our previous paper (FARKAS *et al.,* 1997). pH values were determined by mixing a portion of the vegetables with approximately equal portion of distilled water and by measuring with an electrical pH-meter.

Changes in the purchase acceptability of the prepackaged vegetable samples were followed subjectively as a function of radiation treatment and storage on the basis of scores on appearance, discolouration and softness by touching, ranging from score (5) as excellent to score (1) as non-marketable. The time period elapsed until the scores declined to the level of (2) ("still acceptable") was considered as a limit of the sensorial keeping quality.

Results and discussion

The APC and the viable cell count of *L. monocytogenes*on on shredded cabbage are shown in **Figure 1** as a function of the radiation treatment, storage temperature and time. The sensorial shelf-life values are also marked on the graphs. The radiation treatment reduced the total APC by almost three log-cycles while the viable cell count of *L. monocytogenes* was diminished more then 4 log-cycles by the radiation treatment. *Listeria* grew readily on the heavily inoculated unirradiated shredded cabbage even at 5 °C. At the end of the respective sensorial shelf-life periods the APC of untreated samples were around 10^8 CFU/g while the APC of irradiated samples were only around 10^5 CFU/g. In irradiated samples, no recovery of *Listeria* was observed at 5 °C. At 10 °C, growth of *Listeria* started only when the samples were already of unacceptable sensorial quality. However, at 15 °C recovery of the surviving *Listeria* was noted within two days in the irradiated samples and at least four log-cycle growth occurred until the end of the shelf-life.

The fate of Enterobacteriacea is shown in **Fig. 2**. As compared to the total aerobic plate counts, Enterobacteriaceae were not the dominant spoilage bacteria on the aerobically packaged shredded cabbage. The limiting factor of the sensorial shelf-life was mainly a dark discolouration. Its intensity was strongly enhanced by the increased storage temperature. The Enterobacteriaceae were radiation sensitive. While they grew quite readily even at the lowest storage temperature on the untreated samples, no recovery of Enterobacteriaceae were noted in the irradiated batch during the entire storage temperature period at 5 °C.

Lactic acid bacteria (not shown here) formed only a small fraction of the native microflora of shredded cabbage. They were less sensitive to radiation treatment than the main components of the native microflora because they recovered at each storage temperature, although they grew slowly and could not compete well with other components of the spoilage flora.

The fate of microflora on untreated and irradiated radish samples is illustrated by **Fig. 3** and **Fig. 4** in the same way as above. Reduction of viable cell counts as an effect of irradiation was similar to what has been observed with shredded cabbage. The results of the microbiological analyses during storage showed also similar pattern. However, recovery of *Listeria* was slightly better in the irradiated radish samples than in irradiated cabbage samples. However, their counts fluctuated more among the irradiated samples than on the untreated ones. In general, *Listeria monocytogenes* growth was less pronounced in the unirradiated sliced radish than on the shredded cabbage and at 5 °C the initial *Listeria* population remained practically unchanged during storage in untreated radish samples. Enterobacteriaceae and lactic acid bacteria played even less role in the spoilage of pre-cut radish than in case of shredded cabbage.

The initial pH was pH 5.54 an 5.81 for untreated and irradiated shredded cabbage, respectively. pH-increases of 0.4 - 0.7 were noted until the end of their respective shelf-life while slight decreases of 0.04 - 0.3 pH values were observed in the irradiated samples at various storage temperatures. In sliced white radish the initial pH of untreated and irradiated samples were pH 6.29 and 6.14, respectively. Here some pH-decrease (0.06 at 5 °C, 0.2 at 10 °C, and 0.4 at 15 °C) was measured in the untreated samples during their shelf-life, whereas insignificant (0.01 - 0.16) pH-decreases were found in the irradiated samples.

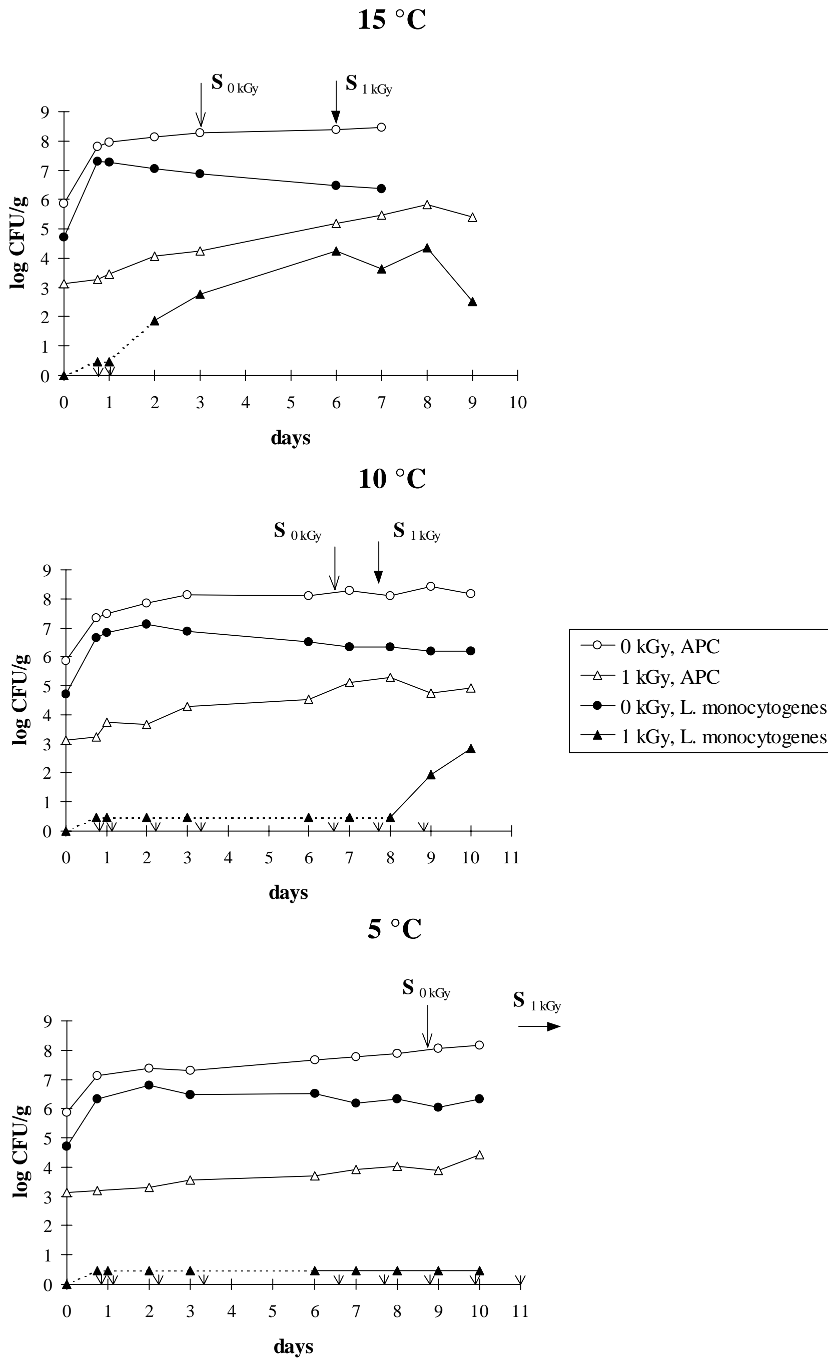

Figure 1: Comparison of the total aerobic plate counts (APC) and the viable counts of *Listeria monocytogenes* on untreated and 1 kGy-irradiated shredded packaged cabbage at various storage temperatures. Sensorial shelf-life periods (S) are indicated with arrows over the growth curves.

The ascorbic acid and dehydroascorbic acid contents of both vegetables were determined at the beginning and at the end of their shelf-life. The vitamin-C content decreased as an effect of the 1 kGy dose by maximum 13 % in shredded cabbage, and by about 30 % in the sliced radish. Until the end of the sensorial shelf-life, no significant further decrease of the vitamin-C content was observed neither in the irradiated cabbage, nor in the irradiated radish samples. Vitamin C content of untreated radish samples diminished by 13 - 30 % during their respective shelf-life at various storage temperatures (FARKAS, 1998).

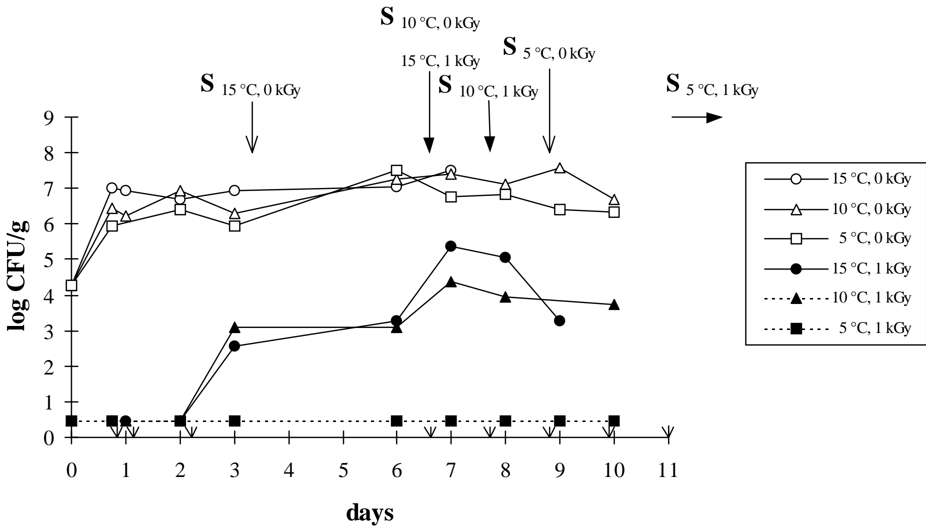

Figure 2: Effects of the storage temperature and time on the growth of Enterobacteriaceae counts of unirradiated and 1 kGy-irradiated shredded cabbage. Sensorial shelf-life periods (S) are indicated with arrows over the growth curves.

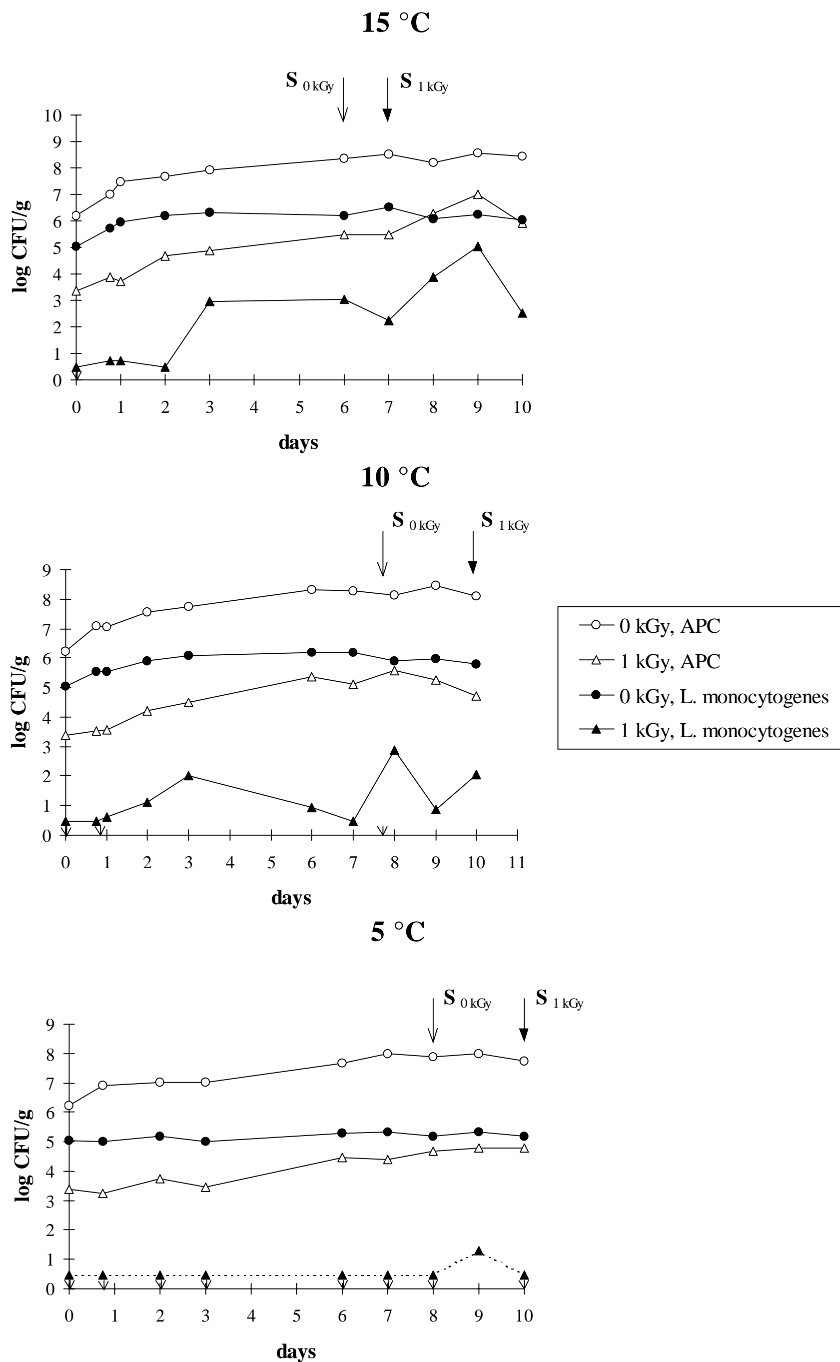

Figure 3: Comparison of the total aerobic plate counts (APC) and the viable cell counts of *Listeria monocytogenes* on sliced packaged radish. Shelf-life periods (S) are indicated with arrows over the growth curves

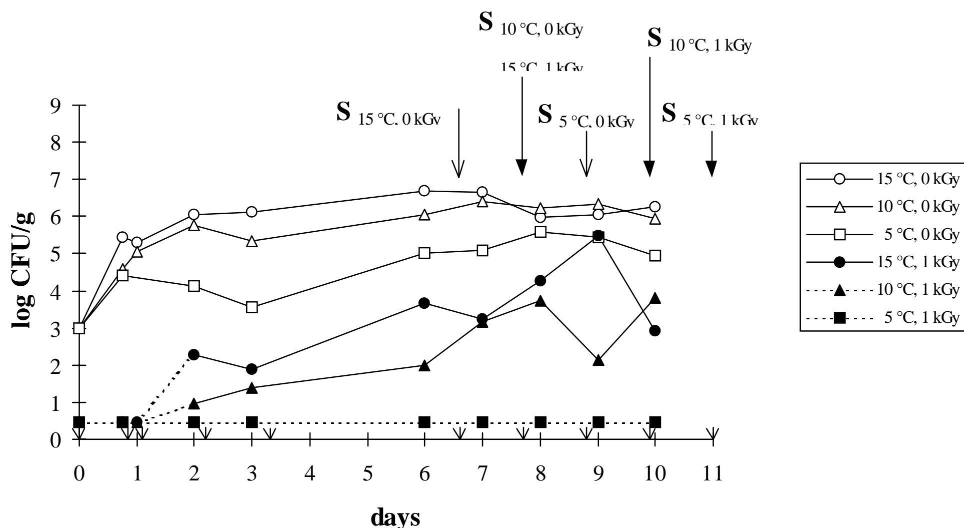

Figure 4: Effects of storage temperature and time on the Enterobacteriaceae counts of unirradiated and 1 kGy-irradiated sliced packaged radish. Shelf-life periods (S) are indicated with arrows over the growth curves

Conclusions

These results together with those described in our previous reports (FARKAS *et al.,* 1997; FARKAS, 1998) show that low-dose irradiation is able to improve the microbiological safety and stability of specific pre-cut, pre-packaged vegetables. The studies also underline the importance of good temperature control in the chill chain. The radiation treatment diminished remarkably the population of *L. monocytogenes* inoculated onto the prepared vegetables. In agreement with our previous model studies (FARKAS *et al.,*1995) and due to its radiation damage the surviving population of the *Listeria* inoculum in the irradiated samples seems to be also more susceptible to low temperature than its unirradiated population. Losses of the vitamin-C content as a direct effect of low dose irradiation seem to be not higher than those which occur in the unirradiated samples during their shelf-life. One may assume that low-dose irradiation would be effective also at inactivating some common parasites which may gain access to press produce before harvest (LOAHARANU & MURREL, 1994). However, package conditions of minimally processed, low-dose irradiated vegetables must be aerobic enough to prevent growth of non-proteolytic *Clostridium botulinum* whose spores are not destroyed by low-dose irradiation.

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Review of decontamination techniques

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Summary

Growing incidences of food poisoning outbreaks throughout Europe and increasing demand for 'natural' minimally processed foods has focused attention on food decontamination.

A perfect decontamination system for raw materials or processed products should completely eliminate food pathogens whilst leaving the organoleptic properties of the food in an unchanged state. Much laboratory research has been carried out on a range of decontamination methods which normally rely on either physical removal of bacteria, or thermal or chemical destruction of bacteria. However, the potential of a method is often not realised in the practical situation due to insufficient thought on the method of application within an industrial environment.

The application of organic acids offers the best potential of the chemical treatments. Reductions of up to 3.5 log units in bacterial numbers have been achieved in laboratory trials. Organic acids have the advantage of being a natural component of many foods and are not thought of as a chemical additive by many consumers.

Thermal destruction using condensing steam is the most promising of the physical methods. Reductions of up to 6 log units have been reported in investigations without irreversible changes to the surface of foods. Steam based beef carcass decontamination units are in industrial use in the USA.

Introduction

There is no terminal step (such as cooking) to eliminate pathogenic organisms from many raw products such as fish and red and white meat until it reaches the consumer. In the case of salad vegetables and most fruit, such a step is not even performed by the consumer. Several of the pathogens present on these types of food are psychrotrophic and can grow at refrigeration temperatures. Centralised processing and preparation of these products are growing, increasing the distance and time between initial preparation and the consumer, thus increasing the risk of growth of pathogens. Ideally some form of terminal step should be introduced, failing that, any step which reduces the microbial load would be advantageous to public health and of economic significance to the industry. That is provided such a step did not change the intrinsic nature of the food, i.e. the 'raw' produce or meat must remain 'raw'.

Many decontamination techniques have been suggested and studied over the last thirty years. Many of these have only been attempted on a laboratory scale. The purpose of this paper is to discuss some of the practicalities of decontaminating raw food products on a commercial scale.

The difference between decontamination methods and treatments

There is rarely any distinction made in the literature between 'decontamination methods' i.e. the whole decontamination system and 'decontamination treatments'. This often clouds the practical

issues of decontamination. There is often too much emphasis placed on the treatment rather than the method of application.

Decontamination is not a matter of simply dipping or spraying the product with chemicals or water, or giving it a quick flash of light. For example, many factors affect the efficiency of aqueous spray systems. In automated spray cabinets the position and number of the sprays, the shape of the spray, and spray pressures, all have a significant effect on the treatment irrespective of the nature of the substance being pumped through the sprays. Many studies have shown that the method of decontamination is often more important than the treatment.

Most abattoirs have relied in the past on manual sprays to wash red meat carcasses, thus automated spray cabinets have been a natural development. Some studies, however, have shown that a deluge method of application where the carcass is passed under a waterfall offers a more effective method of coverage (1).

Fruits and vegetables are usually washed mechanically after harvest to remove physical contaminants such as soil and leaves. The increased awareness of microbial contaminates has led to the addition of chemical sanitisers to this washing. In many cases these are ‘drop-in’ additions to the washing process rather than an integral part of the washing system. There is often little significant difference between different chemicals when compared using the same method of application. Zhang and Farber (2) evaluated a wide range of chemicals, including chlorine and organic acids, against *L. monocytogenes* on fresh-cut vegetables and concluded that one could only ‘expect a 1 log reduction....regardless of the disinfectant used’.

Heat treatments, with or without chemicals, are very reliant on the method of application. To prevent cooking the product, such treatments have to provide a uniform heating of all surfaces for a short period. This is not particularly difficult to achieve on a laboratory scale, spraying or dipping small samples using hot water for example. Similarly, laboratory studies using steam have shown that if very high temperatures are applied for very short times, followed by cooling the surface rapidly, high bacterial reductions can be achieved on meat without effecting the surface appearance. However, successfully applying such techniques to carcasses in an abattoir, for instance, presents many engineering challenges.

The problems of decontaminating raw meat and produce

Fruits, vegetables and animal carcasses are not ideal shapes to decontaminate. Most decontamination treatments rely on physical contact and uniform coverage of the product’s surface. This is difficult, as the surface of many produce and whole animal carcasses are very irregular. For example the outer surface of a poultry carcass has many crevices and folds. These areas are very difficult to treat and provide protection to attached bacteria. Stem scars and growth cracks of tomatoes have been shown to provide similar protection (3). They slow down the penetration of aqueous and gas treatments and cause shadowing problems for radiation treatments such as ultra-violet (UV) light. As well as protecting bacteria, these areas often clog up with physical contamination, such as dirt and hair, and do not drain well. Pools of water or chemical solutions lying in these areas can have detrimental effects on the product’s appearance and cause difficulties in controlling residence time.

Some produce, such as strawberries and raspberries, are not amenable to the physical stresses associated with standard washing treatments and therefore must be decontaminated using other treatments, such as UV radiation or gas-based disinfectants.

There is much evidence that the time at which products are treated greatly affects the efficacy of decontamination processes. The longer bacteria reside on product surfaces, the more difficult removal becomes, because of the ability of bacteria to attach to tissue. Bacteria differ in their ability to attach to different surfaces and the time they require to become fully attached.

The formation of bio-films may increase the resistance of bacteria to disinfectants such as chlorine (4). Surfactants such as 'Tween 80' have been used to increase surface wetting, in theory allowing the disinfectant to 'get at' the bacteria. 'Tween 80' is not used for food production because it causes unacceptable organoleptic changes. Two surfactants, 'Orenco Peel 40' and 'Tergitol', are used for fruits and vegetables in the USA (2). However, Zhang and Farber (2) found that using either of these surfactants in combination with chlorine not only did not improve the efficacy of chlorine but were antagonistic and lessened the antimicrobial effect of chlorine. Surfactants may also accelerate subsequent spoilage by the removal of antimicrobial barriers (5).

These problems are by no means un-resolvable, but mean that the methods of handling and applying decontamination treatments have to be well thought out.

Methods of decontamination

Washing techniques using water in combination with heat and/or chemicals have been the favoured methods of removing bacteria from both raw produce and animal carcasses.

Washing

Washing meat or produce with water can effectively remove physical contaminates such as soil, hairs and other debris, however, its affect on bacterial numbers is marginal. The temperature at the surface and the method of applying the water are the two most important factors in bacterial removal.

Washing with water alone usually obtains a reduction of 1 to 2 log units on both (6) and meat (7). Increasing the temperature of the water increases the reduction. However, a spray jet rapidly loses heat, primarily by evaporation. Studies have shown that the maximum impact temperature on the carcass of a spray placed 300 mm away and supplied with water at 90°C is approximately 63°C (7). Abattoirs have always been worried about the effect of hot water on the appearance on carcasses. However, studies have shown that treatments of 80°C for 10 s not only significantly reduce bacterial levels but do so without any permanent damage to the surface tissue (8).

Automated washing systems for meat carcasses have long been seen to be the way forward. The most comprehensive studies to date have been on the CAPER (Carcass Acquired Pathogen Elimination Reduction) system developed in the USA (9) and the Australian 'Deluge' system (1). The Australian system (Figure 8) solely depends on the action of hot water to decontaminate. While the CAPER system has been designed as a two stage process involving a water stage to remove physical contamination and organic acids to sanitise the carcass. However, both systems remain to be implemented on a commercial scale.

Chemical treatments

Many studies have been carried out to test groups of chemicals for antimicrobial activity against specific pathogenic and food spoilage organisms. A wide range of chemicals are known which will destroy or severely limit the growth of pathogenic and spoilage bacteria. However, the number of chemicals that are likely to be approved for use on meat is severely limited, not least because of legal restrictions. Chlorine has been an accepted part of washing fruits and vegetables for many years. Chemical washing of meat has not generally been accepted but chlorine and more recently trisodium phosphate (TSP) have been used for poultry. There is also growing interest in the use of ozone (10, 11, 12) and naturally occurring antimicrobials (13, 14) for both meat and produce.

The effectiveness of most chemical treatments depends on concentration, application temperature and exposure time.

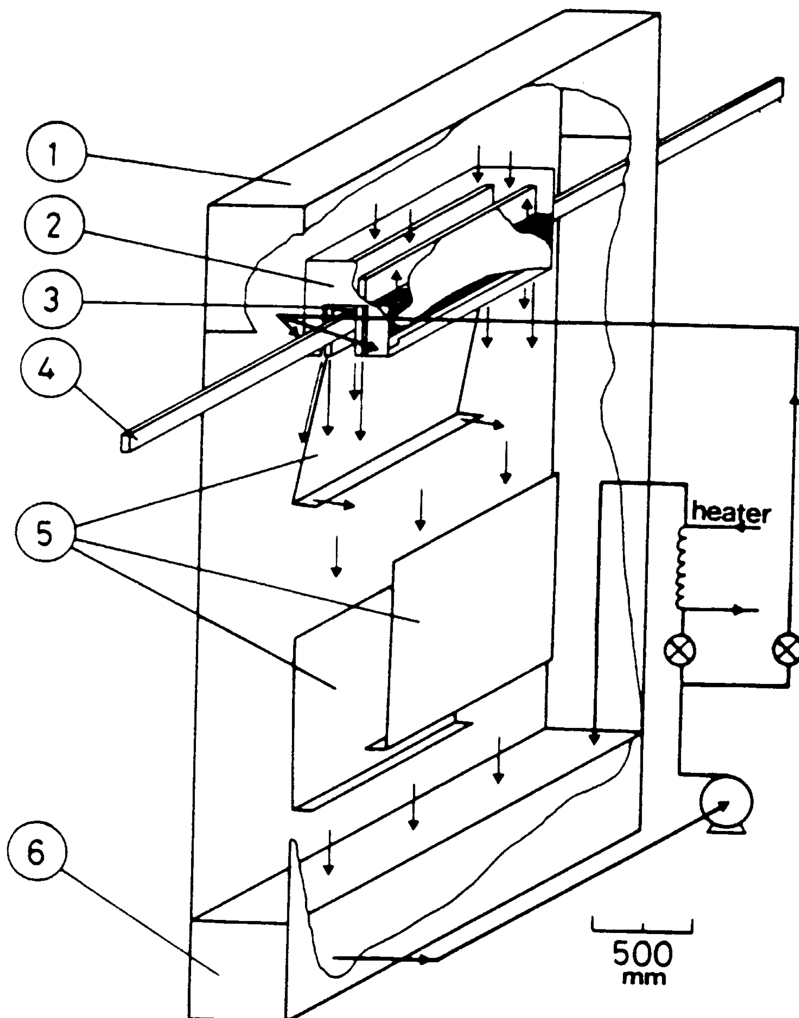

Figure 8: Distributor cabinet: 1. cabinet; 2. distributor; 3. horizontal support for distributor; 4. dressing rail; 5. baffles; 6. recirculation tank (Davey and Smith, (1))

Chlorine

The various forms of chlorine are probably the most widely used sanitisers in the food industry. Many studies have shown that applying chlorine at concentrations of 200 ppm and above to meat carcasses can produce a 2 log reduction in bacterial numbers. Similar reductions have been achieved for produce. These reductions can be further increased by raising the temperature of the chlorine (7, 15). Most pathogens can be readily controlled, though not eliminated, by chlorine but some would require concentrations higher than 200 ppm (16) meat. These upper levels can also cause adverse discolouration and leave off-flavours in fresh produce (17).

Numerous concerns are increasingly being expressed about the use of chlorine. Amongst these are that chlorine reacts with phenolic compounds and that the resultant chlorophenols can cause tainting at very low concentrations (18), as well as possible human health risks associated with chlorinated lipids and proteins (19). There are also many practical problems in terms of control of chlorine levels, protection of delivery systems from corrosion, etc.

Organic acids

Washes and sprays containing organic acids have been successfully used in decontaminating beef, lamb, pork and poultry carcasses. However, their use for fruits and vegetables has not been fully evaluated. Researchers agree that organic acids can reduce the number of pathogenic and spoilage organisms on meat by typically 1 to 3.5 log micro-organisms g^{-1} producing an extension of shelf life of 7 to 17 days respectively. In investigations where the temperature of the acid is varied, greater reductions in bacterial numbers are achieved at higher temperatures. However, in many cases the meat has been immersed in the acid mixture and it is difficult to separate the effect of the temperature from that of the acid.

Studies have generally used concentrations of between 2 to 4% in water with some as high as 24% and it is not clear what the maximum concentration should be. In some studies concentrations of 2% acetic acid were reported to produce discolouration on pork loins (20). In others, at 3% no adverse effects were found on lean samples but slight off-flavours and grey discolouration were reported on fats (21). Overall, treatment with 2% lactic acid solutions applied to meat with a surface temperature of 37°C, have been described as optimal (22). Some researchers advocate a mixture, others a single acid.

It is disappointing that the reductions produced in commercial trials are often significantly lower than those found in laboratory studies. In laboratory trials the samples have often been inoculated with high levels of bacteria and in these situations the acids may be more effective. Also, producing an even surface coverage of acid is for example, far easier on a small sample in the laboratory than over a whole carcass in the abattoir.

Trisodium phosphate (TSP)

Trisodium phosphate was developed in the US for the control of salmonella on poultry (23). It has been shown to be equally as effective in controlling salmonellas on tomatoes (24) and for reducing total bacteria on beef (11). However, Dickson *et al.* (25) and Zhang and Farber (2) reported TSP to be ineffective against *L. monocytogenes* on beef tissue and lettuce, respectively.

Ozone

Ozone (O_3) is a water-soluble, naturally occurring gas which is a powerful oxidising agent. and very unstable, on exposure to air and water it rapidly decomposes to form oxygen. Due to this instability generation is usually at the point of use.

Ozonated chiller water containing levels of aqueous ozone between 3.0 to 4.5 ppm was found by Sheldon and Brown (26) to be more effective at reducing microbial counts on poultry carcasses than non-ozonated water chilling. No significant carcass skin colour losses, lipid oxidation, or off flavours were found on poultry carcasses treated in this way. Both aerobic and psychrophilic counts remained lower on ozone-chilled carcasses than on those water-chilled during shelf life studies at 4.4°C.

Steam

One treatment gaining acceptance is the use of steam. A number of processes using steam for treating meat have been devised and some used commercially with varying degrees of success.

Steam at 100°C has a substantially higher heat capacity than the same amount of water at that temperature. If steam is allowed to condense onto the surface of meat then it has the ability to rapidly raise the surface temperature of the meat. Direct application of steam to pork carcasses has been shown to reduce total bacterial counts by 6 log micro-organisms cm^{-2}. However, the steam marred the appearance of the pig carcasses (27).

Steam can be produced under vacuum at temperatures substantially below 100°C without substantially reducing its heat capacity. It has been shown to be an effective way of decontaminating poultry drumsticks and carcasses, surface temperatures of 75°C for 4 minutes achieving reductions of the order of 5.5 and 3 log, respectively (28). Similar reductions have been achieved using very short treatments of steam under pressure (140°C for 50 ms). Over-heating of the meat was prevented by repeated cycles of heating and cooling (29). One very attractive feature of condensing steam is its ability to penetrate cavities and condense on any cold surface.

The most successful steam process yet, in terms of industrial application, has been that developed by Kansas State University together with Frigoscandia and Cargill Inc. (30). Studies on this commercially available system for treating red meat carcasses have been conducted by Nutsch *et al.* (31, 32) and Phebus *et al.* (33). Significant reductions of the order of 3.5 log-units for specific bacteria have been reported.

Novel methods

A whole range of more novel techniques, such as microwaves (34) ultra-violet (UV) light (35) or visible light (35), have been suggested for treating produce and meats, and in some cases demonstrated to be viable alternatives. Most of these methods depend on heat to destroy the bacteria present though a number of non-thermal treatments have been proposed (36, 37). High voltage pulsed electric field (PEF) treatment relies on the lethal effect of strong electric fields for the inactivation of micro-organisms and has been proposed as a potential non-thermal food preservation technique. Another non thermal technique involving the inactivation of micro-organisms by subjecting product to one or more pulses of an oscillating magnetic field. This was described in a world patent assigned to Maxwell Laboratories Inc. of San Diego, California (38). The mechanism of microbial inactivation, as described by the authors, is due to effects caused to magneto-active

(magnetic dipoles) parts of large biological molecules by the introduction of oscillating magnetic fields. When a large number of magnetic dipoles are present in one molecule, enough energy can be transferred to the molecule to break a covalent bond. It is assumed that in a micro-organism these would be the DNA or proteins, hence destroying the micro-organism or at least rendering it inactive.

Pothakamury et al. (39) concluded that OMF treatment potentially provides three advantages in treating food:

1. minimal thermal denaturation of nutritional and organoleptic properties.
2. reduced energy requirements for adequate processing.
3. potential treatment of foods inside a flexible film package.

At present this would seem to an area of study rich in potential but lacking in research.

Future progress

Meat carcasses typically contain between 1 log and 4 log micro-organisms g^{-1} (40). Fruits and vegetables often contain populations of 4 log to 6 log micro-organisms g^{-1} (41). To achieve any significant improvement in the microbiological condition of such products we require a 4 log-unit reduction in total bacterial numbers. To date no adequate method of achieving this has been found without effecting the sensorial quality of raw produce or meat (Table 3). No treatment, as yet, can be relied upon to eliminate all pathogens.

Table 3: Typical bacterial reductions achieved by various decontamination treatments

Treatment	Range of log reduction	Treatment	Range of log reduction
Water treatments		Chemical treatments	
'Cold'	< 0.5 to < 1.4	Organic acids	1.2 to 3.5
'Hot'	< 0.5 to > 3	Chlorine	< 2
Steam	3 to 6	Trisodium phosphate	2 to 2.5
Ultraviolet	2 to 3	Hydrogen peroxide	2 to 3
Visible light	0.5 to 1.5	Ozone	0.3 to 3
Microwave radiation	~2		

Studies have shown that the application of water can be an effective method of reducing the number of pathogens and food spoilage micro-organisms on the surface of raw produce and meat carcasses. Relatively small reductions of up to 1 log-unit can be achieved using unheated water. This reduction is probably due to physical removal of the micro-organisms from the surface. Using hot water much higher levels, a reduction of over 3 log-unit, has been found. The degree of reduction can be directly related to the time/temperature conditions that can be achieved at the surface of the food. Hot water treatments producing surface temperatures of 80 to 90°C for 10 s would appear to be very effective on meat. Less severe temperature/time treatments result in much lower levels of bacterial reduction. More severe treatments produce adverse and irreversible changes to the appearance of meat. Little comparable work seems to have been carried out on the effect of short time high temperature washing of fruits and vegetables.

The first problem that has to be addressed with all the chemical treatments is not technical but legislative. Provided they meet the approval of legislative authorities, any chemical decontaminant has to be safe and not impair the quality of the meat, vegetable or fruit at the concentrations required to be effective.

It is clear from the published studies that a number of chemicals have much to offer in reducing the levels of contamination on raw food products. There are a number of technical areas that need to be investigated before such chemicals could be recommended for wide commercial use. These are:

- Quantifying the optimum concentration and composition of the chemical for different applications.
- Defining the best method of application including time, duration and temperature.
- Quantifying the cost and cost effectiveness of chemical application.

Further studies on some chemicals, such as TSP and ozone, are likely to reveal specific applications where their particular attributes will be useful.

In all cases, with the exception of a few of the chemicals, the industrial use of decontamination treatments lags far behind the laboratory studies and effort should be directed at developing systems that meet the exacting requirements of commercial users.

Condensing steam under vacuum or pressure has been shown to be very effective with red meat and poultry. The short times and high temperatures required for such treatments, coupled with the need for even exposure and fast, controlled cooling, pose many engineering challenges. Despite the fact that there are commercially available systems on the market many of these challenges remain to be fully conquered.

The Food Refrigeration and Process Engineering Research Centre (FRPERC) are conducting projects sponsored by the UK Ministry of Agriculture Fisheries and Food (MAFF) and EU to examine the decontamination of food products using combinations of condensation at sub-atmospheric pressure and organic acids. The EU project under the FAIR (Agriculture and Fisheries) programme is being carried out by a team from FRPERC, MATFORSK in Norway, the National Food Centre in Ireland, Katholieke Universiteit Leuven in Belgium and the University of Vienna in Austria. Both projects are examining the effect of rapid temperature cycling on micro-organisms present on the surface of different fruits, vegetables, meats and meat products. A further project at FRPERC funded by MAFF is studying the application of steam under pressure and at atmospheric pressure for treating red meat primals.

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Phase transitions in rectified grape must. Influence of pectin.

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Abstract

The use of fruit juice concentrates, such as grape must, in manufacturing of different kinds of foods (for instance, marmalades, confectioneries, low and intermediate fruit products, etc.) is an interesting alternative which may be very appreciated by consumers. Phase transitions in foods or ingredients in the temperature or moisture ranges of product handling or processing greatly affect its properties and stability. The freezing behaviour and glass transitions of rectified grape must were analysed, as affected by moisture content and pectin adding, and the state diagrams were obtained. Grape must without and with pectin have very similar state diagram for moisture contents higher than 10 %, but glass transition temperature increases at lower water content when must contain pectin.

1 Introduction

Concentrate fruit juice is used in food industry as ingredient in manufacture of different kinds of products (jams and marmalades, confectioneries, high and intermediate moisture fruit products etc.). Juice composition in rectified products is like sugar solutions, in many cases fructose and glucose being the major components. In some cases, they are industrial sub-products such as grape must from wine cellars and the industrial use allows us to take advantage of more integral processes. Incorporation of thickeners (starch, gums, proteins,...) to juice concentrates allows us to modify their functional properties and to extend the use range.

Phase transitions in foods or ingredients in the temperature or moisture ranges of product handling or processing greatly affect its properties and stability (White and Cakebread, 1966), which depend of the product composition. For aqueous matrix phase transition temperature can be described in a state diagram as a function of moisture content. State diagram is a useful tool to predict phase transitions in a product during processing (drying, freezing, heating,...), handling or storage (Roos, 1993, 1995; Slade and Levine, 1991) and hence to assure a better quality product since undesirable phase transition may lead to unacceptable product properties.

In this work, the freezing behaviour and glass transitions of rectified grape must were analysed, as affected by moisture content and pectin concentration, and the state diagrams were obtained.

2 Materials and methods

2.1 Materials

The samples used for the experiences were rectified grape must (M0), and rectified grape must with HM pectin at different level: 2.5 (M2.5) and 5 (M5.0) g/100g of dry solids. Samples were frozen at -40°C, and afterwards lyophilised at 0.1 Pa.

2.2 Methods

2.2.1 Sample hydration

Lyophilised samples were moisture conditioned at 35°C at different levels in closed chambers with constant and controlled relative humidity (by using saturated salts with a_w from 0 to 0.75) for about 30 days till constant weight was achieved. At this time, moisture content was measured. Samples with a_w>0.75 were prepared by vacuum concentration at 60°C of the initial solutions at different degrees. In all cases, thermograms of the samples by DSC were obtained.

2.2.2 Analytical measurements

For all samples water content was quantified by vacuum drying the samples at 60°C until constant weight was achieved (AOAC 20013). Water activity was measured in a hygrometer Decagon CX-1 at 35 °C.

2.2.3 Glass transition temperature (Tg) and ice melting enthalpy (ΔH)

Tg (onset (on) and midpoint (mp)) and ΔCp values were analysed by differential scanning calorimetry (DSC 220CU SII, Seiko Instruments Inc., Chiba, Japan). Heating rate was 5°C/min and the temperature range was between –100 and 60°C. To analyse ice melting enthalpy, glass transition temperature of the maximally cryo-concentrated aqueous phase (Tg') and incipient melting temperature (Tm'), annealing was carried out at Tg'<T<Tm' for enough time to achieve the maximal cryo-concentration of the sample.

2.2.4 Tm determination

Initial freezing point (Tm) of samples with different water content were estimated out by using Robinson and Stokes equation (Leung, 1986) from their respective a_w values.

3 Results and discussion

3.1 Glass transitions

The Tg (onset and mid point) and ΔCp values obtained for low moisture samples with different a_w are shown in tables 1 to 3. The Gordon and Taylor (1952) model (Eq. 1) properly fit the Tg development with water content (r^2>0.804), the k value being 2.36, 3.41 and 3.65 for M0, M2.5 and M5.0 samples, respectively. In Fig. 1 and 2 the obtained curves and the experimental points can be seen for samples M0 and M5.0. As can be observed in these Figs. and tables 1 to 3, differences among the sample Tg behaviours were found only in the very low moisture range (lower than 10 %). The polymer addition to the must implied an increase in the average molecular weight of the solids. Nevertheless, this mean an increase of the Tg values only at low water content. From x_w>0.10 the water plasticising effect (Roos and Karel, 1991) smoothes any differences among samples. This behaviour was also reflected in the Tg of the maximally cryo-concentrated solution (with x_w>0.10) where no differences in the Tg' values for three samples were observed. Tables 4 to 6 show the Tg' values obtained for the samples, a mean value of –55 °C may be considered for all cases and no evidences of the pectin cryo-stabilising effect (Simatos and Blond, 1993; Levine and Slade, 1988) were detected as the expected Tg' increase was not promoted.

$$Tg = \frac{(1 - x_w)Tg_s + kx_w Tg_w}{(1 - x_w) + kx_w} \quad (1)$$

Table 1: Glass transition temperature (onset: $Tg^{(on)}$ and mid point: $Tg^{(m.p.)}$) and ΔCp values for grape rectified must (M0) at different water contents

a_w	x_w (kg water/kg sample)	Tg $^{(on)}$ (°C)	Tg $^{(m.p.)}$ (°C)	ΔC_p (J/g°C)
0	0	-1.0 ± 0.8	2.3 ± 0.9	0.116 ± 0.005
0.225	0.034 ± 0.002	-3.1 ± 0.6	0.4 ± 0.4	0.158 ± 0.008
0.432	0.081 ± 0.008	-28.5 ± 1.1	-25.4 ± 1.0	0.156 ± 0.011
0.675	0.233 ± 0.009	-52.9 ± 0.3	-49.4 ± 0.1	0.22 ± 0.04
0.753	0.263 ± 0.007	-67.26 ± 0.11	-65.1 ± 1.6	0.26 ± 0.02

Table 2: Glass transition temperature (onset: $Tg^{(on)}$ and mid point: $Tg^{(m.p.)}$) and ΔCp values for grape must with HM pectin (M2.5) at different water contents

a_w	x_w (kg water/kg sample)	Tg $^{(on)}$ (°C)	Tg $^{(m.p.)}$ (°C)	ΔC_p (J/g°C)
0	0	20 ± 2	23 ± 3	0.066 ± 0.003
0.225	0.037 ± 0.011	-0.28 ± 1.4	2.8 ± 1.1	0.20 ± 0.02
0.432	0.10 ± 0.03	-293 ± 1.2	-259 ± 1.6	0.160 ± 0.007
0.675	0.219 ± 0.017	-52.0 ± 0.8	-49.0 ± 0.8	0.166 ± 0.018
0.753	0.255 ± 0.008	-68 ± 2	-65 ± 2	0.18 ± 0.02

Table 3: Glass transition temperature (onset: $Tg^{(on)}$ and mid point: $Tg^{(m.p.)}$) and ΔCp values for grape must with HM pectin (M5.0) at different water contents

a_w	x_w (kg water/kg sample)	Tg $^{(on)}$ (°C)	Tg $^{(m.p.)}$ (°C)	ΔC_p (J/g°C)
0	0	27.4 ± 0.6	30.5 ± 1.0	0.1918 ± 0.00
0.225	0.035 ± 0.003	0.63 ± 0.11	3.73 ± 0.03	0.093 ± 0.004
0.432	0.070 ± 0.013	-31.4 ± 0.3	-26 ± 3	0.13 ± 0.02
0.657	0.236 ± 0.002	-52.80 ± 0.17	-49.73 ± 0.15	0.154 ± 0.002
0.753	0.258 ± 0.006	-69 ± 2	-66 ± 2	0.21 ± 0.02

3.2 Phase transitions related to freezing

Tables 4 and 5 show the characteristic parameters of the ice melting endotherm (ΔH and Tm') for samples with freezable water, as well as the Tg' values and the associated ΔC_p. No significant differences among Tg' values for samples with different water content or pectin concentration were observed. From the Tg' value and Gordon and Taylor model the water content of the maximally cryo-concentrated solutions (Wg') were estimated. Likewise, this value was obtained from the correlation of melting enthalpies and water content of the samples by means of Eq. 2 obtained from

a balance in the frozen system at T<Tm'. The plot ΔH vs.: x_w for each sample allows us to obtain straight lines from which the Wg' can be obtained when ΔH=0. The Wg' obtained from the two procedures are coherent taking into account the standard deviation of each estimation. When the Wg' values were compared for must with and without pectin no significant differences were observed, the average value being 0.22 g/g solution. The incipient melting temperature Tm' of the different samples (Tables 4 and 5) seems to show slightly lower values in samples with pectin but difference is the order of 1-2 °C.

$$\Delta H = \left(\frac{\Delta H_w^0}{1 - Wg'}\right) x_w - \left(\frac{\Delta H_w^0}{1 - Wg'}\right) Wg' \tag{2}$$

were:

ΔH^0_w = Melting enthalpy pure water (335 J/g).

ΔH = Experimental melting enthalpy (J/g).

Wg' = water content of the maximally cryo-concentrated solution ($g_{water}/g_{solution}$).

The initial sample freezing point (T_m) was calculated, in an approximated way, from the sample a_w values at 35°C by using the Robinson and Stokes equation. Very similar sample's behaviour was observed when compared the T_m - water content relationship (Figs. 1 and 2) for must with and without pectin, as expected from the small influence of polymers on the freezing point depression of solutions.

Table 4: Glass transition of the maximally cryo-concentrated solutions (onset: T_1 and mid point: Tg') and ice melting properties of grape must (M0).

x_w	T_1 (°C)		Tg' (°C)		ΔC_p (J/g·°C)		Tm' (°C)		ΔH (J/g)	
0,506	-59,1	±0,1	-55,4	±0,2	0,46	±0,01	-39,4	±0,2	131	±1
0,684	-53,2	±0,2	-51,2	±0,1	0,17	±0,04	-40,7	±0,9	182	±9
0,851	-54,2	±0,8	-51,8	±0,7	0,10	±0,01	-40	±1	288	±9

Table 5: Glass transition of the maximally cryo-concentrated solutions (onset: T_1 and mid point: Tg') and ice melting properties of grape must with HM pectin (M5.0).

x_w	T_1 (°C)		Tg' (°C)		ΔC_p (J/g·°C)		Tm' (°C)		ΔH (J/g)	
0,546	-57,7	±0,4	-54,7	±0,2	0,32	±0,04	-38,3	±0,3	127	±2
0,700	-58,5	±0,1	-55,1	±0,1	0,26	±0,01	-38	±1	192	±11
0,838	-57,7	±0,5	-53,0	±0,5	0,14	±0,01	-36,9	±0,5	259	±20

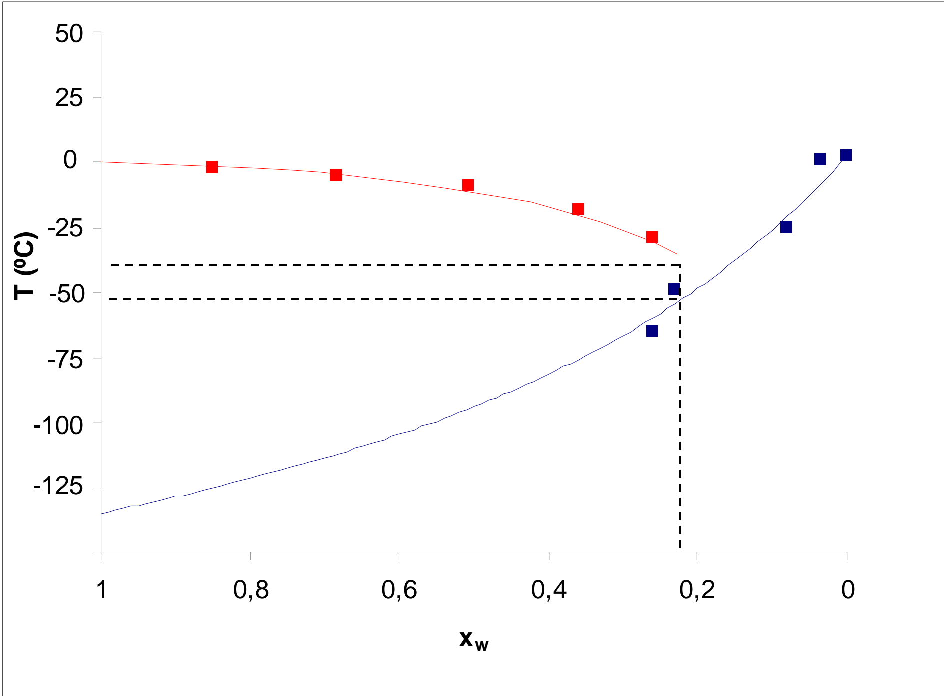

Figure 1: State diagram of rectified grape must.

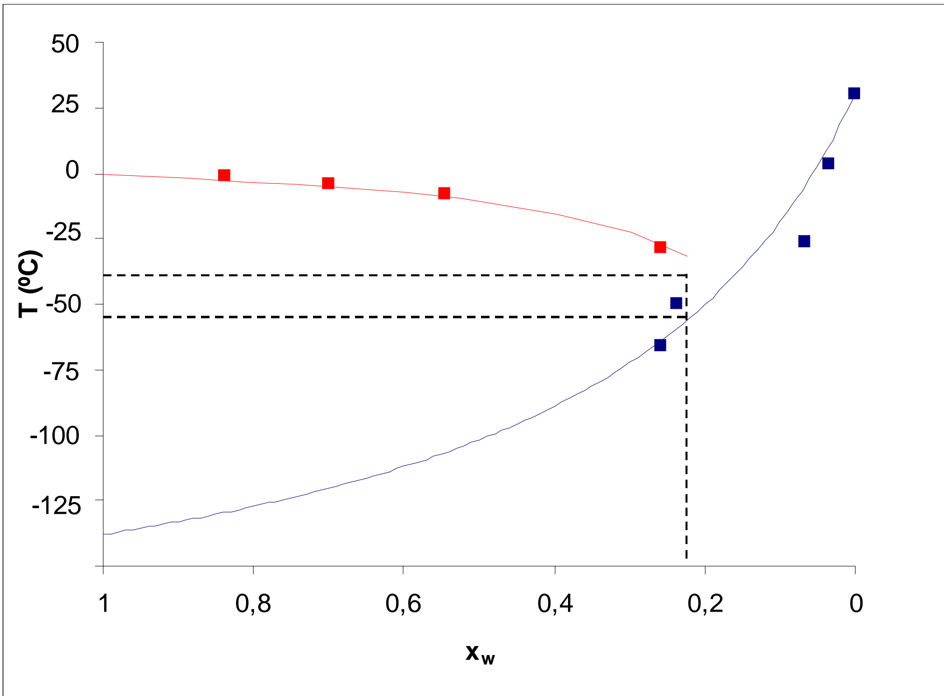

Figure 2: State diagram of rectified grape must with HM pectin (M5.0).

4 Conclusions

Grape must without and with pectin have very similar state diagrams. The only difference appears at low moisture content (lower than 10%) in the glass transition curves. Therefore pectin adding in low moisture must products will increase the critical moisture contents for the abrupt changes in product properties (texture, crystallisation rate, collapse,...) but no great cryo-stabilising effects of pectin may be expected in must as no increase in the Tg' or decrease in the Wg' values were detected.

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Scuffing - Resistance of glass bottles to internal pressure and shock impact

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Abstract

Scuffing is the appearance of wear marks on glass bottles and plays an important role when filling beverages in returnable bottles. In relation to different laws and standards (i. e. German Product Liability Law, ISO 9000, HACCP) it is important to know how the scuffing influences the resistance of a glass bottle to internal pressure and shock impact. On the other hand scuffing is an unwanted appearance for the marketing of high quality products. In this study a portable instrument was developed in co-operation with bottling line constructors and the German Brewers Association that allows the detection and classification of scuffed bottles with respect to their degree of scuffing.

Examinations on the strength of glass bottles showed that the internal pressure stability and the shock resistance of returnable glass bottles first decreases with an increasing wear mark height or area. Then a stagnation of stability can be found for both characteristics with increasing degree of scuffing. It could also be shown that the technical standard and the arrangement of the machinery in a filling line influences the theoretical number of runs. The results from this study will help legislators or associations to determine criterions for guidelines and regulations with respect to consumer safety or bottle appearance.

Introduction

Returnable glass bottles can be severely damaged and their appearance may be unacceptable for the consumer by careless handling in filling lines after a few number of trips. Referring to different standards and product safety (German product liability law, HACCP) damaged bottles play an important role when filling carbonated drinks in returnable glass bottles. In this study some strength properties of glass bottles are tested.

The surface of glass bottles gets mechanically and chemically damaged when passing through bottling lines. During their transport on conveyors or in individual machines within the bottling line the bottles can get wear marks (scuffing) or even more severe surface injuries. On conveyors the bottles get mechanically scuffed especially by bottle-to-bottle pressure before glideliners, in combiners, accumulation tables and on the load of the bottle washer and the inlet of the packer. Dirt and water on the glass surface bottles accelerates the mechanical damage. These effects are due to frictional forces between the bottles which also increase with increasing scuffing.

The bottle surface gets damaged by attacks by chemical action during the washing process. The glass surface is injured and made more sensitive for mechanical stress. Certain detergents like caustic soda, free phosphates, and carbonates attack the surfaces. The effects are influenced by detergent concentration, water hardness, exposure time, and temperature. There are some additives which can reduce the corrosive effect of the lye. But no significant further improvement can be expected because of the required cleaning effect.

1 Theoretical background

The mechanical strength of a glass bottle is mainly influenced by its surface conditions like damages, scratches or micro-breakages in the surface of the glass. Therefore bottles having damages must be eliminated out of the filling line and an all surface empty bottle inspection is essential.

The mechanical strength of a glass container is influenced by serveral factors like bottle shape (especially largest diameter), absolute wall thickness, wall thickness variations, scratches, injuries, or discontinuities on the glass surface [1]. The actual strength of a single bottle cannot be predicted, even considering most of the parameters mentioned above. Some investigations [1] describe methods to predict the strength span of bottles with a given bottle design and manufacturing process. But these methods are mainly suitable to optimise a bottle design for specific strength requirements.

There are different parameters which describe the strength of a glass bottle: internal pressure stability, shock resistance, vertical load, and resistance to thermal shock. In this study the resistance to internal pressure and shock impact in relation to the degree of scuffing was examined. For this purpose the herein before mentioned strength parameters of differently scuffed bottles were measured. Therefore genuinely used bottles are tested, hence the number of trips is not known. But over concurrent made tests the correlation between scratched area and age of a bottle can be confirmed.

2 Materials and methods

For all tests first the degree of scuffing of each bottle was determined. Then the strength stability of the bottle was tested either the internal pressure stability or shock impact stability at the upper or lower shock band.

2.1 Degree of scuffing

To determine the degree of scuffing of the tested bottles two portable test rigs were developed in co-operation with the companies Heuft Systemtechnik, Burgbrohl, and Krones, Neutraubling, Germany. A schematic overview of the test arrangement is shown in figure 1. A CCD-camera takes a picture of the shock bands, the bottle area where the scuffing can occur. The projected grey level picture from the camera is evaluated in an attached computing unit. The degree of scuffing is classified and the data are stored for each bottle. The calculated wear mark area can be output to a personal computer for further statistical evaluation.

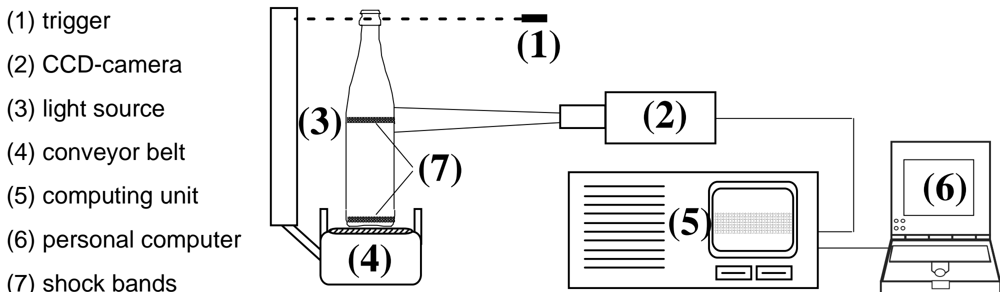

Fig. 1: Scheme of the test rig evaluating the degree of scuffing

For each bottle the degree of scuffing z was determined by rotating of the bottle before the camera evaluating 6 measuring fields (field width about 3.2 cm) along the range of the upper shock band. From the average value of these 6 measurements z was calculated and standardised for a field width of 20 mm. The degree of scuffing is indicated in mm^2 scuffed area within the standardised computed field.

2.2 Internal pressure

The internal pressure resistance of the scuffed bottles was measured with a Ramp Pressure Tester of the company AGR, Butler, USA, according to the American standard ASTM C 147-91 [2]. Herein hydrostatic pressure is built up and electronically controlled in the bottle by the Ramp Pressure Tester. The pressure is initially atmospheric pressure which increases at a uniform rate (4.1 bar/s) until the bottle breaks or the maximum pressure (60 bar) is reached. For test duration t between 3 and 60 s, the actual pressure p_t can be converted into the equivalent "1-min-pressure-resistance" p_{60} to meet ASTM C 147 (equation 1). All results in this study referring to internal pressure resistance are 1-min-pressure-resistance values.

$$p_{60} = \left(\frac{7.97 + 1.53 \cdot \log(t)}{10.69} \right) \cdot p_t \qquad (1)$$

2.3 Shock resistance

Usually pendulum impact tests at glass containers are executed according to a German standard DIN 52295 [3]. DIN 52295 describes the testing method as well as the measuring instrument. In this study the resistance of the bottles to shock impact was measured by an in-house developed pendulum impact tester. This is especially designed to simulate the impact stresses to a bottle on a conveyor belt colliding with other already stopped bottles. The used pendulum impact tester differs in some details from the pendulum impact tester described in the German standard. The counter bearing of the tester is heavier and consists of two brass bodies shaped like the tested bottles with a comparable modulus of elasticity with glass. A bottle was repeatedly stroken by a hardened steel ball at the upper or lower wear mark area until it broke. Between two strikes the bottle was rotated 30° and the shock impact increased, i. e. increasing the angle of the pendulum in steps as shown in table 1.

The magnitude of the angle of the pendulum determines the impact speed of the steel ball right before striking the bottle. The transferred kinetic energy to the bottle and thus impact impulse affecting the bottle can be calculated knowing the geometry of the tester. Because of the shock impact being a comparatively slow event the entire kinetic energy is converted into deformation energy of the bottle. In this study the impact impulse in SI base unit kgm/s is used to describe the shock impact on the bottles.

2.4 Materials

In this study 0.5-l-NRW("North Rhine-Westphalia")-bottles for filling beer were examined. But there are similar results for other types of bottles. The test bottles were selected in a brewery between the bottle-washer and the empty-bottle inspector. For these tests only bottles which obviously showed scuffing were selected. But the bottles do not have any other damages.

3 Results and conclusion

3.1 Internal pressure stability

According to their degree of scuffing z the bottles were divided in 6 classes. In the classes 1-6 are increasingly strongly scuffed bottles. Class 0 contains only new bottles, class 0* new bottles which passed the bottle washer once. The boundaries of the respective classes, the number of bottles per class, and statistical sizes are represented in table 1.

Table 1: Classification of the bottles, number of bottles per class n, mean values of z and $p_{60,}$ and standard deviation of p_{60}

class	0	0*	1	2	3	4	5	6
z in mm^2	-	-	0.0-10.0	10.1-20.0	20.1-30.0	30.1-40.0	40.1-50.0	≥50.1
$\bar{z}$ in mm^2	0	0	1.9	15.2	25.5	34.6	44.8	55.8
n	104	118	227	88	131	81	70	38
$\bar{p}_{60}$ in bar	40.7	25.2	15.3	15.1	15.2	14.6	14.8	14.6
$s(p_{60})$	10.3	4.2	3.5	2.6	2.6	2.6	2.2	2.0

Table 1 shows the decrease of the mean value of 1-min-pressure-resistance $\bar{p}_{60}$ from 41 ± 10 bar for new bottles (class 0) to 25 ± 4 bar for once cleaned bottles (class 0*). With only few scuffed bottles (class 1) $\bar{p}_{60}$ still reduces to 15 ± 4 bar. However $\bar{p}_{60}$ as well as $s(p_{60})$ of the scuffed bottles of the classes 1 to 6 do no more differ significantly (with a significance level $\alpha = 0.05$), so that an increasing degree of scuffing does not exert further strength-reducing influence on the glass bottle.

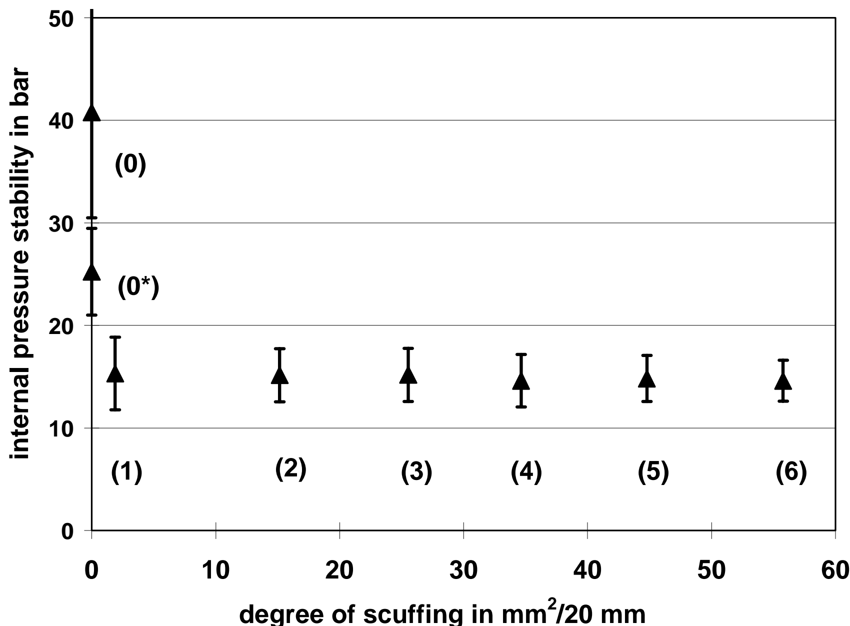

Fig. 2: Relation between degree of scuffing and internal pressure resistance

The above results are further highlighted in figure 2 where the internal pressure stability is plotted versus degree of scuffing. The respective mean values of z and p_{60} of each class are entered. The error bars represent the standard deviations $s(p_{60})$ of the individual classes 0-6. The stagnation of the internal pressure stability on a pressure level of approximately 14 bar can be confirmed from figure 2. Similar examinations of other types of bottles, as for instance the 0.5-l-Ale and 0.7-l-GDB-bottles, led to the same result. Such a stagnation of the strength starting from a certain flaw depth is described also with Gliemeroth [4].

The strength of the bottles decreases to a limiting value on the first initial trips through the filling line, where frictive damage begins. In further trips the damages got more extensive but not in depth only the injured area increases. The standard deviation is very high for the new and only lightly damaged bottles, which refers to a various severity in depth of the surface damages and wide variations in hot and cold end coating quality. After several trips (class 1) when the most severe scratch is reached the standard deviation halves.

3.2 Shock impact resistance

The results of the shock impact resistance i are here presented in table 2 and 3 as well as in figure 3 and 4. The tested bottles were classified in 8 classes separately for the upper and lower wear mark area. The starting impact impulses i_0 and the increase of the impulse Δi after each measurement are indicated in the tables 2 and 3. Here too the classes 0 or 0* contain new or once cleaned bottles. In the classes 1-6 differently strong scuffed bottles are classified regarding their degree of scuffing, with increasing scuffing from class 1 to 6.

Table 2: Upper wear mark area: classification, number of bottles per class n, initial impulse i_0, increase of impulse Δi, mean values of i and z, standard deviation s(i), and tolerance limits $T_U(i)$

class	0	0*	1	2	3	4	5	6
z in mm^2	-	-	0.0-10.0	10.1-20.0	20.1-30.0	30.1-40.0	40.1-50.0	≥50.1
$\bar{z}$ in mm^2	-	-	4.1	14.5	25.0	35.0	45.2	56.8
n	100	100	148	103	92	99	125	113
i_0 in kgm/s	1.18	1.18	0.80	0.80	0.80	0.80	0.80	0.80
Δi in kgm/s	0.27	0.27	0.13	0.13	0.13	0.13	0.13	0.13
$\bar{i}$ in kgm/s	3.03	1.64	1.23	1.17	1.17	1.15	1.12	1.13
s(i) in kgm/s	0.29	0.11	0.16	0.10	0.12	0.11	0.11	0.11
$T_U(i)$ in kgm/s	2.55	1.46	0.96	1.00	0.97	0.97	0.94	0.95

Table 3: Lower wear mark area: classification, number of bottles per class n, initial impulse i_0, increase of impulse Δi, mean values of i and z, standard deviation s(i), and tolerance limits $T_U(i)$

class	0	0*	1	2	3	4	5	6
z in mm^2	-	-	0.0-10.0	10.1-20.0	20.1-30.0	30.1-40.0	40.1-50.0	≥50.1
$\bar{z}$ in mm^2	-	-	3.6	15.4	25.8	35.1	44.7	54.8
n	100	100	132	93	121	129	136	89
i_0 in kgm/s	1.99	1.30	0.67	0.67	0.67	0.67	0.67	0.67
Δi in kgm/s	0.27	0.27	0.13	0.13	0.13	0.13	0.13	0.13
$\bar{i}$ in kgm/s	2.18	1.57	1.14	0.99	0.98	0.94	0.95	0.94
s(i) in kgm/s	0.27	0.26	0.26	0.15	0.16	0.15	0.15	0.13
$T_U(i)$ in kgm/s	1.73	1.14	0.70	0.74	0.71	0.69	0.70	0.71

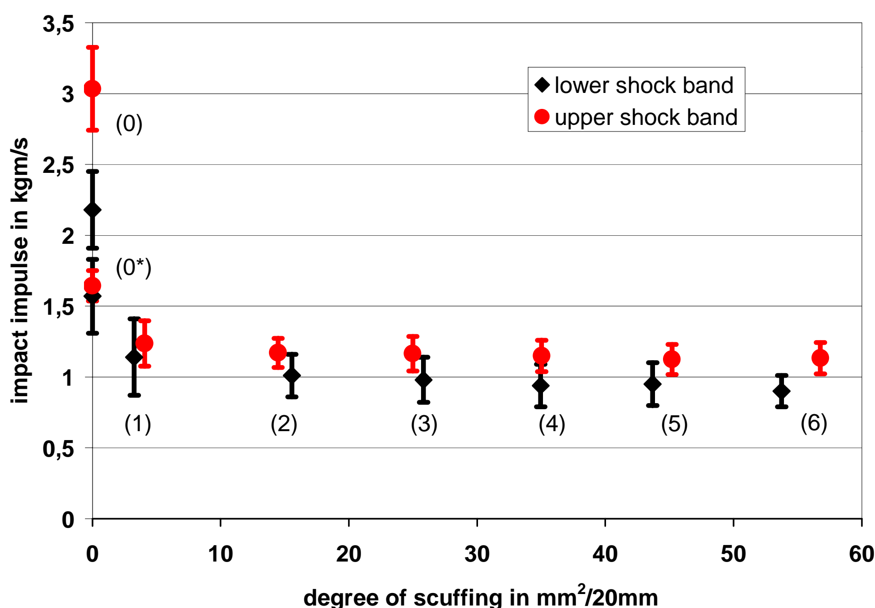

Fig. 3: Relation between shock impact resistance and degree of scuffing

The results are similar to the results of the internal pressure stability. From a very high mean value of the impact strength of the new bottles with 3.03 at the upper and 2.18 kgm/s at the lower wear mark area it comes down to the once washed bottles with 1.64 at the upper and 1.57 kgm/s at the lower wear mark. The quite easily scuffed bottles of class 1 posses a still smaller impact strength with 1.23 upper and 1.14 kgm/s lower wear mark. A small decline is to be registered still with class 2, then however the mean value for the impact impulse of the following classes 2 to 6 oscillates itself at the upper wear mark with approximately 1.12 kgm/s and at the lower shock band with approximately 0.94 kgm/s. A statistical comparison of the average values of these classes 2-6 results in no more significant difference (for a significance level of $\alpha = 0.05$).

The impact strength of the bottles at the lower wear mark area is basically smaller than at the upper wear mark. The transient area between the cylindrical body and the bottom of the glass bottle is particularly sensitive, since in this area with the impact no elastic deforming is possible. A further cause is also the residual tension within the bottle wall from shaping the container.

In the beverage industry of Germany there are guidelines for specific technical conditions for some goods which have to be met by their suppliers, like glassworks. Such a guideline is the STLB for beer bottles [5]. As mentioned in this STLB a lower tolerance limit $T_U(i)$ of at least 60 ips (inches per second) impact strength for new returnable glass bottles on the upper wear mark area is required. According to DIN 52295 this velocity of the pendulum in ips can be converted into an impact impulse of i = 0.756 kgm/s for tests with the AGR Preston Impact Tester. The lower tolerance limit $T_U(i)$ is calculated according to the following equation 2 from the mean value $\bar{i}$, the standard deviation s(i) and a constant k:

$$T_U(i) = \bar{i} - s(i) \cdot k, \qquad k = 1.66 \tag{2}$$

The calculated values for $T_U(i)$ (see table 2) at the upper wear mark area of all classes, thus also of the scuffed bottles, calculated according to equation 2, fulfil the required value. At the lower wear mark area (see table 3) the new bottles achieve this value, only the scuffed bottles of the classes 1-6 did not completely reach the demanded value. Here the values for $T_U(i)$ are situated between 0.69 and 0.74 kgm/s. However the lower tolerance limits in the STLB were determined as already mentioned for new returnable glass bottles.

The impact strength measured with the NRW-bottles are similar to the earlier determined values for the 0.5-l-Euro-bottles (bottle type used to fill in beer before NRW-type in Germany), see table 4. In these investigation in 1991 further classification of the used bottles was not made. Just as the internal pressure stability the resistance to shock impact of the Euro-bottles is smaller than for the NRW-bottles. The cause for this is situated surely in the bottle shape, especially the larger diameter.

Table 4: Former tests on the impact strength of new and used 0.5-l-Euro-bottles [6]

bottles/ wear mark area	new/ upper	used/ upper	new/ lower	used/ lower
$\bar{i}$ in kgm/s	2.21	1.09	1.73	0.96
s(i) in kgm/s	0.50	0.18	0.51	0.19

The following figure 4 shows the cumulative frequency distributions of the impact strength of 0.5-l-NRW-bottles exemplarily at the lower wear mark area. It was differentiated only between new, once cleaned new and used bottles. On the basis of these diagrams, the relatively large standard deviation can be pointed out for the new compared with the used bottles. With the internal pressure stability the standard deviations differ much more.

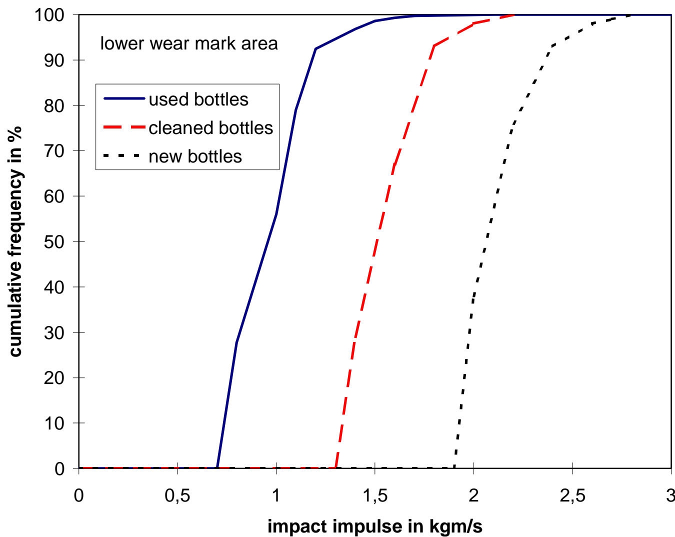

Fig. 4: Cumulative frequency distributions of the impact impulse by new, once cleaned and used NRW-bottles for example of the lower wear mark area

3.3 Conclusion

These examinations show that the strength of a glass container decreases from new to used bottles. But for the used bottles the mean value of strength reaches a constant level which is no more reduced for more scuffed bottles and even the badly scuffed bottles reach the required stability according to STLB [5]. Referring to the standard deviations in strength of new bottles which is as twice as high as for the used ones, particularly weak bottles with only poor strength can be found in each class. For these reasons the bottles have not to be eliminated exclusively due to their degree of scuffing. Nevertheless the degree of scuffing correlates with the age of a bottle. Therefore a badly scuffed bottle is expected to be comparatively old. The older a bottle the more probable it has got some other damages like broken threads or chips. On the other hand aged bottles which are still in the life cycle of returnable containers are the strong ones of the former charge of new from the glassworks. Here a compromise has to be found for the need of an acceptable view of the bottles for marketing and not having oversized costs spending on new glass bottles.

To minimise scuffing there are several possibilities: The bottles get mechanically and chemically damaged when passing through bottling lines. The handling of the bottles in the filling line, on conveyors and in single machines can be approved. The temperature, exposure time and concentration of the lye in the washing machine has to be controlled and minimised. On the other hand the bottles surface can be protected against mechanical and chemical attack by coating after the washing machine. Because of this the number of trips of a bottle without any scuffing can be increased, the life span even doubles. But then you have some additional costs.

Acknowledgement

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Development of ripening in manchego cheese as affected by salt content

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Keywords: manchego cheese, ripening, mechanical properties.

Introduction

Salting of Manchego type cheese is usually carried out by Brine Immersion (BI) for 24-36 hours, depending on the cheese size. Recently a new salting procedure was described (Brine Vacuum Impregnation: VI) which makes the salting process shorter (Chiralt and Fito, 1996, Andrés et al. 1997). This new process is based on the application of vacuum pressure in the salting tank during an initial step. During this period the occluded gas in the pressed curd was expanded and flows out. In a second step, the atmospheric pressure is restored and the residual gas compressed, this leading to the entry of the external brine into the curd pores. In this way, a greater salt uptake occurs in the cheese pieces than in the conventional immersion for the same salting time, and also a different salt profile was achieved (Andrés et al., 1997).

In this paper the ripening development of Manchego cheese as affected by salt content and distribution was studied. Different salt levels and distribution were achieved by using BI or BVI for different times. All cheeses salted in different conditions were stored at 10°C and 85% relative humidity in a ripening chamber for three months. Analyses were carried out immediately after salting and at 1, 2 and 3 months of ripening. Ripening index (lipolysis and proteolysis) and textural properties appear slightly affected by the salt level and distribution, but their respective values were in the normal range for this kind of cheese.

Experimental

Cheese making and salting

Cylindrical cheeses, calf rennet coagulated, 20:80 ewe's and cow's milk (Ewe´s-Cow´s Cheese: ECC) were used. The ECC pieces were supplied by a manufacturer. Cheeses were manufactured according to standard protocol from pasteurised (72 °C for 15 s) milk. Milk was heated to 31°C and 2% lactic starter (*Lactococcus lactis* ssp. *lactis* plus *L. lactis* ssp. *cremonis*; AM Larbus S.A., Barcelona, Spain) was added. After 35 minutes, the milk coagulum was cut into 8-10 mm cubes with a wire knife. The curds were heated at 1 K per 5 min to reach 36 °C and held at this temperature for 10 min prior to wheying. ECC curds (20 cm diam. x 11 cm high) were pressed for 2 h at 0.010 kg/cm^2.

All cheeses were salted using a 24 % (w/w) brine in a stirred tank with temperature and pressure control. Working temperature was 10°C and 50 mbar of pressure was applied during the vacuum periods. Table 1 shows the length of vacuum (t_1) and atmospheric pressure (t_2) steps for the different salting experiments. One cheese was salted for each treatment, but 2 pieces were salted in VI-L treatment (Table 1) to check the reproducibility of the results.

Cheese ripening

After salting a representative sector of each cheese was cut to analyse moisture and salt content and distribution. The cut surfaces were covered with paraffin and cheeses were ripened at 10.0±0.5°C and 85±1 % relative humidity in a maturation chamber of 0.6 x 0.65 x 1.2 m internal dimensions (Ibercex H-900-D) for 90 days.

Analytical determinations

Salt content was analysed in the cheese external zone (to a depth of 1.5 cm from the rind) and in the internal one. The overall content was estimated from these values and the weight fraction of each part. For the sodium chloride determination, samples were homogenised in distilled water at 9000 rpm in an ULTRATURRAX T25 for 5 minutes and centrifuged to remove any fine debris present in the sample. An aliquot of centrifuged sample was taken and titrated in Chloride Analyser equipment (Sherwood Mod. 926). Moisture content was quantified by oven drying to constant weight at 105°C (FIL-IDF 4A:1982). Fat content was determined as described by Nuñez (1986) and total nitrogenous was determined by the Kjeldahl method (IDF, 1993) with a Buchi (Mod.B-426 and B 316, Switzerland) equipment.

Table 1: Salting treatments

Treatment [(a)]	BI-L	BI-S	VI-L[(b)]	VI-S
t_1 (h), P_1=50 mbar	0	0	1.75	1.75
t_2 (h), P_2=1013 mbar	17	5	1.75	0.5

a) BI: Brine immersion, VI: Vacuum impregnation,L: Long time treatment, S: Short time treatment.
b) VI-L treatment was carried out in duplicate: VI-L1 and VI-L2

Measurement of mechanical properties

Mechanical properties of 2 cm side cubes were obtained at 20°C by means of a compression and a stress relaxation test, using a universal test machine (TA.XT2 Texture Analyser, Stable Micro Systems, Haslemere, England). Test conditions are shown in table 2. In both cases, a plane and circular plate 7.5 mm of diameter was used.

Table 2: Test conditions used

Test	Deformation rate	Strain
Compression	48 mm/min	0.75
Stress relaxation	200 mm/min	0.08

From compression test, force distance curves were obtained and characterised parameters were fracture force, fracture distance and elasticity modulus (E). Stress relaxation force-time curves, allowed us to obtain also an apparent modulus function E(t). Moreover, Peleg equation (eq. 1) was fitted to the experimental data and A and B parameters were calculated.

$$\frac{F_{max} - F(t)}{F_{max}} = \frac{A \cdot B \cdot t}{1 + B \cdot t} \qquad (1)$$

Results

Initial cheese characteristics

Fat content from EEC curd was 49.6±3.8 g fat/100 g dry matter (d.m.) and protein contents was 6.1±0.3 g N/100 g d.m.

Salt content immediately after salting in the internal and external parts of the cheese, as well as the overall values, are shown in Fig.1.

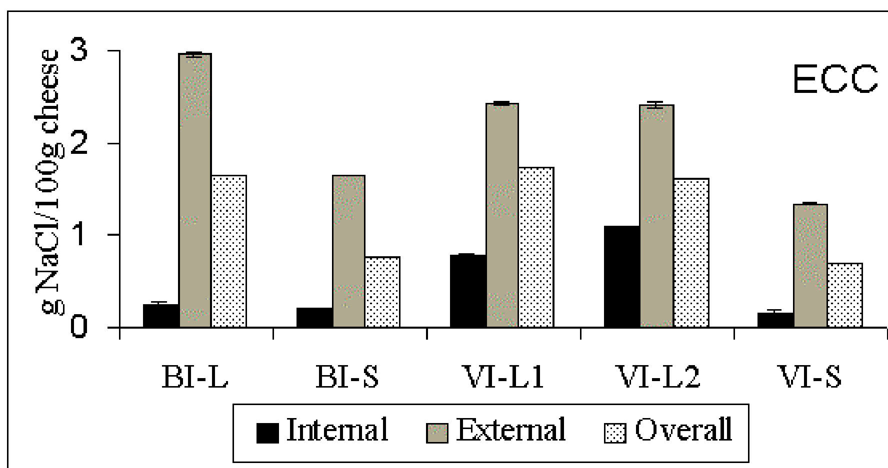

Fig. 1: Salt content and distribution in the internal, external and overall part of the cheese for each salting treatment

A deeper salt penetration was observed for VI treatments, as the achieved internal concentration was higher than those obtained from BI. This occurs to a greater extent when t_2 increases, in accordance with a prolonged action of the hydrodynamic mechanism (HDM), responsible for the vacuum impregnation (Fito, 1994). As has been obtained in previous studies (Chiralt and Fito, 1996, Andrés et al, 1997), VI allows us to reach a similar overall salt content to BI, with a great reduction in the total salting time (from 17 to 3.5 hours in L treatments and from 5 to 2.25 h for S treatments). In BI-L and VI-L treatments led to an overall salt content in cheese of 1.48±0.09 g NaCl/100 g cheese, whereas BI-S and VI-S gave 0.79±0.10 g NaCL/100 g cheese.

Changes in the cheese dimensions due to salting were negligible in pieces salted by BI, whereas an average reduction of 1.5 and 2.0 % were reached in VI treatments for diameter and height respectively. This volume decrease was attributed to the loss of gas and whey pockets during the vacuum step.

Biochemical ripening index : soluble nitrogenous fraction and free fatty acids (FFA).

Soluble nitrogenous fraction development throughout ripening is shown in figure 2. A linear correlation between the levels of nitrogenous fractions and salt concentration has been found at each time of ripening, independently of the initial salt distribution in the internal and external part of cheese (Fig.3). From the obtained correlation, it can be deduced that the lower the total salt content the faster the proteolysis, without significant influence of the initial salt distribution. Nevertheless, at three month ripening no differences in soluble nitrogen was detected among cheeses.

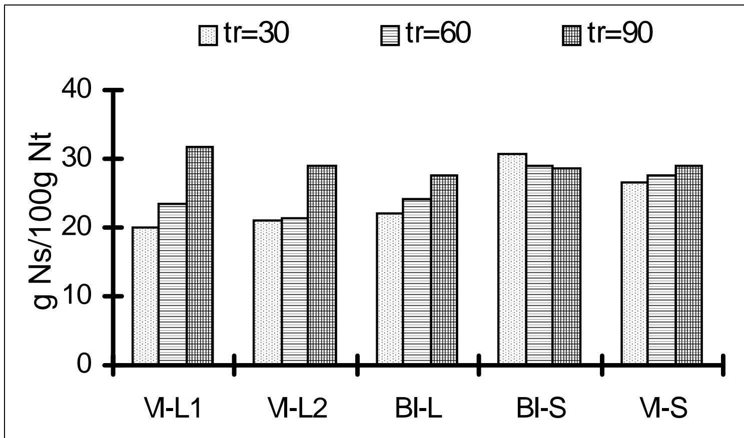

Fig. 2: Soluble nitrogenous at 30, 60 and 90 days of ripening

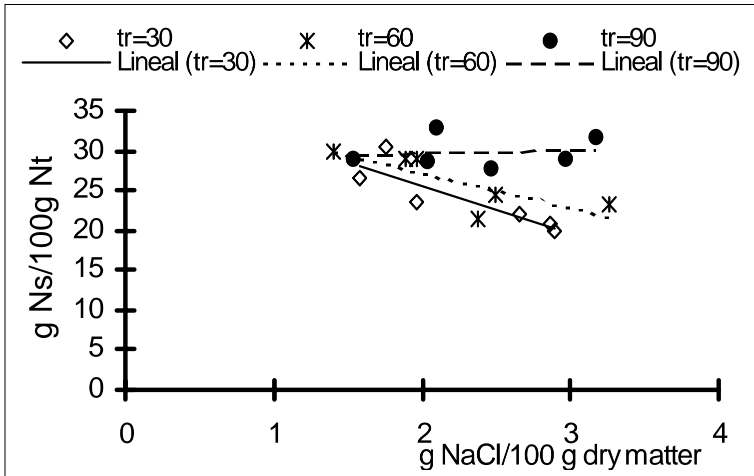

Fig. 3: Soluble nitrogenous vs external salt content

Similar levels of FFA has been obtained for all studied cheeses after 1 month of ripening (mean value = 3.6 meq./100 g cheese). No significant increase of this index was observed afterwards, till the 3 month ripening. It seems that FFA is not a good index to control these cheese development throughout ripening period.

Mechanical properties

Compression test

Compression curves show plastic fracture in ripened cheeses (at 1,2 and 3 moths), but only yield stress was observed for newly salted ones. Plastic fracture become more elastic when ripening and drying is carried on.

Fracture force and distance and apparent elasticity modulus (E).

Almost in all cases, E (Fig.5) and fracture force (Fig.4) increase throughout ripening whereas fracture distance (Fig.4) decrease, so cheeses become more stiff, firm and short throughout ripening (due to structural changes promoted by proteolysis and drying progress). An increase in the toughness is also observed during ripening. The fracture distance (and so cheese shortness) is not significantly affected by salt content and distribution.

Elasticity modulus and fracture force of cheeses salted by BI are affected by salt content. The higher the salt content the higher the stiffness of the cheese. However the tendencies when VI process was applied are not so clear due to the overlapped effect of vacuum treatment in the mechanical properties of cheese piece. It was observed that long vacuum treatments promotes an increase in the fracture force and E values, even for low salt content. Nevertheless, this effect disappears after certain time of ripening. At three months cheeses with lower salt content have lower values of E and fracture force, independently of salting procedure.

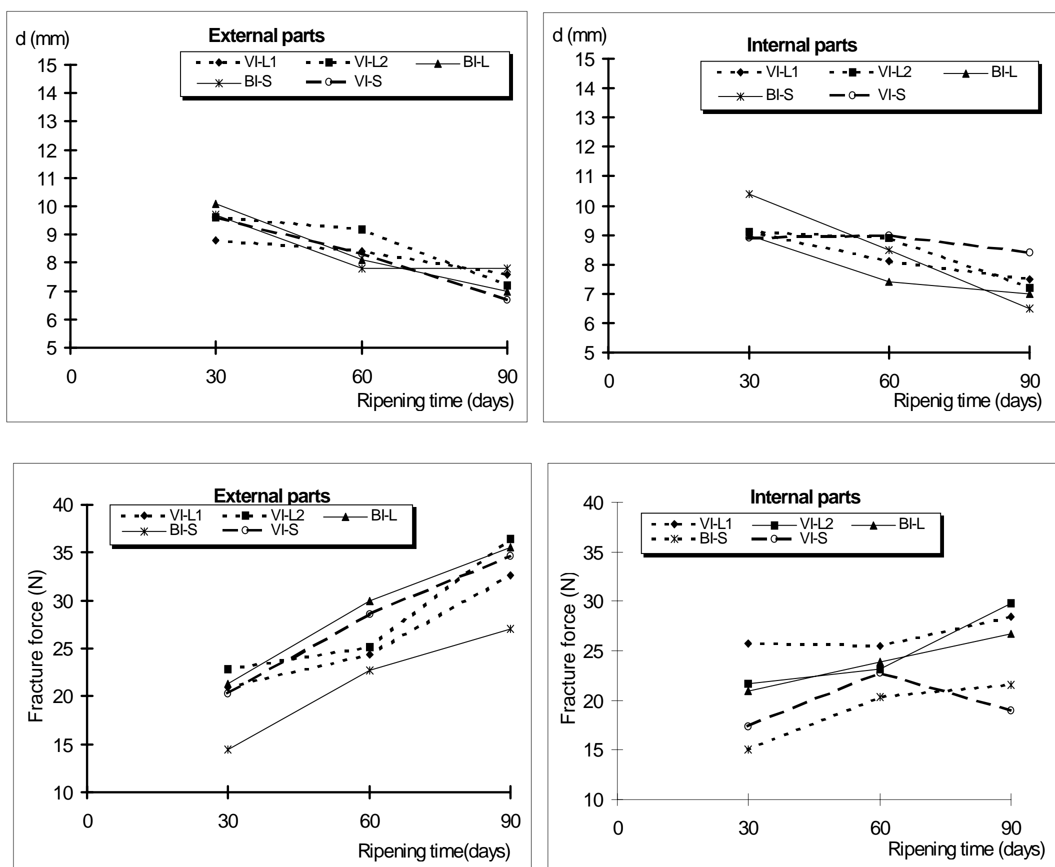

Fig. 4: Development of fracture distance and force during ripening time in the external and internal parts of the cheeses

The influence of vacuum treatments on the mechanical properties of cheeses can be explained in terms of the small spots of air which are eliminated from the curd during the vacuum salting period,

this leading to a more compact cheese structure when restoring the atmospheric pressure (Czulak et al., 1962), whereas the release of whey contributes to reduce the water content, increasing the evenness of moisture contribution (Reinbold and Ernstrom, 1993). When proteolysis progress theses effects are not relevant on mechanical response of cheese as more intense structural changes have been promoted.

The samples from external part have greater variability in the measured mechanical properties because of the less homogeneous water distribution along the cube shaped samples, so values are less useful to observe the effect of salt and salting treatment on mechanical behaviour of cheese.

Differences between the mechanical behaviour of internal and external zones from cheese achieve statistical significance at 3 month ripening, probably due to a less flat water content profiles in the cheeses promoted by drying at this time.

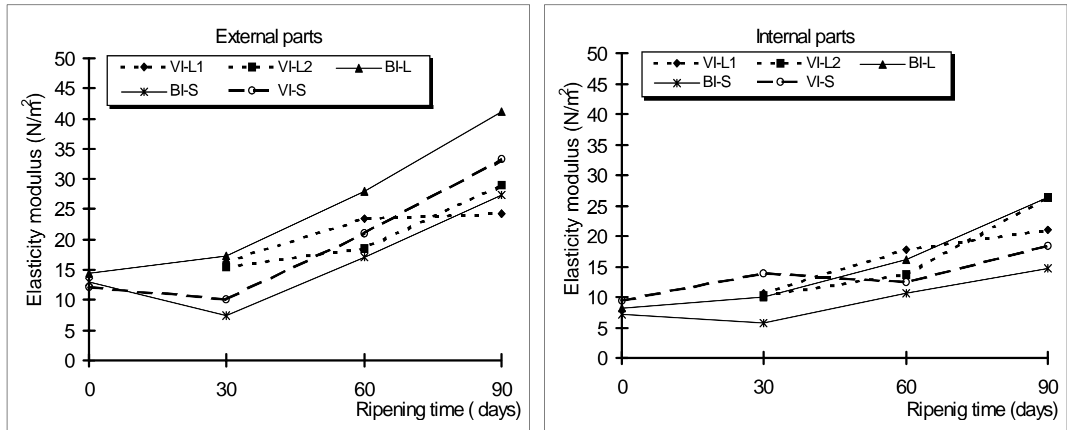

Fig. 5: Elasticity modulus from the initial slope development vs ripening time.

Stress relaxation test

Development of typical relaxation curves during ripening (related directly with the modulus function) are showed in Fig. 6. The increase of the modulus function values (E(t)), observed when ripening time arise, is in agreement with the stiffness increase commented above. The influence of salt content and salting treatment affect the E(t) values at t=0 in the same sense as has been described for E obtained from the compression test.

Both the Peleg (equation 1) and Maxwell models properly fit the relaxation curves, but only Peleg parameters (A and B) are commented due to these are less affected by the mathematical correlation (Fig. 7).

Small changes in the parameter A (level of force reduced during the relaxation) throughout the ripening time were observed in cheeses with higher salt content. Only the cheeses with low salt content show an A decay, principally in the first ripening period (1-1.5 moths). The low salt content will lead to a greater molecular mobility in cheese due to a less water competitive components ratio in the system, and so to a greater stress relaxation.. However the overall structural and compositional changes occurred during ripening seems not affect the A value from a determined salt content or time of ripening.

Relaxation rate (related to B parameter) appears independent from salt content and salting treatment. B values slightly increase in line with the ripening time.

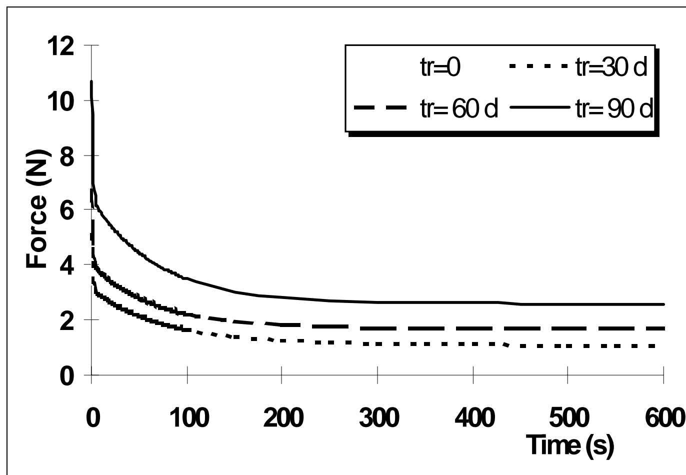

Fig. 6: Typical stress relaxation curves during ripening time

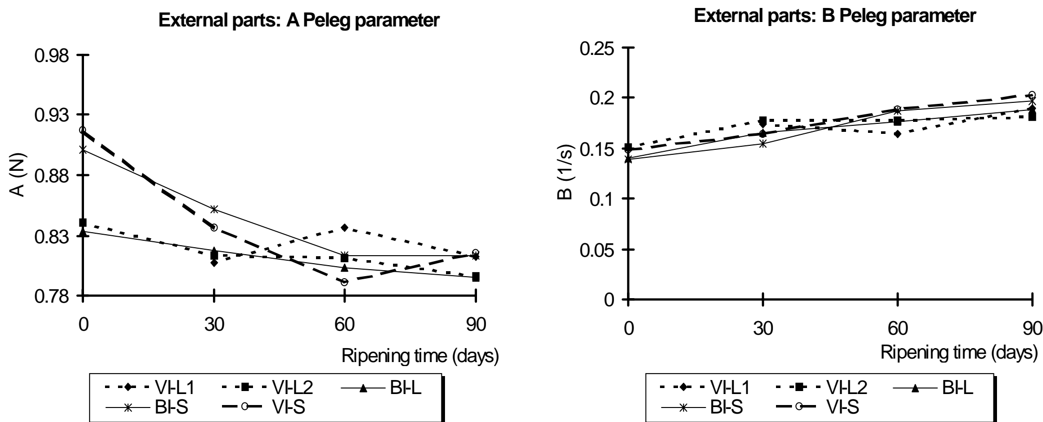

Fig. 7: Development of Peleg parameters during ripening time in the external parts of the cheese.

Conclusions

Differences among cheeses mechanical properties due to salt content or salting method are more important immediately after salting than after the ripening period where intense structural changes occur due to proteolysis and lipolysis progress.

After ripening cheeses become a “more elastic- less viscous” structure as deduced from the increase in the relaxation rate (B) values and the decrease in the relative relaxation levels (A). Moreover, as deduced from the compression test throughout ripening cheeses become stiffer, firmer and shorter.

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Thermal diffusivity of tomato paste

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Abstract

A knowledge of thermal properties is required in all the food processing, storage and preservation processes for optimum design of food processes and processing equipments. In this study thermal diffusivity measurements of tomato paste are carried out by Dickerson's method. Agargel containing 0.3%(w/w) agar was used in order to avoid convective heat transfer within the tube. Thermal diffusivity measurements of the sample at three different levels of moisture content were done with three replicates. Linear equations were obtained for the thermal diffusivity of tomato paste depending on temperature.

1 Introduction

Accurate prediction of temperature history in foods during heating and cooling is of considerable importance for establishing safe and efficient thermal processes. Three fields of application of thermophysical data on foodstuffs are used in different fields of food technology: Design of equipment, plant operation, food quality assessment (Meffert, 1983). Thermal properties are specific heat, thermal conductivity, thermal diffusivity, enthalpy, initial freezing point, surface heat transfer coefficient, etc. It is possible to calculate thermal properties by using prediction equations and to measure them by the application of different methods. Food materials in general, have irregular shapes, non-homogeneous composition and are small. Because of these constraints measurement of thermal properties demands as much ingenuity as basic knowledge in heat transfer. So it is common practice to make simplifying assumptions with regards to sample homogeneity, heat losses, contacts at heat transfer area, similarity to a standard geometry and so on. Thermal diffusivity is an important thermal property in unsteady-state heat transfer applications. This property includes thermal conductivity, specific heat and density, all of which have to be considered for calculations of $\alpha=k/c_p.\rho$. **Table 1.** contains a summary of studies done on thermal diffusivity in the literature.

In this study it is carried out thermal diffusivity measurements of tomato paste by using Dickerson’ s method (Dickerson, 1965).

Table 1: A summary of studies done on thermal diffusivity of foods.

Material	Prediction Model	Reference
corn	$\alpha = 3.095 + 4.22 \times 10^{-6} \times W - 1.06 \times 10^{-7} \times W^2 + 2.15 \times 10^{-7} \times T$	11
wheat	$\alpha = 3.01 \times 10^{-4} + 2.35 \times 10^{-6} \times W + 2.05 \times 10^{-7} \times T$	
barley	$\alpha = 3.09 \times 10^{-4} + 2.84 \times 10^{-6} \times W + 1.98 \times 10^{-7} \times T$	
oats	$\alpha = 3.44 \times 10^{-4} + 1.55 \times 10^{-6} \times W + 1.97 \times 10^{-7} \times T$	
rye	$\alpha = 3.05 \times 10^{-4} + 1.42 \times 10^{-6} \times W + 2.01 \times 10^{-7} \times T$	
rape seed	$\alpha = 1.89 \times 10^{-4} + 7.06 \times 10^{-6} \times W + 2.69 \times 10^{-7} \times T$	
rough rice	$\alpha = 5.52 - 0.201 \times W + 3.96 \times 10^{-3} \times W^2$ (m^2/h)	3
rough rice	$\alpha = 6.4 - 0.279 \times W + 5.64 \times 10^{-3} \times W^2$ (m^2/h)	
wheat	$\alpha = 4.99 - 0.142 \times W + 2.27 \times 10^{-3} \times W^2$ (m^2/h)	
rice flour	$\alpha = 7.21 - 0.329 \times W + 6.12 \times 10^{-3} \times W^2$ (m^2/h)	
sugar cane (AJ-3)	$\alpha = (0.34 + 0.0126 \times W) \times 10^{-7}$	9
sugar cane (trimono)	$\alpha = (0.39 + 0.0119 \times W) \times 10^{-7}$	
sugar cane (common)	$\alpha = (0.35 + 0.0125 \times W) \times 10^{-7}$	
carrot (Pierwszyzbior)	$\alpha = (0.67 + 0.0087 \times W) \times 10^{-7}$	
red beet	$\alpha = (0.63 + 0.0089 \times W) \times 10^{-7}$	
celery	$\alpha = (0.61 + 0.0088 \times W) \times 10^{-7}$	
parsley	$\alpha = (0.61 + 0.0072 \times W) \times 10^{-7}$	
apple GD	$\alpha = 1.46 \times 10^{-7}$ $\alpha = 1.52 \times 10^{-7}$	6
apple cox	$\alpha = 1.34 \times {}^{-7}$ $\alpha = 1.39 \times 10^{-7}$	
swede	$\alpha = 1.38 \times 10^{-7}$ $\alpha = 1.42 \times 10^{-7}$	
onion	$\alpha = 1.41 \times 10^{-7}$ $\alpha = 1.46 \times 10^{-7}$	
apple	$\alpha = 0.14115 \times 10^{-6}$	1
orange	$\alpha = 0.11300 \times 10^{-6}$	
potato	$\alpha = 0.12424 \times 10^{-6}$	
all foods	$\alpha = [0.057363 \times W + 0.000288 \times (T + 273)] \times 10^{-6}$	12
sliced carrot	$\alpha = (1.85 \pm 0.04) \times 10^{-7}$	2
tomato	$\alpha = 1.51 \times 10^{-7}$	7
potato	$\alpha = 1.77 \times 10^{-7}$	
potato	$\alpha = (1.49 \pm 0.08) \times 10^{-7}$	17
pistachio	$\alpha = 51.1 \times 10^{-9} - 0.568 \times 10^{-9} \times W$	8
meat emulsion	$1.168 \times 10^{-7} - 1.245 \times 10^{-7}$ (42-81°C)	14
potato	$\alpha = 0.08$ (cm^2 / min) (10-50°C)	10
emulsion type sausage	$\alpha_L = [2.527 \times W - 0.820 \times \xi - 0.011 \times W^2 - 0.058(\phi)(W)] \times 10^{-9}$ (26-33°C) $\alpha_H = [2.838 \times W + 2.115 \times \xi - 0.014 \times W^2 - 0.059(\phi)(W)] \times 10^{-9}$ (34-40°C)	15
meat analog system	$\alpha = 0.0318 + 0.074 \times W / 100$ (cm^2/min) (71-82°C) $\alpha = 0.0394 + 0.063 \times W / 100$ (cm^2/min) (93°C) $\alpha = 0.0123 + 0.095 \times W / 100$ (cm^2/min) (104-115°C)	16

Note: All the non indicated units are in m^2/s.

2 Material and method

2.1 Material

It is used tomato paste purchased from local market and diluted to different moisture contents by addition of pure water in order to measure the affects of moisture content on thermal diffusivity. Moisture content and ash content of tomato paste is determined by using the methods given in Turkish standards (TSE,1466,1974)

2.2 Method

For the thermal diffusivity measurements it is used Dickerson's method (Dickerson, 1965). A schematic view of thermal diffusivity apparatus is given in **Fig. 1.** It is used a brass sample tube with a diameter of 30 mm ID and 1 mm thickness. Tomato paste samples are filled in the tube by pressing with a paddle in order to place the sample properly. Teflon is used as the material for covers because of its lower thermal conductivity. Between the Teflon cover and the tube ends it is placed silicon seal to prevent water leakage. It is placed T-type thermocouples on the outer surface of the tube and at the centre of the tube through the hole drilled in one of the Teflon covers and the hole is sealed. Water bath with a capacity of 100 L water and with the dimensions of 65*65*33 cm including two heaters (2000 Watts, each) one of which is connected to a thermostat, a stirrer to provide uniform temperature distribution, is used in the system.

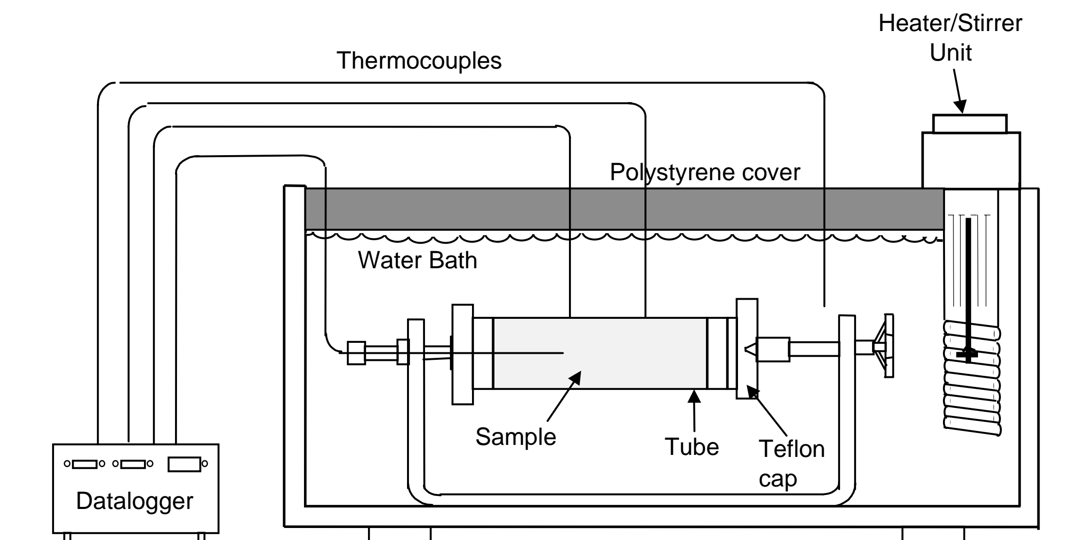

Figure 1: Schematic view of thermal diffusivity apparatus.

During the experiments the tube filled with the material is placed in the water bath and waited until reaching equilibrium temperature. Then it is turned on the heaters and temperatures at the centre and the surface of the tube are recorded. It is plotted temperatures versus time (**Fig.2.)** and using the values taken from the graph it is calculated thermal diffusivity.

Mathematical theory of the calculation depends on the assumption that;

$$\frac{\partial T}{\partial t} = A = \text{const} \tag{1}$$

in Fourier' s unsteady-state heat transfer equation,

$$\frac{\partial T}{\partial t} = \alpha \cdot \left[\frac{\partial^2 T}{\partial r^2} + \frac{1}{r} \cdot \frac{\partial T}{\partial r} \right] \tag{2}$$

The solution of the eq.(2) with the defined initial and boundary conditions results in the following equation giving thermal diffusivity (α), using some experimentally obtained values from **Fig. 2.**. A is the slope of the line surface temperature(°C)-time(s), (T_R-T_0) is the constant difference between the surface and centre temperatures, and R is the radius of the cylindrical tube(m).

$$\alpha = \frac{A \cdot R^2}{4 \cdot (T_R - T_0)} \tag{3}$$

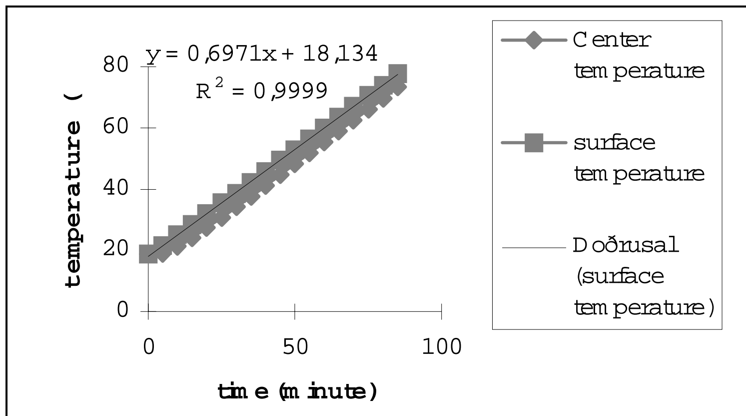

Figure 2: The plot of temperature versus time values recorded during thermal diffusivity measurement experiment.

Sample calculation for agargel in 30 mm diameter tube is shown in **(Fig.3.)**

For the values of A=0,6971 °C/min , R=15 mm, DT= 4,6 °C;

$$\alpha = \frac{0{,}6971 \times (15 \cdot 10^{-3})^2}{4 \times 60 \times 4{,}6} = 1{,}42 \cdot 10^{-7}\ m^2\ /s$$

Figure 3: Sample calculation of thermal diffusivity by the values taken from the graph of temperature versus time of 0.3% agargel.

Heating process takes place up to 85 °C centre temperatures. Calibration of the system is done with agargel 0.3%(w/w).

3 Results and discussion

In order to test the system and to find the effect of the size of the tube, some experiments were carried out with agargel containing 0.3%(w/w) agar in order to avoid convective heat transfer within the tube. The dimensions of the sample tubes made of the same material (brass) used in the experiments and results of the measurements done with agargel and also the comparison of the experimental results with literature value are given in **Table 2.**

Table 2: Effect of the tube dimensions on thermal diffusivity measurements of agargel, and comparison of the results with literature values.

Tube Dimension	Experiment No	Temperature Range (°C)	α (m^2/s)	$\alpha_{average}$ (m^2/s)	Standard Error	Percent Difference (%)
	1	17-83	$\alpha_1 = 1.44.10^{-7}$			
11 mm ID	2	15-80	$\alpha_2 = 1.33.10^{-7}$	$1.40.10^{-7}$	$5.19.10^{-9}$	-9,7
	3	22-80	$\alpha_3 = 1.44.10^{-7}$			
	1	20-73	$\alpha_1 = 1.42.10^{-7}$			
30 mm ID	2	20-80	$\alpha_2 = 1.45.10^{-7}$	$1.47.10^{-7}$	$5.10.10^{-9}$	-2.0
	3	26-80	$\alpha_3 = 1.54.10^{-7}$			
	1	15-86	$\alpha_1 = 1.56.10^{-7}$			
48 mm ID	2	20-86	$\alpha_2 = 1.44.10^{-7}$	$1.49.10^{-7}$	$5.25.10^{-9}$	-0.7
	3	20-83	$\alpha_3 = 1.46.10^{-7}$			

The literature thermal diffusivity value is the one that was calculated by the equation $\alpha = k/\rho.\ cp$, with the values of k, cp and ρ of water at average measurement temperature (Geankoplis, 1993).

The similar results were obtained for the tubes with 30 mm and 48 mm ID and they were in good agreement with the results obtained from the literature. The results for the tube with the ID 11 mm were not found satisfactory because of the large deviation from the reference results. As it requires larger amounts of samples for the with the diameter of 48 mm, it is preferred to use the 30 mm ID tube in the experiments carried out with the tomato paste.

Tomato paste containing 70% water initially, and 10.1% ash (dry basis) was used in thermal diffusivity measurements. It was added water to obtain different concentrations to examine the effects of water content on thermal diffusivity and it was applied oven method to determine moisture contents of samples (TSE, 1466,1974). It was used three levels of moisture content and each experiment was done with three replicates with the same sample. Statistical analysis were applied to obtain standard error of each three replicates. The temperature range was divided into three regions to find the effect of the temperature on the thermal diffusivity. The results were given in **Table 3.** to **5.**

The range of the moisture content of samples was between 73.3-82.8%. It was found insufficient the range selected to understand the effect of moisture content on thermal diffusivity but the effect of temperature was found to be clear. Linear equations were obtained for the thermal diffusivity of tomato paste depending on the temperature. A comparison of linear equations are given in **Table 6.**

Table 3: Thermal diffusivity values of tomato paste having 73.3% (w/w) water and comparison with the literature* value.

Experiment No	Temperature Range (°C)	α (m^2/s)	$\alpha_{average}$ (m^2/s)	Standard Deviation	Percent Difference (%)
1	25-38 41-53 56-72	α_1= 1.11.10^{-7} α_2= 1.26.10^{-7} α_3= 1.46.10^{-7}	1.28.10^{-7}	1.43.10^{-8}	_
2	20-37 40-53 55-68	α_1= 1.11.10^{-7} α_2= 1.26.10^{-7} α_3= 1.43.10^{-7}	1.27.10^{-7}	1.31.10^{-8}	_
3	27-40 43-52 55-74	α_1= 1.36.10^{-7} α_2= 1.43.10^{-7} α_3= 1.55.10^{-7}	1.45.10^{-7}	7.85.10^{-9}	_
average	25-38 41-53 55-72	α_1= 1.19.10^{-7} α_2= 1.32.10^{-7} α_3=1.48.10^{-7}	1.33.10^{-7}	8.26.10^{-9}	-1.5

Literature thermal diffusivity value for tomato paste having %73.3 MC(w/w) ($\alpha_{literature}$=1.35.10^{-7} m^2/s) is calculated by the equation $\alpha = \left[0.0574.W + 0.000288(T + 273)\right] \times 10^{-6}$, W being the % (w/w) moisture content, and T is the average temperature of the product.(Martens, 1980; Magee and Bransburg, 1995).

Table 4: Thermal diffusivity values of tomato paste having 74.7% (w/w) water and comparison with the literature* value.

Experiment No	Temperature Range (°C)	α (m^2/s)	$\alpha_{average}$ (m^2/s)	Standard Deviation	Percent Difference (%)
1	17-36 39-51 54-83	α_1= 1.06.10^{-7} α_2= 1.28.10^{-7} α_3= 1.70.10^{-7}	1.35.10^{-7}	2.65.10^{-8}	_
2	17-35 38-53 56-77	α_1= 1.13.10^{-7} α_2= 1.40.10^{-7} α_3= 1.90.10^{-7}	1.4810^{-7}	3.19.10^{-8}	_
3	18-35 38-51 53-65	α_1= 1.09.10^{-7} α_2= 1.16.10^{-7} α_3= 9.96.10^{-8}	1.08.10^{-7}	6.72.10^{-9}	_
average	17-35 38-51 54-77	α_1= 1.09.10^{-7} α_2= 1.28.10^{-7} α_3=1.53.10^{-7}	1.30.10^{-7}	1.67.10^{-8}	-4.4

Literature thermal diffusivity value for tomato paste having %74.7 MC(w/w) ($\alpha_{literature}$=1.36.10^{-7} m^2/s) is calculated by the equation $\alpha = \left[0.0574.W + 0.000288(T + 273)\right] \times 10^{-6}$, W being the % (w/w) moisture content, and T is the average temperature of the product.(Martens, 1980; Magee and Bransburg, 1995).

Table 5: Thermal diffusivity values of tomato paste having 82.8% (w/w) water and comparison with the literature* value.

Experiment No	Temperature Range (°C)	α (m^2/s)	$\alpha_{average}$ (m^2/s)	Standard Deviation	Percent Difference (%)
	19-31	α_1= $1.27.10^{-7}$			
1	34-50	α_2= $1.36.10^{-7}$	$1.39.10^{-7}$	$1.17.10^{-8}$	_
	53-75	α_3= $1.55.10^{-7}$			
	14-30	α_1= $1.10.10^{-7}$			
2	33-51	α_2= $1.28.10^{-7}$	$1.31.10^{-7}$	$1.85.10^{-8}$	_
	54-71	α_3= $1.55.10^{-7}$			
	16-31	α_1= $9.62.10^{-8}$			
3	34-49	α_2= $1.15.10^{-7}$	$1.19.10^{-7}$	$2.10.10^{-8}$	_
	52-73	α_3= $1.47.10^{-7}$			
	16-31	α_1= $1.11.10^{-7}$			
average	34-50	α_2= $1.26.10^{-7}$	$1.30.10^{-7}$	$8.22.10^{-9}$	-7.1
	53-73	α_3=$1.52.10^{-7}$			

Literature thermal diffusivity value for tomato paste having %82.8 MC(w/w) ($\alpha_{\lambda\iota\tau\epsilon\rho\alpha\tau\upsilon\rho\epsilon}$=$1.40.10^{-7}$ m^2/s) is calculated by the equation $\alpha = \left[0.0574.W + 0.000288(T + 273)\right] \times 10^{-6}$, W being the % (w/w) moisture content, and T is the average temperature of the product.(Martens, 1980; Magee and Bransburg, 1995).

Table 6: Linear equations of tomato paste depending on the temperature.

% MC of tomato paste	a value#	b value#	correlation coefficients
73.3	9E-08	9E-10	0.9983
74.7	8E-08	1E-09	0.9982
82.8	9E-08	1E-09	0.9863

Coefficients of linear equations with the form α=a+b$\times$T .

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Thermal conductivity measurements of granular and powdered foods

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Abstract

Thermal conductivity is one of the important thermophysical properties for the foods subjected to heat transfer operations during storage, or processing. In this study thermal conductivity of different kinds of granular and powdered foods (red lentil and chickpea and their flours in different particle sizes) in bulk are measured. Hot wire prob method was used for measuring thermal conductivity values. It is observed that thermal conductivity of granular and powdered foods increase with increasing bulk density and moisture content. By multiple regression analysis empirical equations between thermal conductivity, moisture content and bulk density are obtained. Experimental results were compared with various porous media thermal conductivity models and it was found that „Parallel“ and „Woodside“ are the most suitable ones for the samples used in this study.

Introduction

A knowledge of thermal properties and prediction of time-temperature histories of granular foods, such as grains, cereals, seed, are required in all the food processing, storage and preservation processes. Such data are essential and useful for optimum design of food processes and processing equipments. Among thermal properties, thermal conductivity of foodstuffs and its relationship to moisture content is one of the most important transport properties required to model processes in which mass and energy are exchanged. Typical examples are drying processes, rehydration processes and moisture pickup by packaged dried foods during storage.

The measurement and modelling of bulk thermal conductivity of granular food materials is a challenge as it depends not only on moisture as well as composition, but on the structure or physical arrangement of the sample (Perez and Calvelo, 1984; Sweat, 1986). The structural characteristics which affect conductivity include the number and size of voids, heterogeneities, impurities, particle to particle contact and fiber orientation. The presence of air voids between grains complicates the process of heat transfer, air impedes the flow of heat and, consequently, the process involves not only thermal conduction but convection and radiation as well. However, it is known that with the value of $(Gr.Pr)<10^3$ convective heat transfer in pores can be neglected (Luikov, 1964). According to Woodside (1958), the effect of convection becomes significant for particles with diameters larger than 1 cm. At temperatures above 200°C (Deissler and Boegli, 1958; Wilhelm et al.,1948), as well as for larger grains, radiation also contributes to thermal conductivity in granular materials. Heat transfer models for granular porous materials are applicable for granular food materials as well, most of these models neglect the effects of convection in pores and the effects of radiation heat transfer between particles. All the models predict the effective bulk thermal conductivity (k_e) of porous materials as function of thermal conductivity of the solid phase (k_s) and fluid phase (k_f) and also of porosity (ε) which is defined as the ratio of the fluid volume to the total volume. In the present study the solid phase is the grain and its thermal conductivity (k_s) may be estimated from the thermal conductivity and mass percent (X) of the constituents by the equation proposed by Dominguez et al., (1974):

$$k_s = 0.61X_w + 0.20X_p + 0.205X_c + 0.175X_{fat} + 0.135X_a \quad (1)$$

Where, the subscripts stand for various components: w=water, p=protein, c=carbohydrate, fat=fat, a=ash and 0.61, 0.20, 0.205, 0.175, 0.135 are respectively the thermal conductivities of water, protein, carbohydrate, fat and ash in W/m.K , at room temperature (25°C). The fluid phase is the air between solid grains and has thermal conductivity k_f=0.026 W/m.K at 25°C. Some of the models take further into consideration such parameters as the shape of the solid particles, the orientation and distribution of the particles, the contact resistance between the particles.

A brief survey of the literature reveals us that thermal conductivity of granular food increases linearly with moisture content according to the equation given below:

$$k_e = a + bX_w \quad (2)$$

where, X_w is the moisture content in wet basis (%), expressed as:

$$X_w(\text{w.b.}) = \frac{\text{moisture weight}}{\text{dry matter weight} + \text{moisture weight}} \times 100 \quad (3)$$

More recent work on thermal conductivity of granular food materials has been carried out by Halliday et al. (1995), Bilanski and Fisher (1976), Tavman and Tavman (1988), Dutta et al. (1988), Sharma & Thompson (1973), Kustermann et al. (1981), Chang (1986), Zuritz et all. (1989), they all determined a linear increase of thermal conductivity with moisture content. Similarly linear equations between thermal conductivity and bulk density are given in the literature (Chang (1986), Chang et al. (1980), Kusterman et al. (1981), Chuma et al. (1981), Tavman and Tavman (1997)).

Conduction models

The maximum value of effective thermal conductivity is estimated by the model in which the solid and fluid phases are arranged in alternate layers parallel to the direction of heat flow. In this model, the effective thermal conductivity is given by the weighted arithmetic mean of the thermal conductivities of the two phases:

$$k_e = \varepsilon.(k_f) + (1-\varepsilon).k_s \quad (4)$$

The minimum value of effective thermal conductivity occurs for the series distribution in which the solid and the fluid phases are in layers normal to the direction of heat flow. In this case the effective thermal conductivity is given by the weighted harmonic mean of the solid and fluid conductivities:

$$k_e = \frac{k_s.k_f}{\varepsilon.k_s + (1-\varepsilon).k_f} \quad (5)$$

The geometric mean model assumes random distribution of phases, the effective thermal conductivity by the weighted geometric mean of the conductivities of the two phases:

$$k_e = k_f^{\varepsilon}.k_s^{1-\varepsilon} \quad (6)$$

Maxwell (1954), using potential theory for electrical conduction through heterogeneous media obtained an exact solution for the conductivity of randomly distributed and noninteracting homogeneous solid spheres in a homogeneous continuous medium:

$$k_e = k_f \cdot \frac{2\varepsilon.k_f + (3-2\varepsilon).k_s}{(3-\varepsilon).k_f + \varepsilon.k_s} \tag{7}$$

This equation is applicable only when the porosity is large, since it was derived on the assumption that the solid spheres are far enough apart so that they do not mutually interact. For granular food materials porosities are generally in the range 0.3 to 0.5 so that the Maxwell equation is not directly applicable for thermal conductivity estimation for such materials.

The model proposed by Woodside and Messmer (1961) is a combination of the series and parallel distributions. They use the electrical conductivity analogy of an aggregate of conductive particles saturated with conducting electrolyte, to derive a modified resistor model equation to predict the effective thermal conductivity of porous media:

$$k_e = \frac{a.k_s.k_f}{k_s.(1-d)+d.k_f} + c.k_f \tag{8}$$

where, $c = \varepsilon - 0.03$, $a = 1 - c$, $d = \frac{1-\varepsilon}{a}$

The expression for "c" is calculated by using the experimental data of Stephenson and Woodside (1958) for a model of a cubic pack of spheres at a porosity of 47.6%.

Krupiczka (1967) derived a numerical solution for effective thermal conductivity of granular materials using first a model made up of long cylinders ($\varepsilon = 0.215$), and then another model made up of spheres in cubic lattice ($\varepsilon = 0.476$). In view of the complicated character of the formulas obtained, he approximated the two solutions by a general correlation which takes into account the effect of porosity:

$$k_e = k_f \cdot \left(\frac{k_s}{k_f}\right)^{A+B.\log\left(k_s/k_f\right)} \tag{9}$$

$A=0.280-0.7571.\log\varepsilon$ $\qquad$ $B=-0.057$

The correlation formula obtained is valid for $0.251 \le \varepsilon \le 0.476$, but it can also be used without too much error for porosities near the region under considerations. For the samples used in this study (porosity between $\varepsilon=0.20$ and 0.6), the effective thermal conductivity values calculated from Maxwell, Woodside and Messmer and geometric mean models, as well as the maximum and minimum values calculated by the parallel and series phase distribution models, are compared for all of the samples. The results of the calculations are given in Table 2. In the region of interest for this study, that is for k_s/k_f about 10, only parallel and Woodside & Messmer models predict acceptable values, comparable to each other.

Material

In this study different kinds of granular and powdered foods (red lentil and chichpea and their flours in different particle sizes) are measured in bulk at different moisture contents and bulk densities. The relationship between thermal conductivity and moisture content and bulk density was obtained by application of regression analysis to the results. Samples of various moisture contents are obtained

by soaking kernels in water and placed in airtight plastic bags for subsequent thermal conductivity measurements. Red lentil seeds used in this study were with 4.5 mm average diameter and 2.7 mm thickness and chickpea was with 8.5 mm average diameter. Red lentil flour (LF) and chickpea flour (CPF) were obtained by application of size reduction process in a laboratory scale hammer mill and then subjected to screening in order to divide into groups of different sizes. The size of each group are given in Nomenclature. Chemical composition of each group of the material are determined by laboratory experiments done according to AOAC, 1990 and given in Table 1.

Table 1: Chemical composition of samples used in this study

FOOD	MOISTURE CONTENT (%)	ASH (%)	FAT (%)	PROTEIN (%)	CARBONHYDRATE (%)
Red lentil seeds	10.23	2.63	1.06	27.86	58.22
LF1	10.54	2.08	0.71	24.15	62.52
LF2	10.67	2.16	0.7	27.57	58.9
LF3	10.35	2.05	0.8	28.91	57.89
LF4	10.77	2.04	0.93	26.81	59.45
Chickpea	9.96	2.95	5.51	20.83	60.75
CPF1	10.06	2.98	3.44	19.09	64.44
CPF2	10.53	2.62	5.31	22.46	59.08
CPF3	9.74	2.42	5.78	21.99	60.08
CPF4	10.85	2.65	4.67	20.26	61.57

Thermal conductivity measurements

A modified hot wire method is used to measure the effective thermal conductivity of the granular food materials. In the conventional hot wire method, a line source, i.e., a thin straight wire through which a constant electric current is passed generating constant heat is embedded in the sample under test. When the sample and the wire is at uniform and constant temperature, constant power is supplied to the heater element and the temperature rise of the heating wire is measured by a thermocouple and recorded with respect to time during a short heating interval, the thermal conductivity of the sample from the temperature-time record and power input as:

$$k = \frac{Q.\ln(t_2/t_1)}{4\pi(T_2 - T_1)} \qquad (10)$$

where, T_1 and T_2 are temperatures at times t_1 and t_2, Q is the heat flow per unit time, per unit length of the heating wire. The values $d(\Delta T)/d(\ln t)$ can be read from the straight line portion of a plot of ΔT versus ln(t) obtained from a test run recording of ΔT versus t. In our experiments, a modified hot wire technique is used, the heating wire is placed between two rectangular shaped materials, the first one is an insulating material of known thermal properties which is a part of the measuring probe and the second one is the granular sample placed in a rectangular shaped sample holder of dimensions 10cm length, 3cm width and 4cm height (Figure 1).

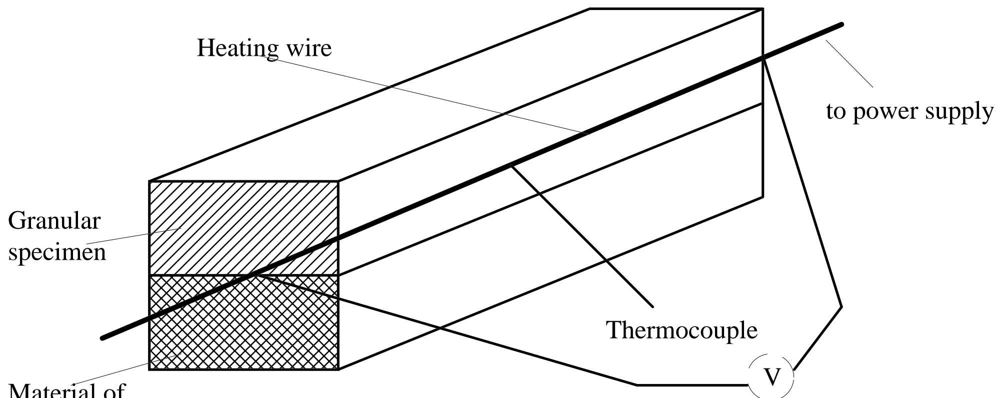

Figure 1: Thermal conductivity measuring probe

The error which may arise by the variation of the resistance of the heating wire with temperature, causing a nonconstant power input, is made negligible by using a heater wire with a low temperature coefficient of resistance. The measurement takes place in 10 to 90 seconds after heating starts at the wire. In this case, the thermal conductivity is given by the following equation;

$$k = F.\frac{Q.\ln(t_2/t_1)}{T_2 - T_1} - H \tag{11}$$

where, F and H are specific constants of the probe to be determined with materials of known thermal conductivities. By this method, the thermal conductivity is measured with an accuracy of ±5% and reproducibility of ±2%. For each specimen the thermal conductivity is measured five times and the mean values are reported.

Results and discussion

The results of thermal conductivity measurements as function of moisture content (w.b.) and bulk density , for the samples are shown in figures 2 to 5. All measurements are performed at room temperature, at about 25°C. For each specimen, the thermal conductivity is measured five times and the mean values are considered. The estimated accuracy of the measurements is ±5% and the reproducibility is within ±2%. The bulk thermal conductivity values of all the samples appeared to be linearly dependent on the moisture content and bulk density therefore, were analyzed by linear regression. The regression equations are given in Table 3.

Table 2: Comparison of standard deviation between experimental thermal conductivities and thermal conductivity models

Food	Porosity	Perpendicular	Geometric	Maxwell	Krupiczka	Woodside	Parallel
Chickpea	0.321	0.090	0.053	0.064	0.067	0.042	0.015
CPF1	0.473	0.055	0.030	0.036	0.037	0.016	0.009
	0.435	0.055	0.027	0.034	0.030	0.013	0.013
CPF2	0.473	0.056	0.032	0.038	0.039	0.017	0.008
	0.435	0.055	0.027	0.034	0.036	0.012	0.013
	0.414	0.053	0.024	0.031	0.034	0.009	0.016
CPF3	0.564	0.050	0.031	0.035	0.034	0.018	0.005
	0.533	0.049	0.029	0.034	0.033	0.015	0.008
	0.497	0.050	0.027	0.033	0.033	0.013	0.011
CPF4	0.650	0.044	0.030	0.033	0.029	0.020	0.002
	0.596	0.044	0.027	0.031	0.028	0.023	0.008
	0.523	0.045	0.023	0.029	0.028	0.016	0.015
Red lentil seeds	0.318	0.077	0.040	0.050	0.054	0.028	0.001
	0.295	0.077	0.037	0.048	0.052	0.027	0.001
	0.296	0.075	0.034	0.046	0.049	0.025	0.002
LF1	0.370	0.051	0.018	0.027	0.030	0.004	0.023
	0.348	0.052	0.017	0.027	0.030	0.005	0.022
	0.325	0.051	0.014	0.024	0.028	0.002	0.025
	0.299	0.050	0.011	0.022	0.025	0.000	0.027
LF2	0.390	0.053	0.021	0.030	0.032	0.007	0.020
	0.347	0.049	0.014	0.024	0.027	0.001	0.026
LF3	0.390	0.055	0.023	0.032	0.034	0.009	0.017
	0.347	0.053	0.018	0.028	0.031	0.005	0.021
LF4	0.525	0.053	0.032	0.037	0.036	0.018	0.007
	0.491	0.053	0.029	0.035	0.035	0.014	0.011
	0.451	0.053	0.026	0.033	0.034	0.011	0.015
	0.405	0.050	0.020	0.028	0.030	0.005	0.021

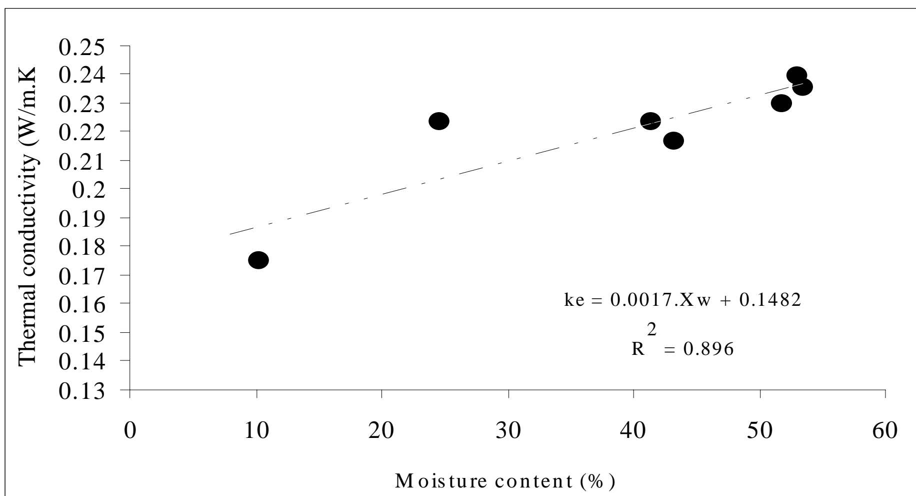

Figure 2: Graph for thermal conductivity vs. moisture content of red lentil seeds in bulk.

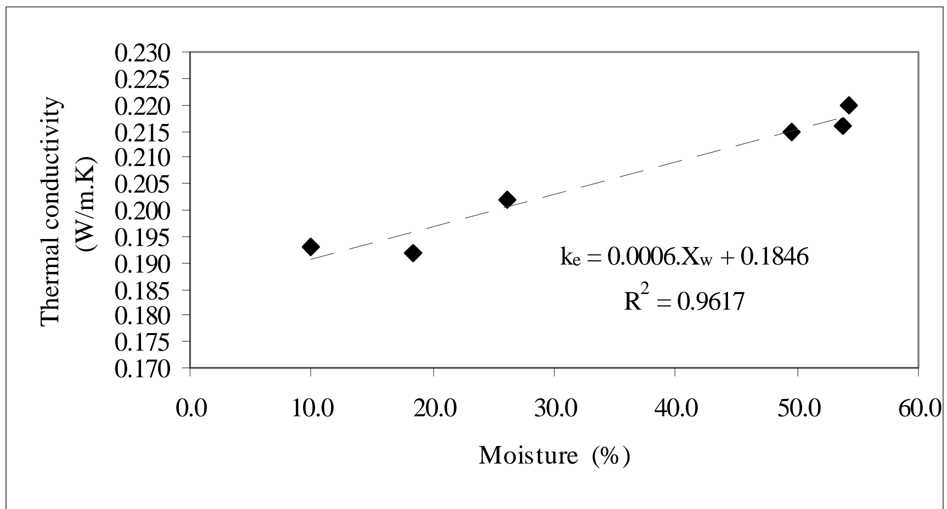

Figure 3: Graph for thermal conductivity vs. moisture content of chickpeas in bulk.

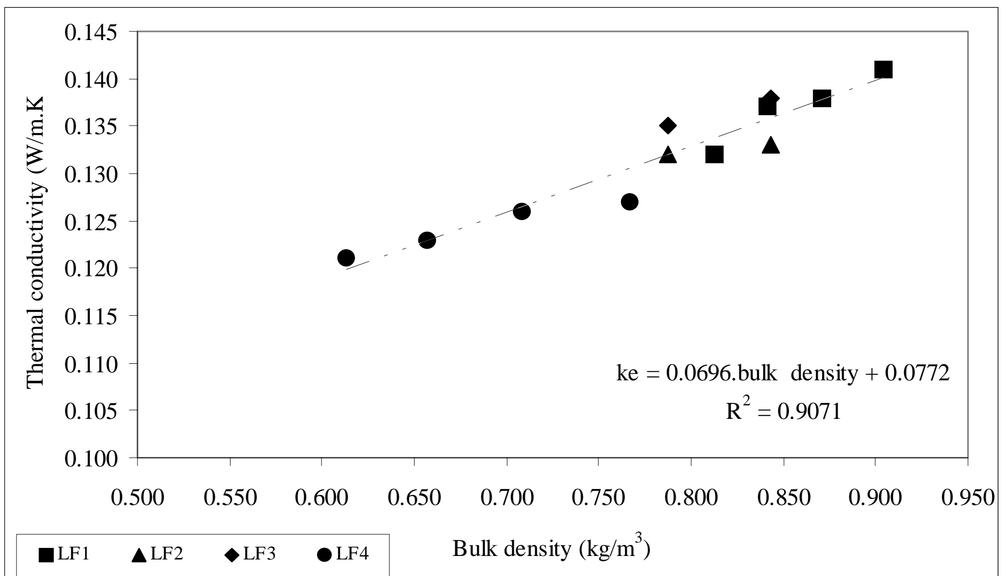

Figure 4: Graph for thermal conductivity vs. bulk density of red lentil seed flours in bulk.

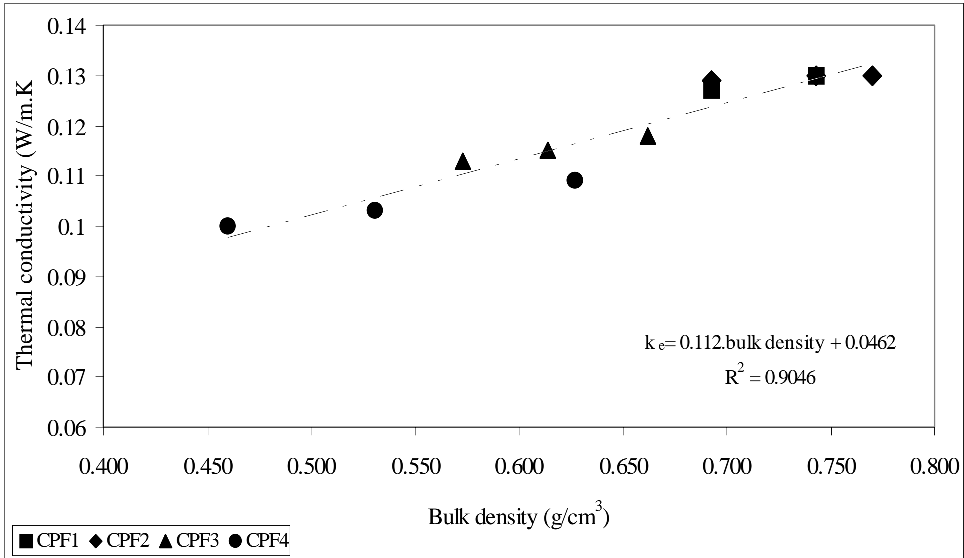

Figure 5: Graph for thermal conductivity vs. bulk density of chickpea flours in bulk

Table 3: Thermal conductivity results and equations of different kinds of granular and powdered foods used in this study

FOOD	X_w (%)	ρ_{bulk} (g/cm^3)	k_e (w/m.K)	EQUATIONS	R^2
Red lentil seeds	10.23	0.880-0.943	0.175-0.181	k_e=0.0901.ρ_{bulk}+0.0961	0.9996
LF1	10.54	0.813-0.904	0.132-0.141	k_e=0.0922.ρ_{bulk}+0.0961	0.8922
LF2	10.67	0.787-0.843	0.132-0.133	*	
LF3	10.35	0.787-0.843	0.135-0.138	*	
LF4	10.77	0.613-0.767	0.121-0.127	k_e=0.0422.ρ_{bulk}+0.00952	0.9654
All red lentil flours	**10.54-10.77**	**0.613-0.904**	**0.121-0.141**	**k_e=0.0696.ρ_{bulk}+0.0772**	**0.9071**
Chickpea	9.96	0.880	0.193	*	
CPF1	10.06	0.693-0.743	0.127-0.130	*	
CPF2	10.53	0.693-0.770	0.129-0.130	k_e=0.204.ρ_{bulk}+0.1145	0.9346
CPF3	9.74	0.573-0.662	0.113-0.118	k_e=0.00549.ρ_{bulk}+0.0813	0.9966
CPF4	10.85	0.460-0.627	0.100-0.109	k_e=0.0564.ρ_{bulk}+0.0734	0.9869
All ckickpea flours	**9.92-10.53**	**0.460-0.743**	**0.100-0.130**	**k_e=0.112.ρ_{bulk}+0.0462**	**0.9046**
Chickpea	9.96-54.27	0.673-0.880	0.193-0.220	k_e=0.0006.X_w+0.1845	0.9582
Red lentil seeds	10.23-53.4	0.700-0.880	0.175-0.236	k_e=0.0017.X_w+0.1482	0.896
Chickpea	**9.96-54.27**	**0.673-0.880**	**0.193-0.220**	**k_e=0.0010.X_w+0.1290.ρ_{bulk}+0.076**	**0.8468**
Red lentil seeds	**10.23-53.4**	**0.700-0.880**	**0.175-0.236**	**k_e=0.0021.X_w+0.1582.ρ_{bulk}+0.010**	**0.9391**

*It was not possible to obtain a correlation between bulk density and thermal conductivity because thermal conductivities for only two bulk density values were available.

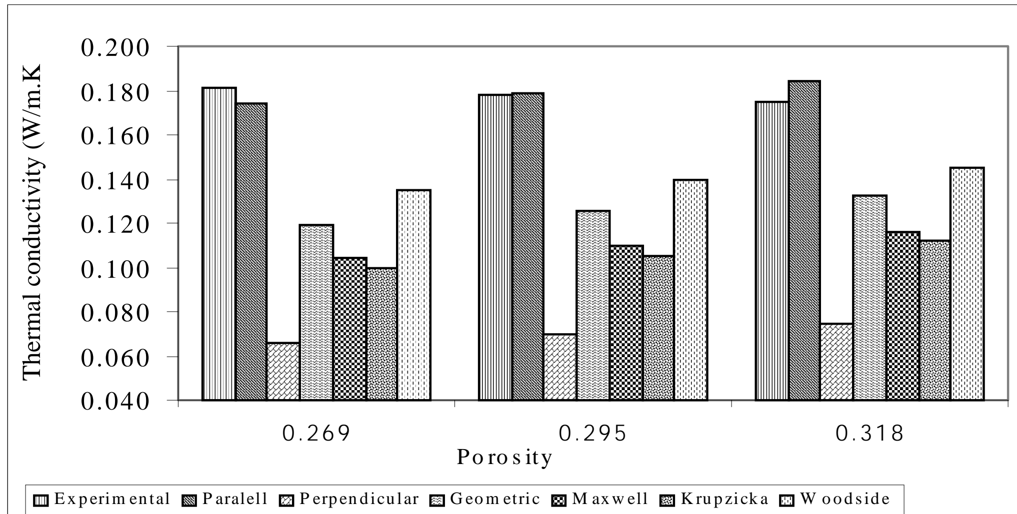

Figure 6: Comparison of experimental thermal conductivities and thermal conductivity models for red lentil seeds in bulk

Conclusions

In this study, the variation of thermal conductivity of red lentil seeds and chickpea are investigated in bulk as a function of moisture content and bulk density. The results show that, the bulk thermal conductivity is a linear function of the moisture content and bulk density. The equations giving correlation between thermal conductivity, moisture content and bulk density are obtained for granular forms of the material. Thermal conductivity models given for porous media are applied to our experimental data and by comparing standard deviations from the models; Parallel model and Woodside & Messmer model was found to be the best ones explaining the dependence of effective thermal conductivity on porosity.

Nomenclature

k :thermal conductivity (W/m.K)
X_w :moisture content, wet basis (%)
Q :heat flow per unit area per unit time (W/m²)
T :temperature (°C)
t :time (s)
X : mass percent
Q :Heat, W
T :Temperature, K
k_e :Efecctive thermal conductivity, W/m.K
ε :Porozite
k_s :Thermal conductivity of solid phase, W/m.K
k_f :Thermal conductivity of fluid phase, W/m.K
ρ_b: Bulk density, g/cm^3
LF1 :Red lentil flour which retains on 355 μm sieve

LF2 :Red lentil flour which retains on 212 μm sieve
LF3 :Red lentil flour which retains on 63 μm sieve
LF4 :Red lentil flour which retains under 63 μm sieve
CPF1 :Chickpea flour which retains on 355 μm sieve
CPF2 :Chickpea flour which retains on 212 μm sieve
CPF3 :Chickpea flour which retains on 63 μm sieve
CPF4 :Chickpea flour which retains under 63 μm sieve

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Determination of heat inactivation kinetic parameters of a *bacillus cereus* pathogenic strain isolated from foods

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Abstract

A *Bacillus cereus* enterotoxigenic strain isolated from cooked chilled foods was characterised in relation to safe food production. Kinetic parameters of the heat resistance of spores were determined by mean of an one step non linear regression and were compared with those calculated by using two linear regression, the traditional method. $D_{90°C}$ value was 39 min and the minimal temperature growth was 10°C. The results showed that this new methodology of analysis applied in this study can improve the accuracy in the estimation of D and z values and, therefore, the characterisation of these high heat resistant pathogenic strains in order to improve the development of heat processes for foods.

Introduction

Bacillus cereus has been established as a food poisoning agent, implicated in two types of foodborne illness, diarrhoeal or vomiting (Gilbert, 1979, Tunrbull, 1981). The vomiting type is caused by an emetic toxin which is produced when cells grow in the food. The diarrhoeal type is attributed to the production of an heat labile enterotoxin. Some authors have reported that this toxin is produced during the vegetative growth of *B. cereus* in the small intestine rather than by preformed enterotoxin, (Gilbert 1979, Thompson et al 1984, Shinagawa et al 1991). The toxin production has to be avoided in the product and hygienic points to prevent the conditions which allow *B. cereus* spores to germinate and multiply. Main factors contribute to *B. cereus* food poisoning in food processing and distribution chain are: (1) inadequate heating process which is insufficient to destroy most of *B. cereus* spores, (2) maintenance of processed products without adequate refrigerator temperature, (3) temperature abuse of foods which are prepared several hours in advance of serving and stored at unsatisfactory refrigeration and (4) post-contamination of the processed food, either by the environment or especially when add ingredients that are subjected to a minimal heat treatment (Kramer and Gilbert 1989).

Presence of certain pathogenic strains resistant to pasteurisation temperatures and able to grow at 6-8°C (Van Netten et al., 1990, Dufrenne et al, 1995) is of increasing concern to the food companies. Until now, little information has been available about the properties of pathogenic strains of *B. cereus* which both, are able to grow at refrigeration temperatures and are high resistant to heat. There is therefore the necessity to develop predictive models of inactivation and growth and to improve the way for the quantitative analysis of the risk in order to define the probability that a particular hazard occurs. For developing of predictive models of inactivation by heat, appropriate kinetic data are necessary. Currently, kinetic parameters of thermal inactivation of microorganisms are calculated by means of two linear regressions. In the first step, D value is obtained, and in the second step, from another linear regression, z value is deduced. Parameter estimation can be improved by a non linear regression instead of the traditional two-step method. The two-step method

is mathematically more simple, but one-step method gives a smaller confidence interval because there is a more complete utilisation of raw data for estimating kinetic parameters (Arabshahi and Lund 1985).

In the present work, the heat inactivation, under isothermic heating conditions, of one pathogenic strain of *Bacillus cereus* spores isolated from foods was studying. The obtained data were analysed by the traditional methodology, two lineal regressions, and by 'one step non linear regression'.

Materials and methods

Bacterial strain and sample preparation

In the present study, a *Bacillus cereus* strain, AV Z421 isolated from cooked chilled foods and provided by the Station de Technologie des Produits Vegetaux (Institut National de la Recherche Agronomique, Avignon, France) was used. This strain was positive for toxin production by TECRA and OXOID tests. According to these tests this strain is diarrhoeal type.

Spore suspension was prepared on fortified nutrient agar (FNA) (Jonhson *et al* 1982, Mazas *et al* 1995) slightly modified, as sporulation medium, when achieved 90% the sporulation rate, spores were harvested and stored at 4°C in distilled water.

Minimal growth temperature

B. cereus strain AV Z421 was grown in TSB at 30°C for 24 h and 0.05 ml of the culture growth was transferred to tubes containing 10 ml precooled Nutrient Broth. The tubes were then placed in a water bath at refrigerator temperatures of 5°C, 8°C and 10°C. After 4 weeks the tubes were visually tested for turbidity. Increase in number of *B. cereus* was checked on Nutrient agar plates incubated at 30°C for 24 h.

Thermal treatment

The thermal treatment was carried out by the capillary tubes technique of Stern and Proctor (1954) suspending the spores in double distilled water as substrate. Samples were heated under isothermal treatment at temperatures ranging between 85 and 105°C. In all cases, spores were activated by a mild heat (15 min 85°C). Viable counts were based on duplicate counts from appropriated dilution in nutrient agar (Gonzalez *et al* 1995) supplemented with starch as recovery medium, adjusted to pH 6.8 (Gonzalez *et al* 1996) and incubated at 30°C for 24 hours.

Estimation of kinetics parameters

D and z values were estimated by non linear regression with equation:

$$\log N = \log No - \frac{1}{D_R \cdot 10^{\left(\frac{T_R - T}{z}\right)}} \cdot t$$

In the non linear regression, an iterative least-squares analysis was used to find the best estimates of the kinetic parameters, minimising the sum of squares until convergence using the following equation:

$$SSQ = \sum_{i=1}^{m} \left[\log\left(\frac{No}{N}\right)_f - \log\left(\frac{No}{N}\right)_m \right]^2$$

where, SSQ= sum of squares; m= number of processes runs; $(N_0/N)_f$= ratio of predicted number of microorganism fitted by the model (at $t=t_f$); $(N_0/N)_m$= number of microorganism measured (at $t=t_f$) and t_f= total process time.

Calculation of joint confidence regions

One of the procedures most commonly used to calculate confidence ranges of parameters estimated by non linear regression is the construction of joint confidence regions for the parameters. This consists of calculating the sum of squares that defines an ellipse where combinations of real kinetic parameters have a determined probability (generally 90 %) of being inside. The sum of squares (SQ) is calculated with the result of the non linear regression according to the following equation (Draper and Smith 1981):

$$SQ = SSQ\left[1 + \frac{p}{m-p} F_{\propto}(p, m-p)\right]$$

where, SQ= sum of squares calculated for a given confidence level; SSQ= optimum sum of squares residual; p= number of parameters; m= number of experimental data; $F_{\alpha}(p, m-p)$= F distribution with p and m-p degrees of freedom at α probability level (e.g. 90% probability gives α=0.1).

Results

Table 1 shows the D and z values of the strain INRA AV Z421 estimated by means of both procedures, two lineal regressions and one step non linear regression. The predicted D and z values using both methodologies were similar. However, the 95% confidence interval was smaller for one step non linear regression. Predicted data by one step non linear regression fit appropriately to experimental ones, as it can be appreciated in the graphical representation of the goodness of fit (Figure 1). $D_{90°C}$ value for this strain sporulated in both FNA modified and J-Agar was 39 and 6.4 min, respectively.

Table 1: Predicted kinetics parameters for the pathogenic strain of *B. cereus* studied.

Temperature	D value (min)	
(°C)	Linear	Non linear
90	40±20[a]	39±3[a]
95	11±3	9.8±0.5
100	2.5±0.4	2.48±0.06
105	0.60±0.19	0.63±0.03
z (°C)	8.0±0.6	8.4±0.2

[a] D value ± confidence interval (95%)

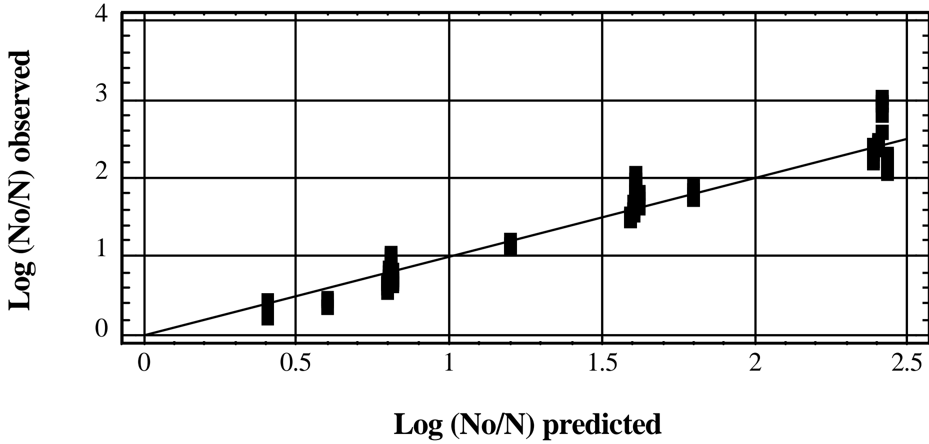

Figure 1: Graphical representation of the goodness of fit of predicted data by non linear regression to observed data for strain *B. cereus* AV Z421.

Figure 2 shows the distribution of residuals experimental data and predicted data by one step non linear regression. This analysis indicated the model and the applied regression procedure was statistically correct. The distribution of the residuals can be adequately modelled by a normal distribution (90%) centred in zero.

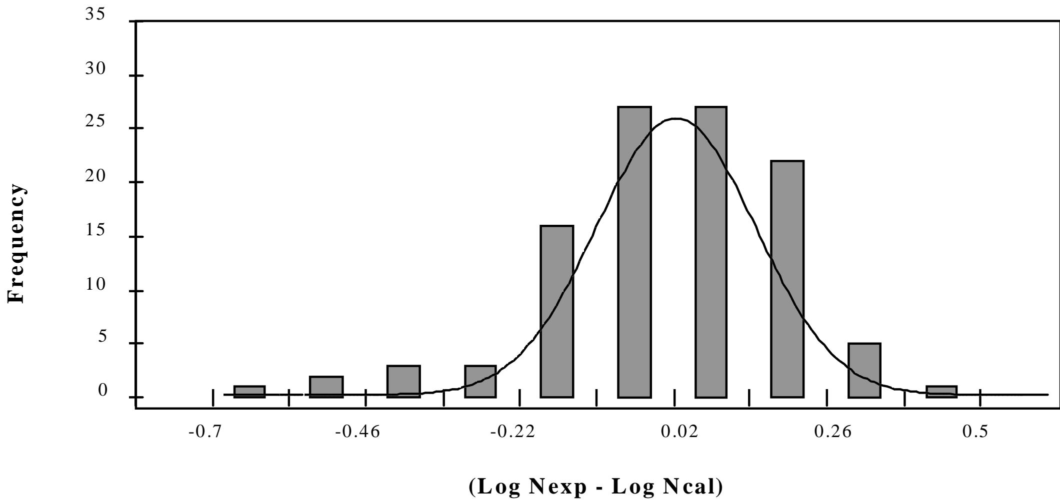

Figure 2: Frequency histogram of the residuals between experimental data and predicted data for *B. cereus* strain AV Z421.

The joint confidence regions (Figure 3) indicates if there are significant differences between the different thermal resistance parameters. D value at each temperature is perfectly separated, presenting a good visualization of the effect of temperature on this parameter, while the z value remains constant at each temperature analyzed.

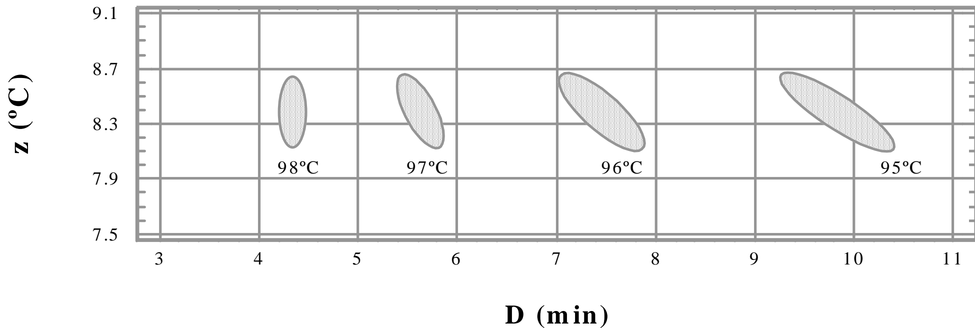

Figure 3: Joint confidence regions (90%) for *B. cereus* strain AV Z421 at 95, 96, 97 and 98°C.

Discussion

There is a growing interest in the application of risk assessment in the context of production of safe food to protect the consumer. One of the stages of the risk assessment in foods is the characterisation of the risk. Risk characterisation is the process of identification and quantification of the factors implied in the risk of being to exposed to unacceptable levels of illness agent, in this case *B. cereus* or its toxin, at time of consumption of the food (Notermans et al 1997). The initial count after pasteurisation or cooking is one of the factors that clearly determines the exposition risk to *B. cereus*. Consequently, it is necessary to define the thermal process treatment correctly, using accurate data for the most heat resistant strain implied in the spoilage of food. One step non linear regression methodology improve the estimation of the kinetic parameters, providing more exact data that those obtained by the traditional two linear regressions (Table 1); on the other hand, the model and the regression procedure applied are statistically correct (Figure 1 and 2).

B. cereus is a pathogen microorganism which presents a wide range of inactivation kinetic parameters between different strains. Thermal resistance parameters obtained, in the present work for the strain studied, were in the range of data published in the bibliography (Parry and Gilbert 1980). $D_{90°C}$ value was 39 min for the strain AV Z421, additionally, this strain was able to grow at 10 °C. These high heat resistant and able to grow at low temperatures strains, make that pasteurisation treatments applied and heat treatments proposed for ready to eat food (90 °C for 10 minutes) could be insufficient to destroy enough spores, if raw material is high contaminated. The problem is particularly serious when diarrhoeal *B. cereus* strains, as AV Z421, are implicated. Although the refrigeration conditions can not permit development of the surviving spores to the thermal treatment, if the concentration of spores is high this microorganism can grow in the intestine and produce the toxin.

The presented results, confirm the great influence of the sporulation medium on the thermal resistance of the spores. Spores sporulated in the J-agar were less resistant to the heat than those sporulated in the sporulation medium used in the present work (Mazas et al 1995). It is necessary to have conservative thermal resistance data in order to deduce safety thermal processes for foods, mainly when there is the possibility of pathogenic microorganisms contaminating the food product. That is why it seems convenient to study and use those sporulation and recovery media producing high thermal resistant spores and high levels of recovered spores, including those that were heat injured or damaged.

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Application of computational fluid dynamics (CFD) to hygienic design of food processing equipment

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Abstract

Clean-in-place (CIP) utilises the flow of hot detergent solutions in processing equipment to effect sufficient shear forces at the fluid/equipment interface. Computational Fluid Dynamics (CFD) offers the process plant designer the opportunity to evaluate the potential cleanability of equipment by predicting wall shear stress to test a variety of design features. Experimental cleaning trials on a purpose built ‘test-cell’ demonstrated an increased cleaning effect of the order of 2 log reductions in bacteria by increasing the detergent velocity from 0.5 $m.s^{-1}$ to 1.5 $m.s^{-1}$. A further increase in velocity to 3.5 $m.s^{-1}$ only slightly increased the bacterial removal. The standard and low Reynolds number k-ε models for turbulent flow were used to demonstrate that wall shear stress increased with average velocity within the ‘test-cell’. CFD model results for flow over forward and backward facing 45° and 90° steps, similar to those present at pipe couplings and valve fittings, are presented. The predicted slow flow highlights the unhygienic nature of these design features, demonstrating the usefulness of CFD for assessing cleanability.

Introduction

It is essential that food processing equipment is regularly cleaned to a high standard to produce consistently high quality safe food. A CIP operation involves flowing a detergent solution through the system, in order to achieve a minimum velocity of 1.5 $m.s^{-1}$. The shear forces of the cleaning fluid at the fluid/equipment interfaces are an important cleaning mechanism when used in conjunction with detergent, high temperatures and time. As far back as the 1970s, it was recognised that CIP should be considered at the beginning of the design stage (Lloyd, 1972). The aim of this work was to show that CFD offers the process plant designer a chance to evaluate equipment design before it is built, giving the opportunity to test a variety of alternative design features.

Modelling criteria and methods

Cleaning trials were carried out on a ‘test-cell’ constructed from a 47.6 mm diameter circular pipe, with four 10$\times$150 mm removable plates cut into the length. A biofilm of *Pseudomonas fragi* was grown on each of the four removable plates in a buffered suspension of the micro-organism for one hour, followed by four hours submersion in a growth medium. The assembled cell was then fitted to a test-rig with sufficient pipe length upstream to allow the flow to be fully developed on entry to the cell. Cleaning trials were conducted by recirculation of fluid through the test cell and, by taking bacterial counts before and after cleaning, the cleaning effect was quantified.

Wirtanen *et al* (1996) found that biofilms were cleaned most effectively from unsoiled surfaces with a simple rinsing procedure, without the aid of heat or chemicals. This showed the mechanical forces of the flow (shear stress) to be very important in cleaning. However, an initial investigation revealed that a flow of water at 1.5 $m.s^{-1}$ for 10 minutes did not induce significant bacterial removal, demonstrating that shear stress cannot be isolated as a cleaning mechanism. To aid cleaning, a

standardised cleaning fluid, the European Hygienic Equipment Design Group (EHEDG) Test Cleaner, defined by the Test Methods subgroup (Holah *et al*, 1992), was used at a temperature of 60-65 °C in a series of studies in which this fluid was circulated through the system at 0.5, 1.5 and 3.5 $m.s^{-1}$ for 10 minutes. Prior to cleaning, a bacterial count of 5-9×10^7 per cm^2 was present on each of the four test plates.

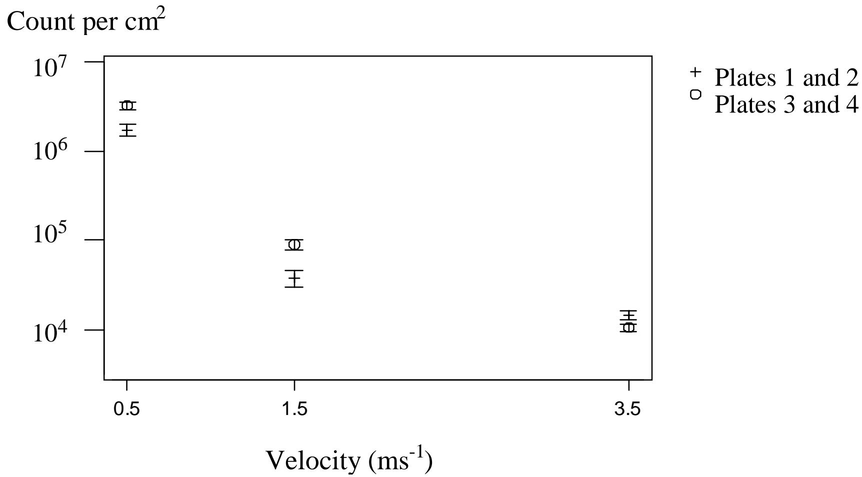

Figure 1: Mean bacterial counts showed a difference between ground-finished (plates 1 and 2) and mechanically polished (plates 3 and 4) surfaces.

Of the four removable flatplates, two were ground-finished and two were mechanically polished, both with 0.4-0.5 μm roughness average. Analysis of variance revealed a significant difference at the 1% level between the bacterial counts for the two surface finishes. Results presented in Figure 1 showed that the ground finished surfaces were easier to clean, any difference at the higher velocity being mainly due to experimental variation. Analysis of variance also showed (at the 1% level) that the bacterial count decreased as the velocity increased, with a cleaning effect of the order of 1 log reduction at 0.5 $m.s^{-1}$ and 4 log reductions at 3.5 $m.s^{-1}$.

A computational model of the 'test-cell' was developed using the commercial CFD software CFX4.1 (CFX International, AEA Technology). The fluid of interest was a detergent solution, with rheological properties similar to those of water, flowing in a turbulent flow regime. Bergman and Tragardh (1990) have shown that the removal rate of deposits can be scaled by using the mean wall shear stress. For this work, the standard k-ε model (Launder and Spalding, 1974) was used to predict mean wall shear stress as a first step towards characterising its relationship with bacterial removal. The k-ε model is computationally efficient as it makes use of wall functions which prescribe the velocity distribution in the laminar sublayer. As a check, the wall shear stress was also calculated using a low Reynolds number k-ε model, which integrates model equations right up to the wall. Wall shear stress predictions by both methods were in good agreement.

Predicted mean wall shear stress values for the removable plates are presented in Table 1 for a range of average velocities. These figures are in agreement with those quoted in other studies carried out for similar geometries and velocities (Bergman and Tragardh, 1990 and Powell and Slater, 1982).

Table 1: CFD predicted wall shear stress within a 'test-cell' for a range of velocities.

Average velocity ($m.s^{-1}$)	CFD predicted wall shear stress ($N.m^{-2}$)
0.5	0.7
1.0	2.3
1.5	4.7
2.0	7.9
3.5	21.6

The results in Table 1 demonstrate that the wall shear stress increases with the average velocity, whilst Figure 1 shows that bacterial removal increases with average velocity. At present, a target minimum average velocity of 1.5 $m.s^{-1}$ is used in CIP systems. It is likely, however, that it is velocity variation close to the wall that truly influences cleaning efficiency, and this is directly related to wall shear stress.

Figure 2 shows CFD predicted velocity vectors for flow over a forward and backward facing right-angled step. This 0.5 mm protrusion into the 47.6 mm diameter 'test-cell' is representative of an obstacle caused by a pipe coupling, for example. As the fluid approaches the protrusion, it slowly moves to avoid the obstruction (slow flow is characterised by shorter vectors). As the fluid flows over the end of the protrusion, a slow moving region of reverse flow is apparent. These model results show qualitative agreement with experimental studies (Adams and Johnson, 1988, Driver and Seegmiller, 1985), although the standard k-ε model is known to underestimate the reattachment length and negative velocities (Driver *et al,* 1985, Rodi, 1991), as the assumptions of the wall function approach are not valid in separated flows (Rodi, 1991). Low Reynolds number versions of the k-ε model offer an alternative with the advantage that equations are integrated right up to the wall, but these have been little tested on separated flows (Rodi, 1991) and also require greater computer resources.

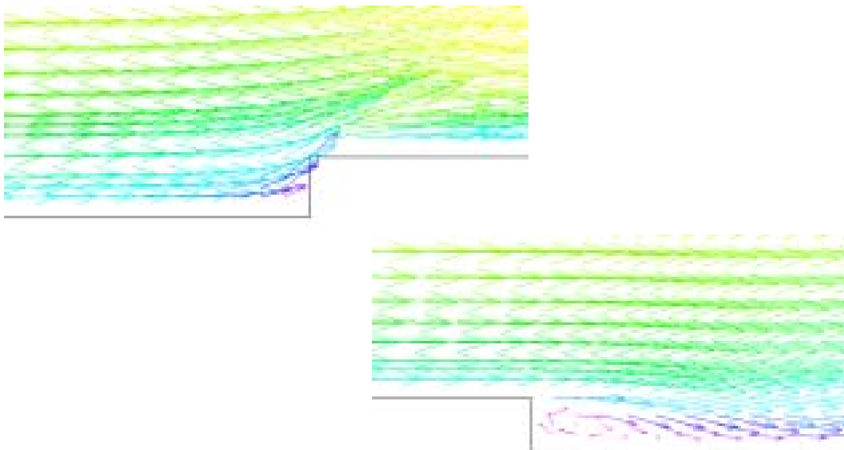

Figure 2: Velocity vectors show typical flow patterns as detergent solution flows over a 0.5 mm right-angled step similar to those caused by pipe couplings.

As well as right angled steps formed at pipe couplings, chamfered steps often appear if rubber seals are squeezed where valves are attached. Figure 3 shows predicted flow patterns for detergent solution over a 45° step. As with the right angled step depicted in Figure 2, the fluid is slowed down on approaching the obstacle, and a re-circulation zone is apparent at the backward facing step. Once again cleaning effect is likely to be lessened by the slow flow associated with this type of design feature.

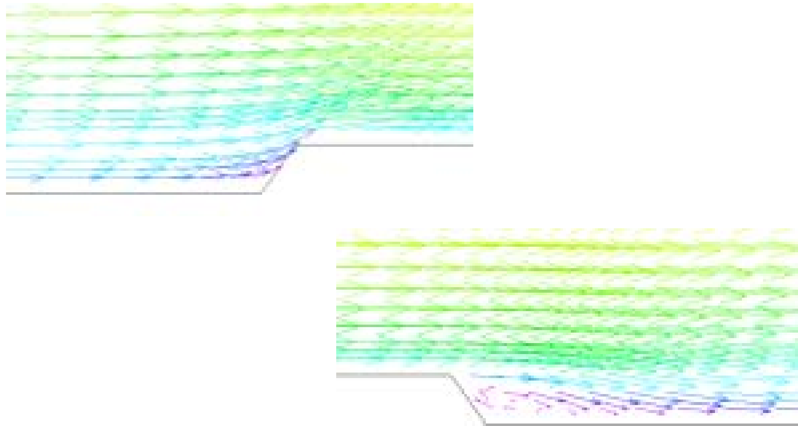

Figure 3: Velocity vectors show typical flow patterns as detergent solution flows over a 0.5 mm 45° step similar to protrusions formed where valves are inserted between pipes.

Conclusions

Experimental cleaning regimes and CFD predicted flow patterns within a 'test-cell' have been presented. Comparison of these results has shown that bacterial removal rates are related to wall shear stress. Quantification of this relationship would enable the process plant designer to use CFD as a tool to ensure that equipment had the potential to be cleaned-in-place. As an illustration of an unhygienic design feature, CFD has been used to predict detergent flow over 45° and 90° forward and backward facing steps. This type of flow obstacle is often present with standard pipe couplings and valve fittings. Flow was shown to be slow before and after the protrusions, with re-circulation zones present, corresponding to a reduced cleaning effect.

Acknowledgements

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Improvement of the microbial safety during the processing of frozen fish from extra-European exporting countries

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Introduction

Imported frozen seafood represents a large percentage of products consumed in Europe. The non-European exporting countries are: Argentina, Somalia, Thailand, India, Equador, Mauritania, Japan, Ivory Cost. The Netherlands, moreover, imports fish from China. After harvesting, the prepared fish is decapitated, eviscerated, washed, frozen etc.

Such operations, which are very critical from the hygienic view point and, when not properly performed, can lead cross contamination and pathogen proliferation. Microorganisms more frequently associated to these products are *Staphylococcus aureus*, *Listeria monocytogenes*, *Vibrio colerae*, *Vibrio parahaemoliticus*, *E. coli* O157:H7.

The process performed on board ship often includes freezing and packaging.

These microorganisms may become a problem during transportation if seafoods are not cooled properly at some time after harvest. Chilled products should be loaded at internal temperatures below 4°C and frozen products at, or below, -18°C.

The process operations in the various European countries are now regulated by specific guidelines concerning the phases from harvesting to distribution.

The proliferation of pathogens and the risks associated with biogenic amines can be effectively prevented when the seafood harvest, processing and distribution are subjected to a proper application of the HACCP system up to the point of consumption.

However it is often difficult to know and control the progress history of frozen fish. Therefore the quantitative risk assessment for pathogens or toxins cannot be accurately performed because of data inconsistencies and because of inadequacy of seafood consumption survey data.

In general, the imported frozen fish is partially reprocessed in the importing country by second transformation companies. The process generally includes the following steps: partial thawing, washing in water bath in order to create a thin “glassy” film which confers a pleasant appearance and limits the dehydration. Such operations are unable to improve the microbial safety of fish, and, when not properly carried out can contribute to the proliferation of the contaminants.

In this preliminary work we studied a possible application of peracetic acid used in the treatment of water for the processing of frozen fish.

The flow diagram of the reprocessing of frozen fish includes the following steps: storage of raw material, partial thawing of the products, peeling, portioning, freezing, deeping in water in order to create a glassy watery which reduces dehydration during storage.

Material and methods

Data was carried out by plate count methods in standard selective means.

Results

A survey on 2 hundred samples of the microbial quality of calamary, mussels cod fish, shrimp, smooth hound, sward fish, seppia, clams. imported from different countries was carried out.

The microbial analyses included: T.M.B., coliform, E. coli, S. aureus, Salmonella, Listeria monocytogenes, Vibrio cholerae and parahemolyticus.

The level of mesophilic bacteria ranged between 1 and 8 log CFU/g, and that of coliforms between 1 and 5 log CFU/g.

The geographic origin was very important for the hygienic properties of the samples. Salmonella was absent in all the samples while Listeria monocytogenes was present in samples from India and Spain. Also for the most part the fish from Spain often has extra-European origins and it is purchased at auctions an international markets.

The process sequence adopted for the reprocessing seems to have an important role on the microbial proliferation. Therefore the final microbial load of frozen fish accounts for both an uncontrolled and unpredictable initial contamination as well as the contamination during the reprocessing operations.

In order to reduce the contribution of this phase, the effectiveness of the added peracetic acid to water bath for the glassy formation, was compared to Cl_2 .

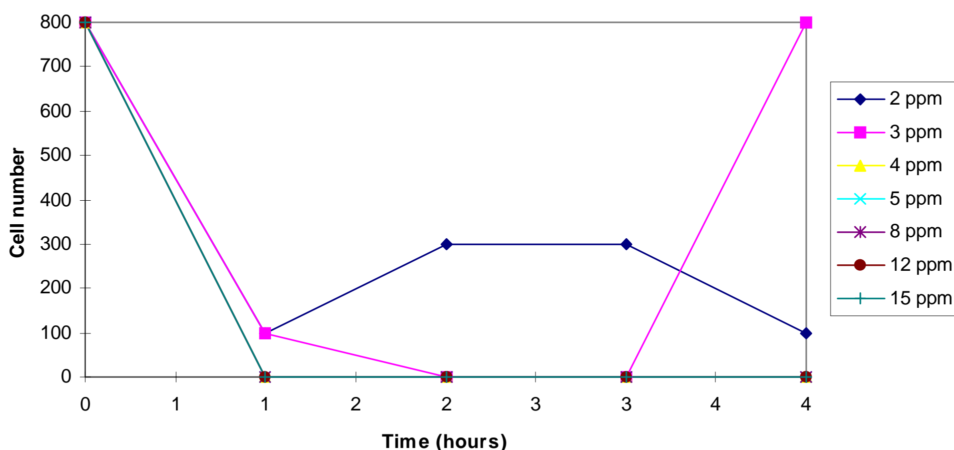

Fig. 1: Evolution of Listeria monocytogenes in water bath added with different concentrations of peracetic acid

The cool water (4°C) used for the washing and glassy phase was added with various doses of peracetic acid (2-15 ppm) (fig.1). The water bath containing organic residues of 1 cycles deeping had been inoculated with 800 cell/ml of Listeria monocytogenes whose growth was evaluated over time for 4 hours with respect to the water added with different doses of Cl_2 (as natrium ipochlorate) of 0.1, 0.2, 0.3 ppm (fig.2).

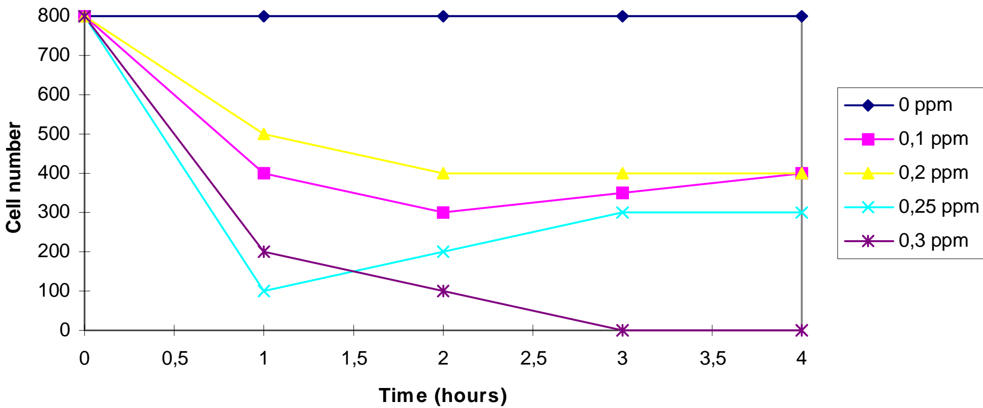

Fig. 2: Evolution of Listeria monocytogenes in water bath added with different concentrations of natrium ipochloride

Although the quantities of Cl_2 did not correspond to the peracetic acid due to the safety regulations which limit the Cl_2 employment, the peracetic acid can be considered as a potentially efficacious molecule. Its presence, which induces a rapid viability decrease of Listeria monocytogenes at the concentration of 3-4 ppm, produces the pH decrease as a side effect. Infact, the molecule combines an oxidative effect with acidification properties. When peracetic acid was added to more contaminated water (containing a level of residues from 5 deeping cycles), higher doses of peracetic acid must also be added as shown by (fig. 3).

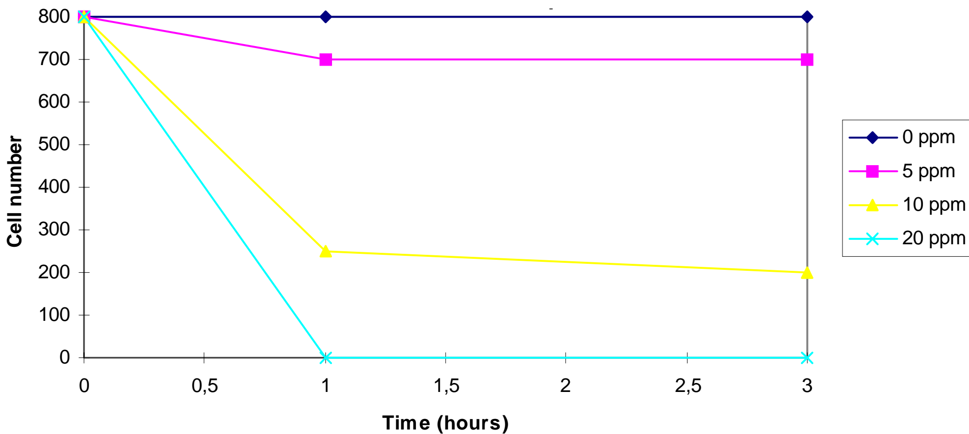

Fig. 1: Evolution of Listeria monocytogenes in water bath added with different concentrations of peracetic acid

The results showed that peracetic acid has a good potential as a chemical agent capable of reducing contamination level and controlling the proliferation of spoilage and pathogenic bacteria during the reprocessing phase of commercial frozen fish products. However the complexity of the operations, the impossibility of controlling raw material safety and the management of the reprocessing technology which does not include any critical control points, makes the implementation of correct procedure, and an effective temperature control in all the steps involved, even make urgent.

Effects of novel mild preservation technology on the growth of *Listeria monocytogenes* on ready-to-use lettuce

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Abstract

The effects of novel mild preservation technologies on the survival and growth of *Listeria monocytogenes* on ready-to-use lettuce were investigated. Populations of *L. monocytogenes* on undipped lettuce stored at 3°C decreased ($P<0.05$) during a 14-day storage period. At 8°C, populations on lettuce held in air also decreased ($P<0.05$) during storage, but populations on lettuce stored in product modified atmospheres did not change significantly. Dipping lettuce in a chlorine or citric acid solution followed by storage at 8°C resulted in a significant increase in *L. monocytogenes* populations compared with undipped samples. Flushing packages of lettuce with 100% N_2 combined with storage at 8°C also resulted in significant growth of *L. monocytogenes*.

Introduction

L. monocytogenes is a foodborne pathogenic bacterium and is a frequent contaminant of vegetables. It has been shown to survive or grow on a range of minimally processed vegetables, such as shredded iceberg lettuce (Steinbruegge *et al*., 1988; Beuchat & Brackett, 1990) and cut cabbage (Kallender *et al*.,1991; Beuchat *et al*., 1986) at refrigeration temperatures.

Washing of prepared vegetables to reduce microbial load is a common commercial practice. The efficacy of washing is often improved by the inclusion of antimicrobials in the wash water. If pathogens, such as *L. monocytogenes* are present on vegetables, they may not be fully eliminated by commercial washing and disinfection treatments.

In addition, contamination by *L. monocytogenes* may occur after washing and disinfection; scrupulous hygiene must be applied at all levels of processing. Disinfection of salad leaves with chlorine (200mg/l) prior to inoculation with *L. monocytogenes* did not affect its growth during storage at 5°C or 10°C (Beuchat & Brackett, 1990). By contrast, Bennik *et al*. (1996) found that *L. monocytogenes* inoculated onto disinfected leaves gave higher counts after seven days at 10°C than on leaves rinsed with water.

Modified atmosphere packaging in combination with refrigeration is increasingly used as a mild preservation technique for minimally processed vegetables. However, the potential effects of this novel technology on the survival and growth of pathogens are poorly understood. Beuchat & Brackett (1990) showed that populations of *L. monocytogenes* on lettuce significantly increased during storage but that modified atmosphere (3%O_2, 97% N_2) packaging did not influence the rate of growth. Ringlé *et al*. (1991) found that *L. monocytogenes* did not grow at either 8°C or 4°C on shredded chicory salads packaged in air using a semipermeable film, but did grow when the product was packaged after flushing with nitrogen. Such inconsistencies in the literature emphasize the need of more work in order to ensure the safety of minimally processed modified atmosphere packaged vegetables.

The aim of this work was to examine the effects of (1) washing treatments, (2) package atmospheres and (3) storage temperatures on the survival and growth of *L. monocytogenes* on shredded lettuce.

Materials and methods

Preparation of lettuce

Samples of ready-to-use lettuce were prepared using a sequence of processing steps (Figure 1). The effects of employing variations in washing, packaging and storage steps on the growth of two strains of *L. monocytogenes* were determined.

Processing steps	Variations
Manual trimming and coring (outer leaves removed using a sharp knife) ↓	
Shredding (into 10 mm strips using a sharp knife) ↓	
Washing ↓	a. In a 100 ppm chlorine solution b. In a 1% citric acid solution c. In distilled water
Spin-drying ↓	
Packaging (25 g in 35 μm oriented polypropylene bags) ↓	a. Sealed bags b. Perforated bags c. Bags flushed with 100% N_2 before sealing
Refrigerated storage (14 days)	a. 3°C b. 8°C

Figure 1: Processing steps and variations used in the production of MA packaged ready-to-use lettuce.

Inoculation of lettuce

L. monocytogenes strains ATCC 19114 and NCTC 11994 were activated by three successive loop-transfers at 24h intervals (37°C) in tryptone soya broth and were centrifuged. The supernatants were discarded and the cells were washed twice in sterile distilled water, resuspended and diluted to desired concentrations to allow for contamination of lettuce at an initial level of approximately 10^4-10^5 cfu/g of shredded lettuce.

A portion (0.1ml) of the cell suspension was added to each package containing 25g shredded lettuce. All packages were sealed using a hand-held heat sealer. Lettuce was stored for a period of 14 days and the survival and growth of *L. monocytogenes* on lettuce was followed at regular intervals throughout the storage period.

Results

The fate of *L. monocytogenes* on shredded lettuce

Similar results were obtained with the two strains of *L. monocytogenes* tested (Figure 2). Populations of *L. monocyogenes* on undipped lettuce stored at 3°C within product modified atmosphere packages gradually decreased (by 1-1.5 log cycles) during a 14-day storage period. Counts on Days 9, 12 and 14 were significantly (P<0.05) less than counts on Days 0, 2 and 6. By contrast, counts on undipped lettuce stored at 8°C did not change significantly over the period.

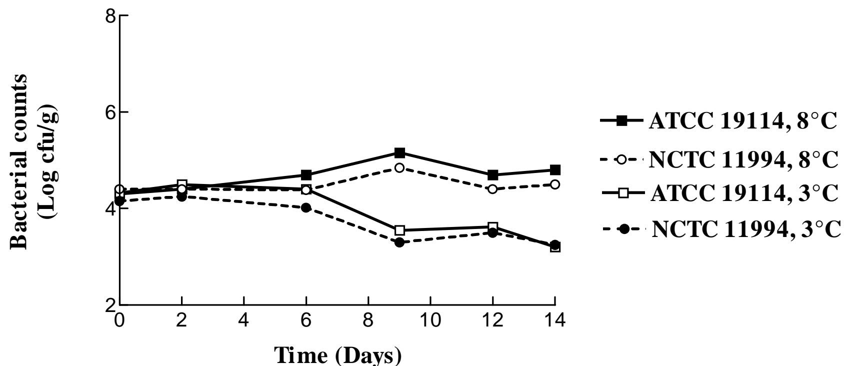

Figure 2: Growth of two strains of *L monocytogenes* on MA packaged shredded lettuce during storage at 3°C and 8°C.

Effects of antimicrobial dips

Similar results were obtained with chlorine and citric acid dips. The effects of chlorine dip and storage temperature are shown in Figure 3. Populations of *L monocytogenes* on chlorine dipped lettuce stored at 3°C were higher than on undipped samples throughout storage. At 8°C, populations of *L monocytogenes* on undipped lettuce did not change significantly during the storage period. However, on chlorine or citric acid dipped lettuce, *L monocytogenes* grew better particularly on extended storage, with final populations in the 10^6-10^7 cfu/g range.

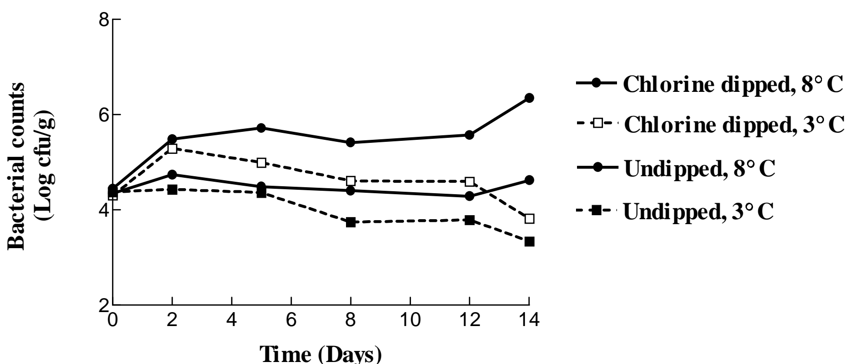

Figure 3: Effects of chlorine dip treatment of shredded lettuce on the survival and growth of *L. monocytogenes* during storage at 3°C and 8°C.

Effects of storage atmosphere

Nitrogen flushing may be used in the processing of ready-to-use lettuce, in order to prevent enzymatic browning at cut surfaces. The effects of storage atmospheres and time on the survival and growth of *L. monocytogenes* on shredded undipped lettuce stored at 3°C are shown in Figure 4. At 3°C, numbers on lettuce decreased ($P<0.05$) between Days 6 and 14 for all treatments. However, nitrogen flushed samples gave higher counts during storage compared with product modified atmosphere samples or samples stored in air.

The data for storage of lettuce at 8°C is shown in Figure 5. Similar results were obtained with the two strains of *L. monocytogenes*. Populations of *L. monocytogenes* increased rapidly in nitrogen flushed packs and by Day 14 counts had increased ($P<0.05$) by 2 log cycles. Populations of *L. monocytogenes* on product modified atmosphere samples did not change significantly during storage. Populations of *L. monocytogenes* decreased by approximately 1 log cycle in samples packaged in air.

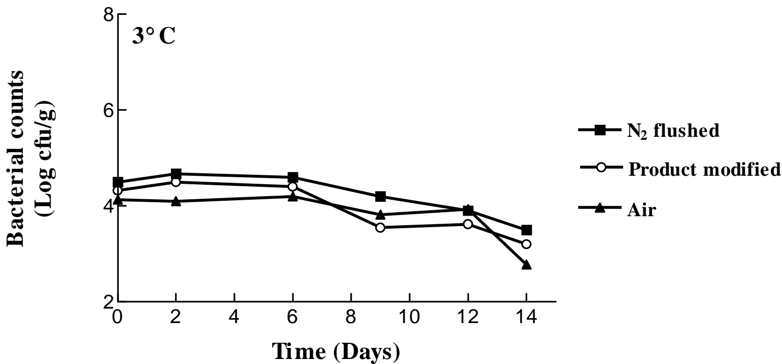

Figure 4: Effects of storage atmosphere on the survival and growth of *L. monocytogenes* on shredded lettuce during storage at 3°C.

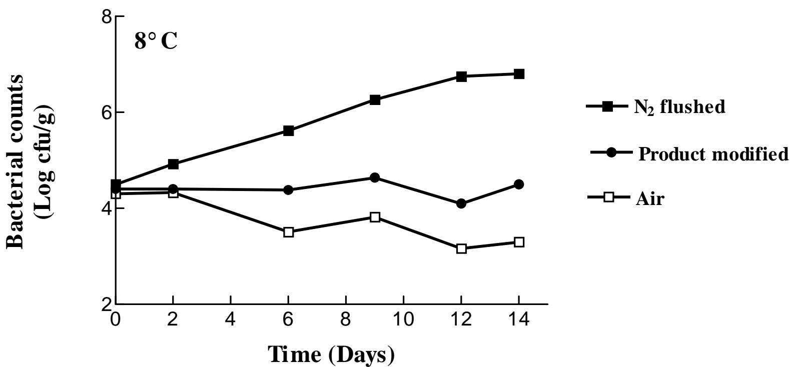

Figure 5: Effects of storage atmosphere on the survival and growth of *L. monocytogenes* on shredded lettuce during storage at 8°C.

Discussion

The use of antimicrobial dips resulted in better survival of *L. monocytogenes* at 3°C. At 8°C, it resulted in both better survival and in significant ($P<0.05$) growth during storage.

The results reported here contrast with the work of Beuchat & Brackett (1990, 1991) who observed that disinfection of lettuce leaves (Beuchat & Brackett, 1990) or tomato slices (Beuchat & Brackett, 1991) with chlorine (200mg/l) prior to inoculation with *L. monocytogenes* did not affect bacterial growth at 5°C or 10°C.

However, the data are supported by the work of Bennik *et al.* (1996). These authors reported that disinfection of minimally processed endive increased the growth of *L. monocytogenes*. The results presented here demonstrate that use of a chlorine or citric acid dip treatment, followed by storage at 8°C resulted in a significant increase ($P<0.05$) in *L. monocytogenes* populations compared with undipped samples.

Package atmospheres affected the survival and growth of *L. monocytogenes.* At 3°C, flushing with nitrogen extended the survival. At 8°C, product modified atmospheres also enabled *L. monocytogenes* to survive better, but nitrogen flushing resulted in significant ($P<0.05$) growth, particularly on extended storage.

These effects of modified atmospheres on *Listeria* survival and growth contrast with the work of Beuchat & Brackett (1990) who reported that growth of *L. monocytogenes* on lettuce was not influenced by modified atmosphere. Steinbruegge *et al*. (1988) observed a variable behaviour of *L. monocytogenes* during storage on shredded lettuce. On most samples, *L. monocytogenes* increased by several log cycles during storage, but it failed to grow on some samples. Carlin and Nguyen-the (1994) observed that growth patterns of *L. monocytogenes* on different types of green salads varied between experiments and that growth was significantly affected by salad type. Carlin *et al.* (1995) showed that the leaf characteristics of broad-leaved endive, such as age, quantity and quality of the background microflora, can also affect the growth of *L. monocytogenes*.

The work reported here is supported by data from Ringlé *et al*. (1991). These workers found that *L. monocytogenes* did not grow at 8°C or 4°C on shredded chicory salads packaged in air, but did grow when the product was packaged under nitrogen.

The data presented are of significant relevance to public health as they suggest that the use of antimicrobial dips and nitrogen flushing increases the potential for *L. monocytogenes* to survive and grow, particularly at mild abuse temperature. The data also emphasise the importance of strict hygiene during processing and packaging in order to avoid contamination of the product after disinfection and highlights the importance of strict temperature control in the chill chain.

Conclusions

Two unit operations commonly used in the preparation of ready-to-use vegetables have been shown to increase the potential for *Listeria* survival and growth, particularly at mild abuse temperatures. The commercial use of antimicrobial dips and nitrogen flushing should be subject to further study and their potential hazards incorporated into HACCP (Hazard Analysis at Critical Control Points) agendas. In particular, the importance of strict hygiene to eliminate the possibility of mid/post process product contamination with *Listeria* should be highlighted. The work also underlines the importance of good temperature control in the chill chain and the risks introduced by products which have excessively extended shelf lives.

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Session 5:

Consumer Perception and Transfer Strategies

The optimization of sweet taste quality (TOSTQ)

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Introduction

A major European Concerted Action programme is underway (PL98-4040) to apply the mechanistic understanding of sweet taste chemoreception to the optimisation of sweet taste quality. The programme lasts two years and involves ten universities, one research association and five companies.

Mechanistic behaviour of sweeteners

The bulk and intense sweeteners differ in many ways and their mechanistic behaviour originates in their interaction with water. This can be quantified by solution chemistry and used to explain their quality and intensity.

Table 1: Synergy of binary bulk intense sweetener mixtures

Sweetener mixture	Blend ratio*	% Synergy	Isentropic specific compressibility (cm^3/g.bar)
Sucrose:Sodium cyclamate	75:25	+13	$-5.705x10^6$
Maltitol:Acesulfame K	75:25	+19	$-5.155x10^6$
Maltitol:Sodium cyclamate	50:50	+27	$-6.508x10^6$
Sucrose:Aspartame	25:75	-33	$-6.286x10^6$

*Blend ratios are calculated as proportions of contributed sweetness by each component

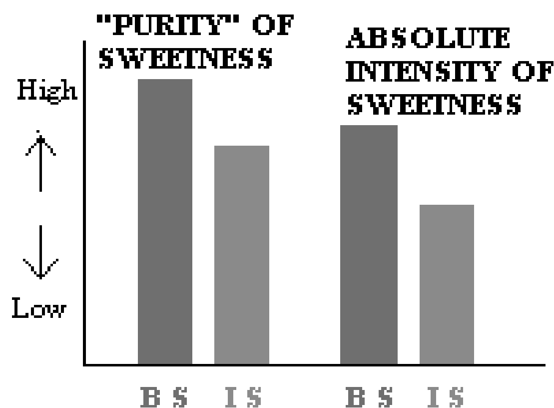

Figure 1: Quality and intensity of sweetness (BS: Bulk sweetener; IS: Intense sweetener)

Synergy

Positive synergy has been shown to occur in binary mixtures of sweeteners, but no addition advantage occurs with three sweeteners (Schiffman et al., 1995; 1996). Some examples of synergy are listed in table 1 (Parke and Birch, 1998). This phenomenon may be explainable by hydration mechanisms.

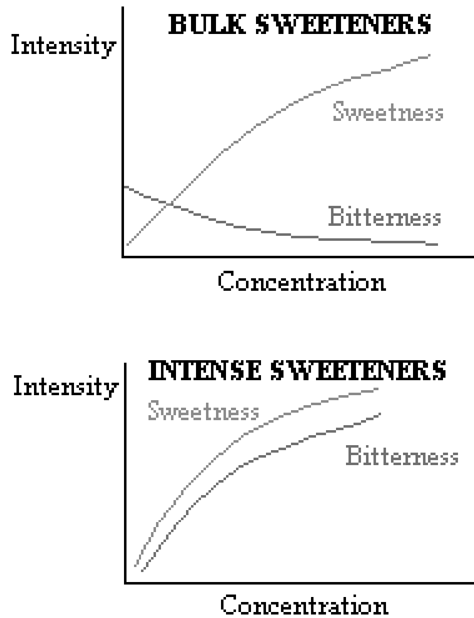

Figure 2: Shapes of psychophysical sweetener curves

Conclusion

Hydration measurements of sweeteners have already been used to interpret their qualitative and quantitative taste properties. This approach is now being directed to food and pharmaceutical products.

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Water sorption and plasticization effect in breakfast cereals. Changes in texture

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Abstract

Corn flakes are a breakfast cereal product whose crunchiness or crispness is considered a primary textural attribute. These properties are highly affected by the physical state (gassy or rubbery) of the product, which is dependent on temperature and water content. Water activity - water content - glass transition temperature relationships for corn flakes have been studied. A combined use of the Gordon and Taylor equation, to model water plasticization effect, and the GAB sorption model, applied to obtained data, allowed the evaluation of the products' stability at various processing and storage conditions. Critical water activity (CWA) and critical water content (CWC) for dramatic changes in the product properties at 35 ºC could be established. CWA and CWC were fixed by means of changes in the mechanical properties of the product evaluated from a penetration test. The shape of the obtained force-distance curves as well as the slope in the first seconds of the test, were considered in mechanical analysis. The critical water activity and water content were respectively 0.603 for and 8.15 g water/100 g sample.

Introduction

Corn flakes are cereal products whose crunchiness or crispness is considered a primary textural attribute. There is a growing literature on the effect of water content on the mechanical properties of different food products. Changes in the physical state of food materials are often nonequilibrium phenomena that are also strongly related to water content and temperature (Roos, 1993). Moreover, the physical state of food components affects its physical properties. In this sense, food texture is a typical example of physical property related to the glass transition temperature (Tg) and it is obvious that crispness of various low moisture foods is lost above Tg.

Prediction of the physical state of food materials at a determined concentration and temperature is often possible if Tg has been determined as a function of water content (state diagram). But also the concept of water activity may be used to explain the textural variations observed in food products when the water content is modified (Bourne, 1987). As proposed by Roos (1993) a combined use of the Gordon and Taylor equation, to model plasticization effect of water, and the GAB sorption model allows evaluation of food stability at various processing and storage conditions.

The aim of the present study was to establish a_w-w-Tg relations for corn flakes in order to determine the CWA and CWC that determine undesirable changes in the mechanical properties of breakfast cereals stored at 35 ºC.

Material and methods

Material

Corn flakes of a commercial brand were purchased at a local supermarket and conditioned immediately after opening at different relative humidities. According to the producer's specifications, the composition of the breakfast cereal was (% w/w): water 0.03, glucids 0.81, protein 0.075; lipids 0.01 and ashes 4.

Moisture sorption isotherms

Sorption experiments were carried out by duplicate at 35 ºC. For moisture conditioning, samples (≈ 2 g) were placed over saturated salt solutions (a_w from 0 to 0.825) in hermetic jars to constant weight. Initial water content of samples was determined by Karl Fisher titration (Scholtz, 1988), also as equilibrium moisture content.

Instrumental texture analysis

Samples with a different moisture content were evaluated using an Universal Texture Analyzer TA.XT2 (Stable Micro Systems). A penetration test (probe 10 mm diameter, strain 95 % and deformation rate 0.1 mm/s) of individual corn flakes was selected to evaluate crunchiness of the product. Although some authors pointed out the difficulty to obtain reproducible results when corn flakes are tested individually (Nuebel and Peleg 1993), enough information to evaluate the physical state of the product was obtained in preliminary assays. The advantage referred to testing them in bulk, as proposed by Nuebel and Peleg, is the small quantity of product necessary for the study.

From force-deformation curves obtained, initial slope and number of break or fracture peaks, related with the degree of jaggedness, were analyzed. The last one is a reproducible parameter that can be used to monitor crunchiness loss as a result of moisture sorption (Barret et al., 1992; Rohde et al., 1993a,b; Wollny and Peleg, 1994).

As one of the manifestations of a particulate crunchiness is an irregular and irreproducible compressive force-deformation curve, enough replicates were made to obtain six similar curves for each moisture level.

Differential Scanning Calorimetry analysis (DSC)

For this analysis, samples prepared for sorption experiment were used. Moreover, in order to have more data, corn flakes with other different moisture levels were dried in a vacuum oven or placed in a hermetic jar with 100 % relative humidity, during different time periods. After that, all the samples were grounded, and moisture content was determined (by Karl Fisher titration) at the same time that ≈ 10 mg were placed into DSC pans for thermal analysis.

A DSC 220CU SII (Seiko instruments Inc.) was used for calorimetric analysis. Heating rate was 5 ºC/min and temperature range (amplitude 40 ºC) varied between -120 and 40 ºC, depending on sample's moisture content.

Results and discussion

Sorption isotherms

Equilibrium moisture content at different a_w of the samples appears in table 1. Sorption data were well fitted to BET ($a_w \geq 0.432$) and GAB models (table 2).

Table 1: Experimental sorption data

a_w	x_w (g water/g dry solids)	w (g water/g product)
0	0	0
0.112	0.030±0.002	0.029±0.002
0.230	0.035±0.014	0.030±0.001
0.320	0.050±0.006	0.047±0.006
0.429	0.055±0.008	0.052±0.008
0.500	0.077±0.007	0.072±0.006
0.675	0.11±0.01	0.10±0.01
0.750	0.14±0.01	0.128±0.009
0.825	0.33±0.02	0.25±0.02

Table 2: Parameter values obtained for the BET and GAB fitted models.

PARAMETERS OF THE BET MODEL			
x_o (g water/g dry solids)	**C**		**r^2**
0.0367	14.51		0.9726
PARAMETERS OF THE GAB MODEL			
x_o (g water/g dry solids)	**C**	**K**	**r^2**
0.0360	16.05	1.0105	0.9502

Mechanical analysis

Fig. 1 shows the different shape of the force-deformation curves obtained from penetration test, as a function of a_w of samples. For the figure, one of the replicates at each moisture content has been selected. Samples with low water activity, showed an irregular shape characterized by a variable number of fracture peaks, which decreased progressively and disappeared at a_w=0.675. So, in a qualitative way an a_w value between 0.500 and 0.675 may be pointed out as the critical value for the crunchiness loss of samples. The corresponding critical water contents were 0.072 and 0.10 g water/ g sample (table 1). Nevertheless, in order to obtain a quantitative parameter to reproduce the textural behaviour, other characteristic parameter of the curves, the initial slope, was evaluated. Initial slope of the force-distance curves has been considered for some authors (Bruns and Bourne, 1975; Katz and Labuza, 1981) as a good indicator of the intensity of the crunchiness of samples.

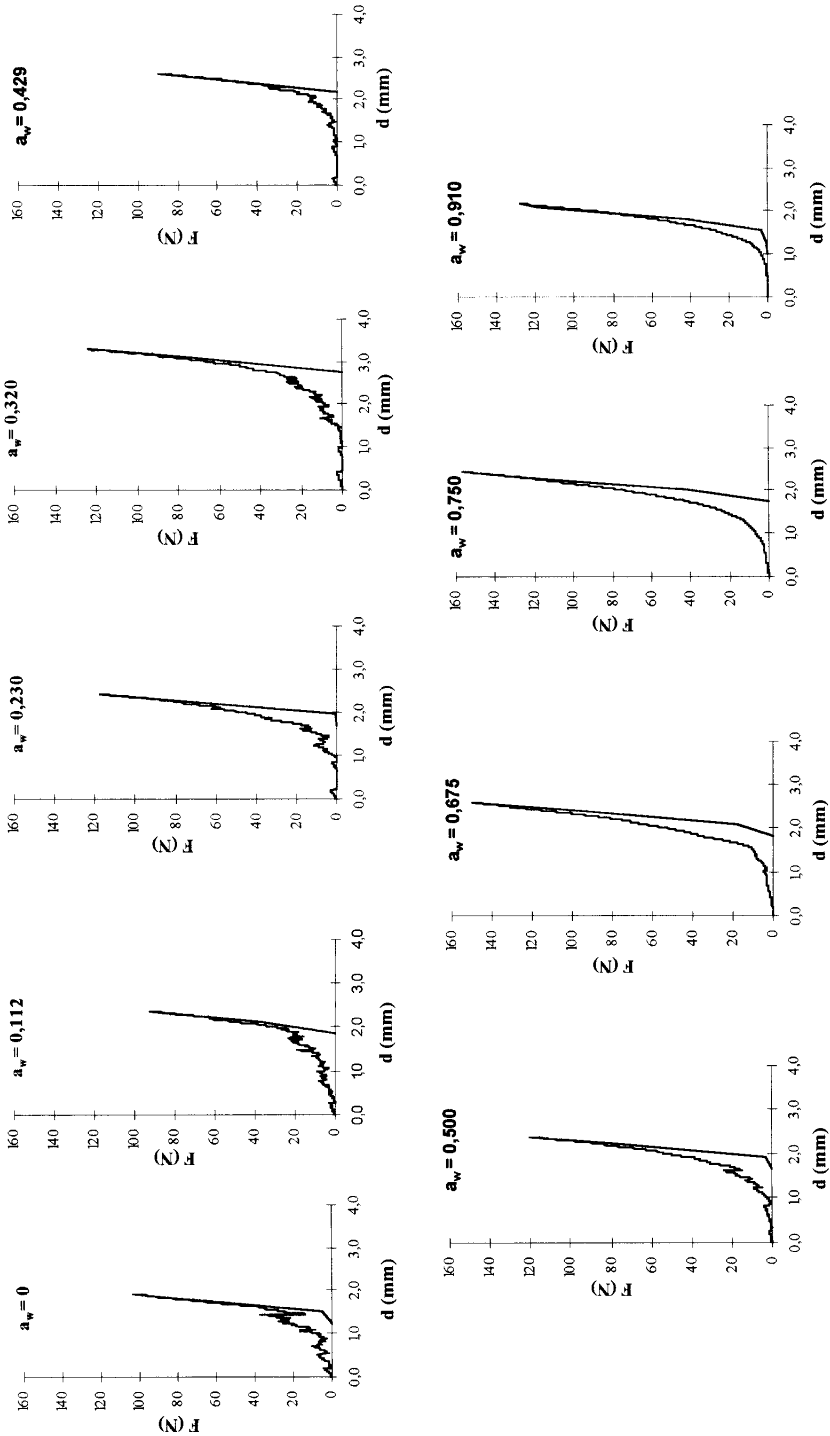

Fig. 1: Force-distance curves obtained for corn flakes with different a_w values.

Fig. 2 shows mean values and standard deviation obtained for this parameter as a function of water activity of corn flakes. It can be observed a decrease in the slope value when a_w increases. Nevertheless, the rate of the diminution is not constant. Until a_w = 0.320, the slope decreases quickly, leading to an asymptotic value. But at a_w between 0.500 and 0.675, a decrease in the slope can be observed, being this the same range than detected qualitatively for critical water activity.

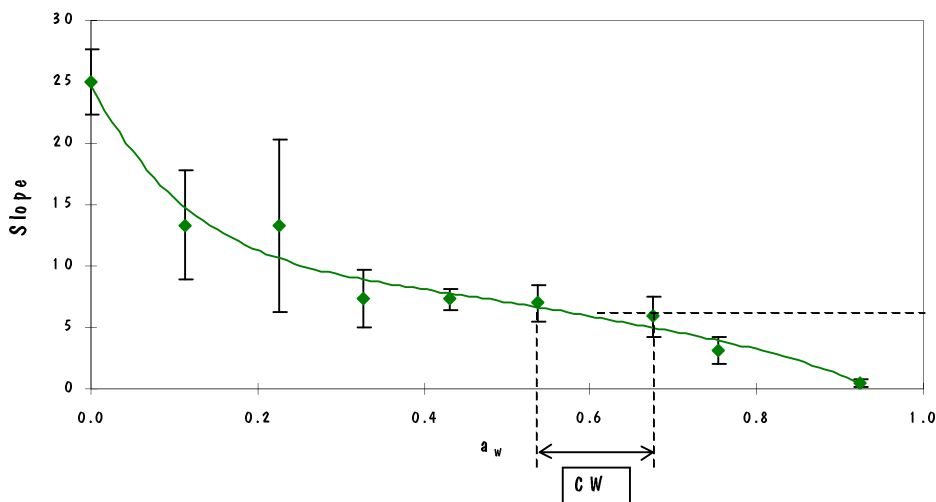

Fig. 2: Initial slope of force-distance curves as a function of a_w of corn flakes. Dashed line denotes the theoretic asymptotic slope value. The decrease in the slope from this point was assigned to the loss of the crunchiness.

A sigmoid shape of the curve was also observed by other authors when sensorial crispness was evaluated for some breakfast cereals (Sauvageot and Blond, 1991), so that they propose to determine CWA as the intersection between two straight lines determined by visual adjustment. Nevertheless, as the variation of crispness intensity versus a_w (or water content) must be found in some internal changes, an accurate method to determine it could be proposed. Possible explanations for physical changes can be found analyzing the effect that an increase of water content can cause on the principal constituents of the product: increase of retrograde amylopectin, protein denaturation and glass transition of the amorphous matrix of the product.

Taking into account the temperature in which the experiment was made (35 ºC) and the fact that the mechanical behaviour (instrumental texture) of many solid foods, including breakfast cereal products (Georget et al., 1995), at and around their glass transition is characterized by a sigmoid relationship with temperature, moisture content or water activity, this one seems to be the more probable reason. In corn flakes, soluble carbohydrates may be in an amorphous glassy state, due to rapid dehydration process occurred during extrusion of the product. An increase in water content will imply the mobility of soluble constituents that became to a rubbery state, causing dramatic changes in texture. In fact, when calorimetric study was done to all the samples, only glass transition was observed as thermal event.

Thermal analysis

Fig. 3 shows thermograms obtained for corn flakes with different water content. Values of glass transition temperature (Tg) at the mid point, can be observed in table 3. Data were fitted to Gordon and Taylor equation (Gordon and Taylor, 1952), obtaining k = 4.1052 and Tg of anhidrous solids = 369.96 K (R^2 = 0.9656) as values of the parameters of the model. It can be observed the depression of the Tg with increasing water content or a_w (plastizer effect of water). Roos (1993) defined the water content and the water activity that depressed the Tg to ambient temperature to be the critical moisture and water activity, respectively. When the water activity level depresses the Tg below the experiment temperature, the product will be in a rubbery state. This will be the critical water activity, and sorption isotherms may be used to predict the corresponding water content.

Table 3: Glass transition temperature for corn flakes with different moisture content

w (w/w)	0.018	0.022	0.027	0.030	0.043	0.055	0.057	0.074	0.094	0.220
Tg (ºC)	81.4 ±0.0	79.3 ±0.4	67.03 ±0.05	66.4 ±0.05	60.6 ±0.1	57.1 ±0.1	53.1 ±0.0	29.4 ±0.1	25.23 ±0.05	-28.8 ±0.2

A combined plot of Tg-a_w-w (Fig. 4) was used to determine the exact CWC and CWA at 35 ºC for the changes in the textural quality of corn flakes. In Fig 4 experimental sorption (x_w-a_w) and glass transition (Tg-a_w) data, also as the corresponding GAB and Gordon and Taylor fitted models can be observed. To determine a_w of samples submitted to thermal analysis, the GAB fitted equation was used. CWA and CWC at 35 ºC can be obtained from this figure, being 0.603 and 8.15 g water / 100 g product, respectively. These values are in the range suggested by the mechanical analysis.

Representations as Fig. 4 allow to obtain CWC and CWA values at any temperature, although the temperature dependence of the water activity must be taken into account in all modelling and interpretation of sorption data.

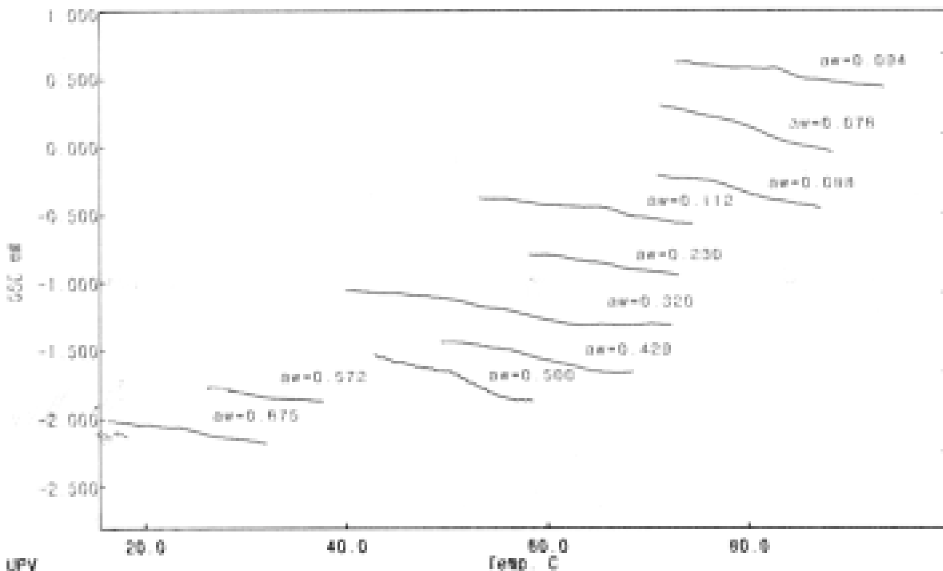

Fig. 3: Thermograms showing the glass transition obtained for samples with different a_w.

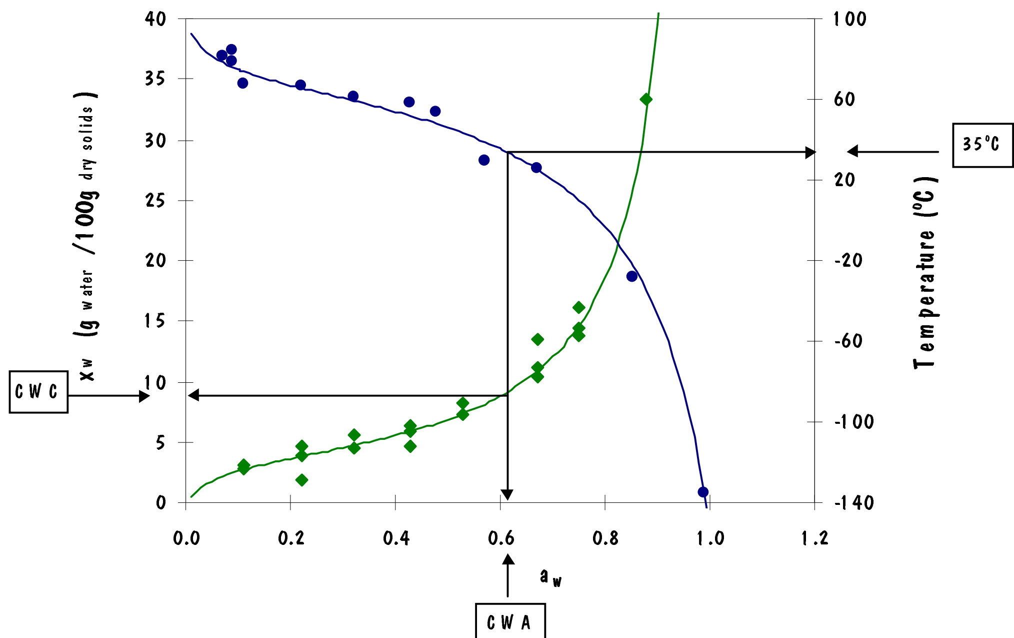

Fig. 4: Glass transition – water activity – water content relations for corn flakes. Arrows show the critical water activity and water content at the temperature of the experiment.

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