Optimal Extraction and technological Revalorisation of bioactive Polyphenols from Grape Pomace

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CONTENTS

ZUSAMMENFASSUNG

Das intelligente Management von Agrar-Abfallstoffen ist auch angesichts eines ständig steigenden Bedarfs an natürlichen Rohstoffen ein essentieller ökonomischer Faktor. Agrar-Abfallstoffe verursachen Kosten und Probleme bei der Entsorgung und Deponierung. Eine Mehrwert schaffende Verwertung, sei sie auch nur partiell, könnte diese Probleme vermindern. Die ökologisch wie auch ökonomisch sinnvolle Alternative, diese Reststoffe aufzuwerten, wäre die gezielte Gewinnung wertvoller Inhaltsstoffe zum Beispiel zur Verwendung als natürliche Zusatzstoffe in Lebensmitteln, Kosmetika oder intelligentem Verpackungsmaterial. Einige solcher Stoffe stehen im Mittelpunkt des Verbraucher-interesses auf Grund ihrer antioxidativen Wirkung und den damit verbundenen möglichen gesundheitsfördernden, protektiven Eigenschaften.

Am Beispiel von Traubentrester (rot, weiß und Kerne) aus biologischem und konventionellem Anbau wurde deren Gehalt an Polyphenolen untersucht. Extrakte aus beiden Anbauarten zeigten eine hohe antioxidative Kapazität und einen hohen Polyphenolgehalt. Erhebliche Menge an Anthocyaninen und Procyanidinen wurden in rotem Traubentrester und Kernen gefunden. Bei Extrakten aus weißem Traubentrester gab es signifikante Unterschiede zwischen dem biologisch und konventionell hergestellten Ausgangsmaterial. Traubentresterextrakte zeigten antibakterielle Wirkung gegen Gram-positive Bakterien, wie z.B: *L. monocytogenes*, *S. aureus*, *E. faecium*, *E. faecalis und B. thermosphacta* aber sie hemmten auch *P. chrysogenum, P. expansum, A. niger, T. viridie* und *A. versicolor* im Wachstum, wobei sich signifikante Unterschiede zwischen biologischen und konventionellen Traubentresterextrakten zeigten. Zurückzuführen waren sie auf den Pestizidgehalt in konventionellen Extrakten, in denen erhebliche Mengen an Fungiziden nachgewiesen werden konnten. Deren Konzentrationen lagen noch unterhalb der gesetzlichen Grenzwerte und stellten somit kein gesundheitliches Risiko für die Verbraucher dar.

Traubenkerneextrakte wiesen den höchsten Procyanidingehalt auf und wurden aus diesem Grund zur Inkorporation in Verpackungsfolien eingesetzt und ihre antimikrobielle Wirkung *in vitro* getestet. Auf stärke basierte Folien, angereichert mit Kernextrakten, zeigten optimale mechanische Eigenschaften für eine Anwendung als Haushaltsverpackungen und inhibierten das Wachstum von *B. thermosphacta* auf Fleischoberflächen während der ersten 4 Tage einer Lagerung bei 4°C. Die Verpackungen sind damit nicht nur bioaktiv, sondern auch vollständig abbaubar, so dass hierdurch eine ökologische Alternative zu synthetischen Folien vorliegt.

Roter Traubentrester, mit seinem höheren Anthocyaningehalt, wurde als Ausgangsmaterial für die Optimierung der Extraktion mittels Ultra-Hochdruck eingesetzt. Verschiedene Parameterkombinationen wurden eingestellt und die beste Extraktionsausbeute wurde bei 600 MPa, 70°C und 50% Ethanol erzielt. Darüber hinaus wurde die Hochdruckextraktion mit anderen Extraktionsverfahren, wie Ultraschall und Einsatz gepulster elektrischer Felder (PEF), verglichen. Bei mit PEF extrahierten Proben war die Ausbeute vierfach, bei Hochdruckbehandlung dreifach, und bei Ultraschallbehandlung doppelt so hoch wie bei konventionell extrahierten Proben. Im Vergleich zur konventionellen Extraktion war die Ausbeute höher, die Extraktionszeit deutlich verkürzt und der Lösungsmittelverbrauch konnte erheblich reduziert werden.

Die Stabilität von Anthocyaninen bei thermischer Belastung unter Hochdruckeinwirkung wurde untersucht, um die optimalen Bedingungen für die Hochdruck-Pasteurisation anthocyaninhaltiger Produkte zu ermitteln. Modell-Lösungen, die Cyanidin-3-O-glukoside enthielten, zeigten nach über 30-minütiger Hochdruckbehandlung bei 400 MPa und 50°C, deutliche Abbauverluste. Diese Reaktion folgte eine Kinetik erster Ordnung. Bei Zusatz von überschüssigem Pyruvat bildeten sich Anthocyanin-Pyruvat-Addukte. Solche Produkte sind aufgrund ihrer Farbmerkmale und sonstigen funktionellen Eigenschaften industriell interessant. Unter den Bedingungen der Hochdruck-Pasteurisation bei 600 MPa und 70°C während 10 Minuten blieben die Anthocyane in ihren komplexen Matrices Wein und rotem Traubentresterextrakt unverändert. Diese Ergebnisse sind insofern sehr vielversprechend, als die Menge an Schwefeldioxid, die normalerweise als antimikrobielles Antioxidantsmittel im Wein eingesetzt wird, ersetzt oder zumindest verringert werden könnte. Hochdruckspasteurisation stellt somit eine sinnvolle und gesunde Alternative zum Einsatz von Zusatzstoffen dar.

Im Zusammenhang mit der Hochdruckpasteurisierung von Wein wurde auch an der Entwicklung von geeigneten Druck-, Temperatur-, und Zeitindikatoren zum Nachweis und Monitoring von Hochdruckbehandlungen gearbeitet. Als besonders geeignet erwiesen sich Diels-Alder-Reaktionen (2+4 Cycloadditionen) von Coenzym Q_0 und Sorbat. Indikatoren auf dieser Basis sind unkompliziert, kostengünstig, nicht-toxisch, und erfordern aufgrund der klaren Farbreaktion keine Auswertegeräte.

SUMMARY

The intelligent management of agricultural wastes in distribution of scarce resources is an important factor in the economics. On one side, agricultural wastes represent a disposal problem for industries due to their large production and limited exploitation. On the other side, public awareness for food issues and improved standard of living have increased public demands for nutritional supplements which fortify their health. Based on these two fundaments, the reutilisation of agricultural wastes as sources of bioactive compounds represents an efficient, inexpensive and environmentally friendly alternative for their utilization as natural additives for food, cosmetic or pharmaceutical products.

In this thesis work, several strategies and methodologies were proposed and investigated to effectively add value to grape pomace extracts. The characteristics and differences of grape pomace extracts from organically and conventionally managed land were studied. Extracts from both, organic and conventional grape pomace showed a high antioxidant capacity and a high content in polyphenols. Red grape pomace and seed extracts represented an important source of anthocyanins and procyanidins, respectively. Significant differences between organic and conventional white grape pomace extracts were observed. Extracts were antimicrobial and inhibited the growth of Gram-positive food-borne pathogens such as: *L. monocytogenes*, *S. aureus*, *E. faecium, E. faecalis and B. thermosphacta*. A considerable inhibition of food related moulds such as *P*. *chrysogenum*, *P*. *expansum*, *A*. *niger*, *T. viridie* and *A. versicolor* was also achieved. A higher inhibition was exerted by conventional extracts. As a result, pesticide loads in grape pomace were estimated and their presence in conventional extracts was confirmed. Pesticide traces found in the extracts were below the permitted levels in grapes. They do not pose a risk for human health but influence extract antibacterial properties.

Considering their highest antioxidant and antibacterial properties, grape seed extracts (GSE) were incorporated into pea-starch films. GSE films showed optimal mechanical properties as home packaging and were antimicrobial. They inhibited *B. thermosphacta* surface loads in meat after four days incubation at 4°C. Biodegradable films improved thus meat quality and extended its shelf life as well as they represent an ecological alternative to polypropylene-based ones.

Based on the high anthocyanin content estimated in red grape pomace extracts, different parameters were selected for the optimization of their extraction by means of high hydrostatic pressure (HHP). Optimal extractions were achieved at 600 MPa, 70°C and 50% ethanol

concentration. Extractions assisted by HHP were compared to emerging potential extraction technologies such as ultrasonics and pulsed electric fields (PEF). Recovery obtained by PEF, HHP and ultrasonics was four-, three- and two-fold higher than a conventional extraction. Significant increase in extraction yields, shortening of extraction times and solvent consumption were observed when compared to common extractions.

The stability of anthocyanins under high hydrostatic pressure was investigated, to determine the conditions at which anthocyanin degradation or condensation reactions occur. These parameters lead to the establishment of optimal pasteurisation conditions for anthocyanin enriched products. Cyanidin-3-O-glucoside in ethanolic model solution was degraded above pressures of 400 MPa, temperatures of 50°C and processing times longer than 30 min. Degradation followed a first-order kinetic, and different hydrolytic products were identified as a result of the process. In the presence of pyruvic acid excess, the formation of condensed anthocyanin-pyruvic-adducts was accelerated under pressure. The resulting compounds presented different colours and functional properties from the genuine ones with major relevance from an industrial or nutritional point of view. The stability of anthocyanins in wine and grape pomace extracts under pasteurisation conditions was further investigated. No significant changes in anthocyanin composition were observed up to 600 MPa, 70°C, 10 min. High hydrostatic pressure was demonstrated an efficient pasteurisation method for wines and opens up meaningful avenues for the replacement of undesirable additives, e.g. Sulfur dioxide to preserve wine quality.

In addition, for traceability of pasteurisation/sterilisation processes under pressure a pressure-, temperature-, and time- indicator (PTTI) was developed based on Diels-Alder-reactions (2+4 cyclo-additions) between potassium sorbate and coenzyme Q_0. Pressure/temperature ideal processing conditions could be monitored by a simple colour change becoming thus a feasible device for monitoring high pressure processes.

LIST OF NOTATIONS

Abbreviations

A	*Aspergillus*
ABTS	2.2 azinobis [3-ethylbenzothiazoline-6-sulphonic acid]
ADI	acceptable daily intake
ANS	anthocyanidin synthase
Ant	anthocyanin
Ant3acgl	anthocyanin-3-O-acylglucoside
Ant3gl	anthocyanin-3-glucoside
Antglu	anthocyanin glucuronide
B	*Brochothrix*
BFR	Bundesinstitut für Risikobewertung
C	cytoplasm
C4L	coenzyme A ligase
CBG	cytosolic ß-glucosidase
CFU	colony forming unit
CHS	chalcone synthase
COMT	catechol-O-methyltransferase
Cy	cyanidin
Cy3gl	cyanidin-3-O-glucoside
Cy3pcmgl	cyanidin-3-O-p-coumaroylglucoside
CY4H	cinnamic acid 4-hydroxylase
DAD	diode array detector
DFR	dihydroflavonol 4-reductase
Dl	delphinidin
Dl3acgl	delphinidin-3-O-acylglucoside
Dl3gl	delphinidin-3-O-glucoside
DM	dry matter
E	*Enteroccocus*
ESI	electrospray ionization
F3H	flavonoid 3´-hydrolase
FCR	folin ciocalteau reagent
FLS	flavonol synthase
GAE	gallic acid equivalents
GPE	grape pomace extract
GSE	grape seed extract
HHP	high hydrostatic pressure
L	*Listeria*
L	Linnaeus
LPH	lactate phlorizin hydrolase
Mv	malvidin
Mv3acgl	malvidin-3-O-acylglucoside
Mv3gl	malvidin-3-O-glucoside
Mv3pcmgl	malvidin-3-O-p-coumaroylglucoside
NOAEL	no-observed-adverse-effect-levels
OP	oxygen permeability
ORAC	oxygen radical absorbance capacity
OTR	oxygen transmission rate
P	*Penicillium*
PAL	phenylalanine ammonia-lyase
Pd	peonidin
Pd3acgl	peonidin-3-O-acylglucoside
Pd3gl	peonidin-3-O-glucoside

Pd3pcmgl	peonidin-3-O-p-coumaroylglucoside
PEF	pulsed electric fields
Pg	pelargonidin
Pt	petunidin
Pt3acgl	petunidin-3-O-acylglucoside
Pt3gl	petunidin-3-O-glucoside
Pt3pcmgl	petunidin-3-O-p-coumaroylglucoside
RGPE	red grape pomace extracts
RH	relative humidity
S	*Salmonella*
S	*Staphylococcus*
SE	seed extracts
SGLT	sodium-glucose cotransporter
SULT	sulfotransferase
T	*Trichoderma*
TE	trolox equivalents
TROLOX	6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid
UDP-GT	uracil diphosphate-glucuronosyltransferase
V	*vitis*
V	vacuole
WGPE	white grape pomace extracts
WVP	water vapour permeability
WVRT	water vapour transmission rate

Symbols

A_F, *A1, A2, A3*	semi axis in field direction or x, y, z direction	
β	isothermal compressibility	Pa^{-1}
ε	dielectric constant	ρ Kg^{-1} m^{-3}
E	electric field strength	kV cm^{-1}
φ	electrical conductivity	µS cm^{-1}
ΔG	activation energy	J mol^{-1}
ΔH	enthalpy	J
K	equilibrium constant	-
k	rate constant	s^{-1}, min^{-1}, h^{-1}
P	pressure	MPa
R	universal gas constant	8.314 J mol^{-1} K^{-1}
T	temperature	°C, °K
ΔV	Volume change	cm^3 mol^{-1}
ΔS	entropy	J K^{-1}

1 INTRODUCTION

Increasing consumer awareness of diet related health problems and the recent upturn in consumer mistrust of synthetic additives is forcing the industry and the scientific community to search for natural food additives to replace existing synthetic hazardous ones (Krishnakumar & Gordon, 1996; Gilbert, 1997; Zink, 1997). Epidemiological studies have pointed out that consumption of fruits and vegetables imparts health benefits, e.g. reduced risk of coronary heart diseases, as well as of certain types of cancer (Knekt et al., 1996; 1997; Yochum et al., 1999; Hirvonen et al., 2001; Garcia-Closas, 2001; Arts et al., 2002; Sun et al., 2002; Mennen et al., 2004). However, a large group of the population lacks a generous intake of fruits and vegetables. Thus, dietary supplements and food fortification may be an alternative to the consumption of minor plant components that may have health benefits. Agro-industrial wastes from fruit and vegetables still maintain part of these valuable compounds (Sudhakar & Maini, 1995; Anand & Maini, 1997; Kennedy et al., 1999a; 1999b; Larrauri, 1999; McKee & Latner, 2000; Shrikhande, 2000; Das, 2001) whose availability, extraction and antioxidant capacity have been the subject of several works (Fox et al., 1991; Bravo et al., 1998; Bocco et al., 1998; Moure et., 2001). As industrialisation continues, agro-industrial wastes will become more concentrated, creating greater quantities of waste at a given location. While this can create greater environmental problems, the concentrated waste could be more easily reassimilated into the food cycle in form of natural food additives and ingredients (Figure 1.1).

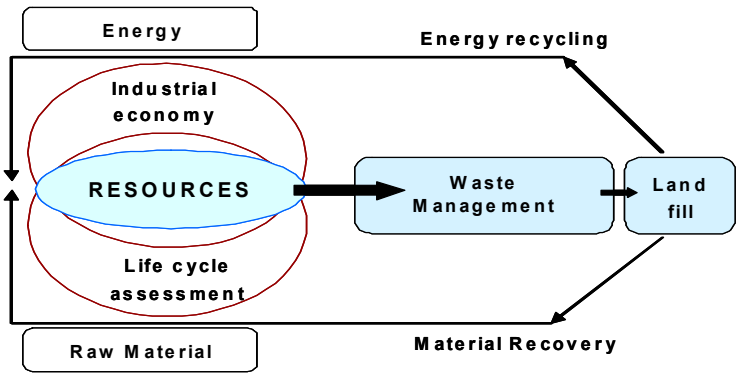


Figure 1.1: Sustainable resource management (Eininio 1999).

Grapes are one of the world´s largest fruit crop with more than 60 million tons produced annually. About 80% of the total crop is used in wine making (Mazza & Miniati, 1993) and pomace represents approx. 20% of the weight of grapes processed. From these data it can be calculated that grape pomace amounts oscillate around 9 million tons per year (Meyer et al., 1998; Schieber et al., 2002). Grape pomace is a great source of different compounds such as polyphenols, pigments, sugars, tartrate, fibers, oils and ethanol (Nerantzis &

Tataridis, 2005), which could be processed and used for compost (Bertran et al., 2004), animal feed (Nicolini et al., 1993), food and nutritional supplements (Hang, 1988; Lu & Foo, 1997; Schrikhande, 2000; Kammerer et al., 2004; Palenzuela et al., 2004; Yilmaz & Toledo, 2006), alcoholic drinks, colorants, antibacterial agents (Ozkan et al., 2004), as leather preservatives, biofuel and fuel additives (Scrase et al., 1993; Leber, 2004), as well as for other forms of bioenergy (Vlissidis & Zouboulis, 1993).

Grape skins and seeds comprise about 13% of the amount of processed berries (Torres & Bobet, 2001) and are specially a rich source of health-promoting polyphenols[1] such as proanthocyanidins, anthocyanins, flavonols, and flavan-3-ols which possess antibacterial, antiviral, antioxidant, anti-inflammatory, anti-cancerogenic properties and can prevent cardiovascular diseases (Renaud & De Lorgeil, 1992; Frankel et al., 1995; Rimm et al., 1996; Bravo, 1998; Shrikhande, 2000; Kallithraka et al., 2005a; 2005b). Grape pomace represents a low-cost raw material for the extraction of value-added compounds (polyphenols) with potential as food additives or nutraceuticals. However, in order to incorporate grape pomace extracts in the food market, it is necessary to estimate quality of the raw material; to undertake different processes to maximise product recovery minimising secondary wastes and to study potential and wider applications of grape pomace constituents for marketability.

Based on these statements this thesis contains a detailed characterisation of grape pomace extracts from conventionally and organically managed land as regarding antioxidant capacity, polyphenol characterisation, antimicrobial and antifungal activities (Chapter 5.1). This part includes also the determination of pesticide loads of both kinds of extracts and their drawbacks within the scope of food safety and public health.

In addition, the potential application of grape seed extracts as additives of bioactive pea starch-based food packaging films was further studied (Chapter 5.2). Physico-chemical characteristics of grape seed extract enriched films were studied as well as their ability to inhibit surface bacteria in experiments *in vitro* with pork loins was investigated.

The optimal process parameters for the extraction of anthocyanins from red grape pomace assisted by high hydrostatic pressure were also determined (Chapter 5.3). This chapter offers an open overview of the feasibility of other environmentally friendly and energy efficient technologies such as pulsed electric fields, and ultrasonics for extraction purposes.

Finally, the stability of anthocyanins in model solutions and food matrices under heat and/or high hydrostatic pressure to determine the parameters for adequate pasteurisation processes was discussed (Chapter 5.4). Therefore, a pressure-, temperature-, and time-indicator (PTTI) (Chapter 5.5) was developed for monitoring optimal pasteurisation conditions.

[1] In this work the term polyphenol includes phenolic acids and flavonoids.

2 BACKGROUND AND LITERATURE REVIEW

2.1 Function and biosynthesis of phenolic compounds in plants

Plants synthesize primary carbohydrates, lipids, and proteins. Secondary plant compounds are synthesized from lipid precursors and aromatic amino acids. Phenolic compounds build an important portion of the secondary metabolites in plants. Within phenolic compounds in plants phenolic acids and flavonoids play a major role in the interaction of plants with their environment (Harborne, 1994). They may attract insects, function as signals between plants (allelopathy), as signals between plant and symbiotic bacteria (N_2 fixing bacteria) or against pathogenic organisms (phytology).

Phenolics display a wide variety of structures, ranging from simple moieties containing a single hydroxylated aromatic ring (phenolic acids) to highly complex polymeric substances (Strube et al., 1993; Harbone, 1994). The biosynthetic pathways of phenolic compounds in plants have been extensively researched (Haddock et al., 1982; Harborne, 1988; Macheix et al., 1990; Strack, 1997) and biosynthesis pathways of some flavonols and phenolic acids are represented in Figure 2.2. The synthesis of phenolics in plants can be endogenously controlled during plant development and differentiation (Macheix et al., 1990; Strack, 1997) or it can be regulated by exogenous factors: biotic, such as insects and diseases and abiotic, such as light, temperature and nutrient stress (Bennet & Wallsgrove, 1994; Dixon & Paive, 1995; Schreiner, 2005). The phenyalanine/hydroxycinnamate pathway is defined as “general phenylpropanoid metabolism” and involves the transformation of L-phenylalanine to the hydroxycinnamates and their active forms (Strack, 1997) catalysed by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CY4H), and hydroxycinnamate-coenzyme A ligase (C4L) (Hahlbrock & Scheel, 1989).

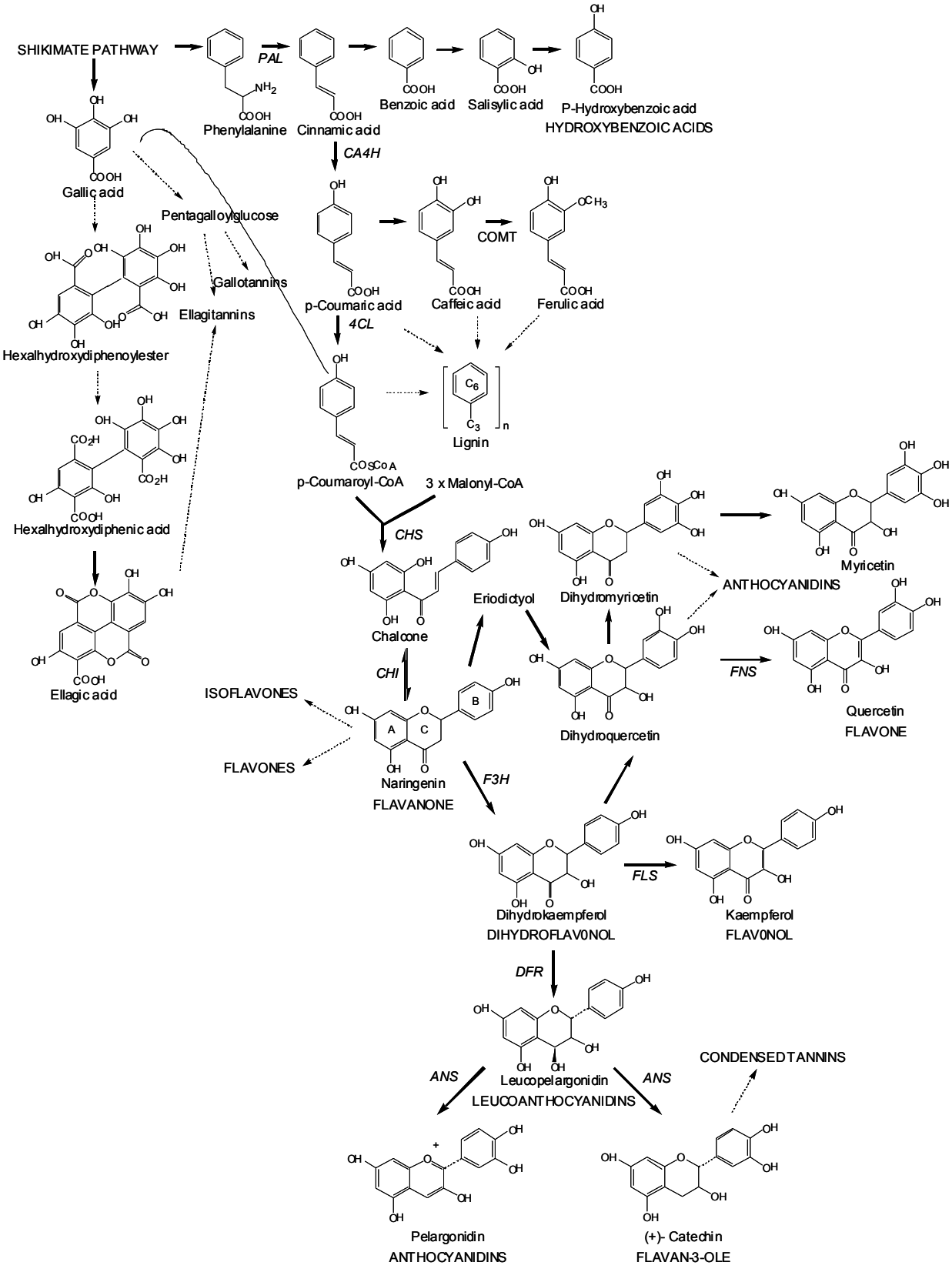


Figure 2.2: Biosynthesis of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids. Enzymes involved: *PAL*, phenylalanine ammonia-lyase; *CA4H*; cinnamic acid 4-hydroxylase; *CHS*, chalcone synthase; *4CL*, 4-coumarate coenzyme A ligase; *CHI*, chalcone isomerase; *F3H*, flavonoid 3´-hydrolase; *FLS*, flavonol synthase; *DFR*, dihydroflavonol 4-reductase; *ANS,* anthocyanidin synthase. Dashed lines represent transformations that require multiple enzymes.

2.2 Major phenolic acids and flavonoids in grapes

The most abundant classes of flavonoids in grapes (*V. vinifera* L.) include the flavan-3-ols, anthocyanins, and flavonols, while the most abundant class of non-flavonoids are hydroxycinnamates. Phenylpropanoid and flavonoid compounds usually accumulate in the central vacuoles of guard cells and epidermal cells as well as leaf subepidermal cells (Moskowith & Hrazdina, 1981; Weissenböck et al., 1987; Schnabl et al., 1986; 1989) and shoots (Ozimina, 1979). Within the grape berry, flavanols are localized mostly in seeds and skins. Flavanol content is higher in seeds than in skins.

Microscopic observations have shown the presence of tannin aggregates in the vacuoles of skin cells. Most epidermal cells but only few hypodermal cells, which are more abundant in the external layers, contain tannins. Tannins have been mainly localised in vacuole membranes and cell walls.

Anthocyanins and flavonols are mainly localized in skins, except in a few varieties, referred to as teinturier that also are contained in the pulp. In the skin, they are present in the first external layers of the hypodermal tissue, and exclusively in the vacuoles (Figure 2.3).

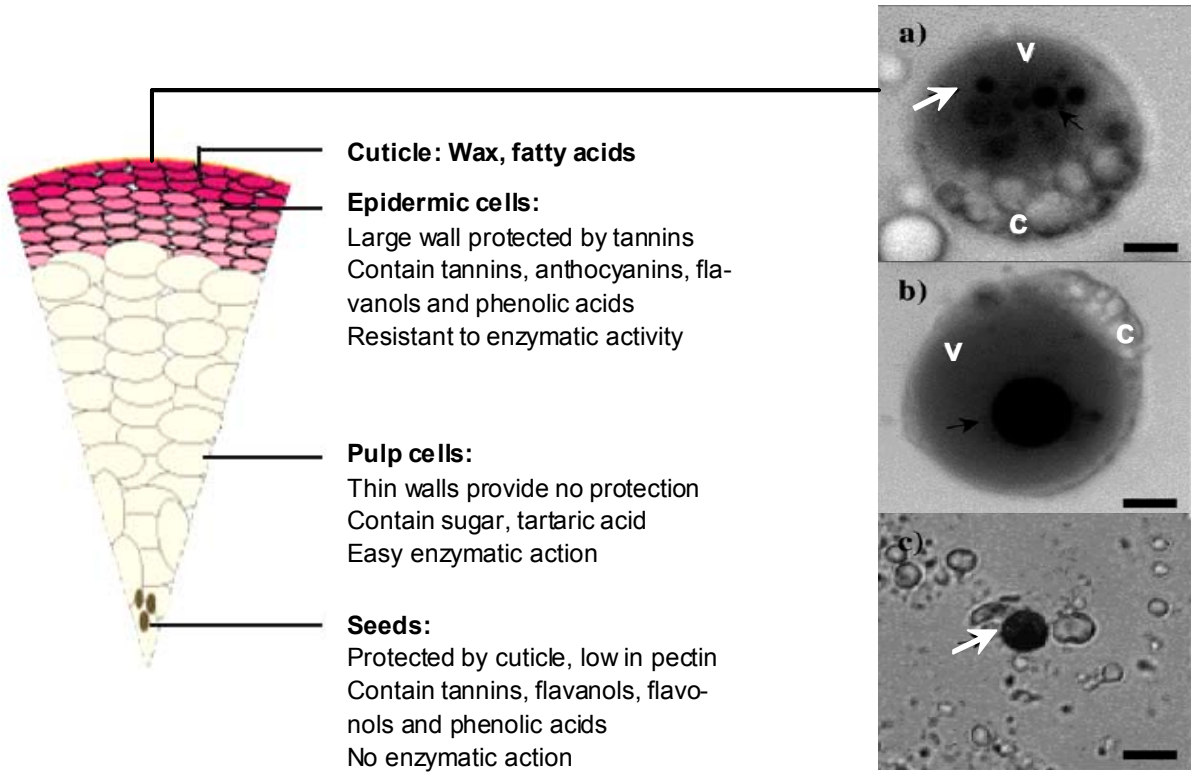
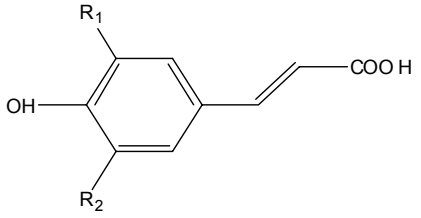


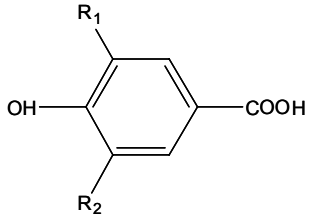
Figure 2.3: Polyphenol location in grapes (Left). Microscopic images of dark-grown *V. vinifera* L. protoplasts under bright field microscopy (a, b) before and (c) after lysis. Arrows indicate anthocyanin vacuolar inclusion; Bar: 10µm, V: vacuole; C: cytoplasm (Right) (adapted from Conn et al., 2003).

2.2.1 Phenolic acids

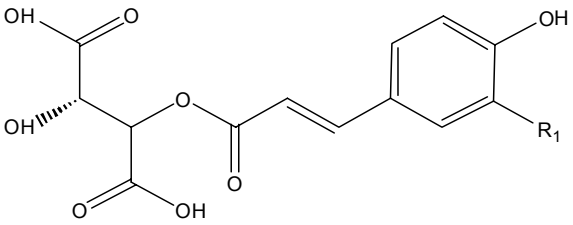
Phenolic acids are classified in function of their carbohydrate backbone in hydroxycinnamic acids (C_6–C_3) or hydroxybenzoic acids (C_6–C_1). The most common phenolic acids found in grapes are represented in Figure 2.4. In addition hydroxycinnamic acid esters such as coutaric, caftaric and fertaric are also often found in grapes (Macheix, 1990).



Hydroxycinnamic acids	R_1	R_2
p-Coumaric acid	H	H
Ferulic acid	OCH_3	
Sinapic acid	OCH_3	OCH_3
Caffeic acid	H	OH



Hydroxybenzoic acids	R_1	R_2
Gallic acid	OH	OH
Protocateic acid	OH	H
Syringic acid	OCH_3	OCH_3
Vanillic acid	OCH_3	H



Hydroxycinnamic acid esters	R_1
Caftaric acid	OH
Coutaric	H
Trans-Fertaric acid	OCH_3

Figure 2.4: Main phenolics identified in *V. vinifera* L. berries.

2.2.2 Flavonoids

The flavonoids are generally categorized as phenolics or polyphenols because of their chemical structure (Figure 2.5). Flavonoids possess a C_{15} (C_6-C_3-C_6) flavone nucleus, two benzene rings (A and B) linked through an oxygen containing pyran or pyrone ring (C).

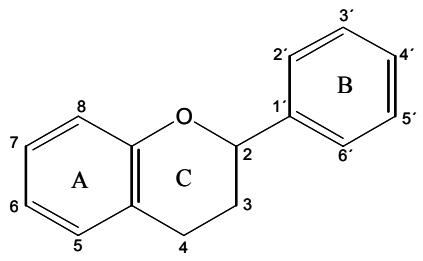


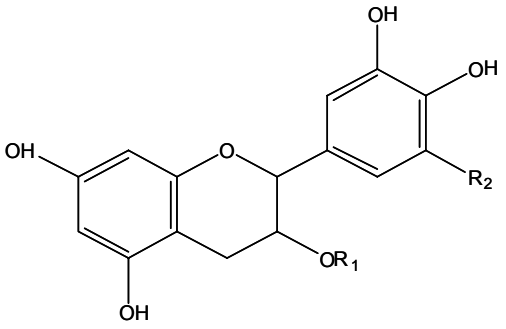
Figure 2.5: Basic monomeric structure of flavonoids.

2.2.2.1 Flavanols, flavonols, flavones and dihydroflavonols

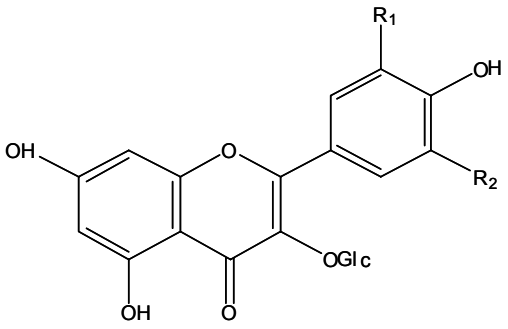
Flavanols, possess the same C_{15} (C_6-C_3-C_6) flavone nucleus as the rest of flavonoids (Harbone, 1988; Macheix, 1990). This is the common structure of 3-deoxyflavonoids (flavones, flavanones, isoflavones and neoflavones) and 3-hydroxyflavonoids (flavonols, anthocyanins, flavan-3,4-diols and flavan-3-ols). Flavan-3-ols are encountered in grape as monomers, oligomers, and polymers and posses a pale yellow colour. Major monomers are (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-gallate. Gallocatechin in *V. vinifera* L. and catechin-3-gallate and gallocatechin-3-gallate in non-*vinifera* grapes have also been reported (Figure 2.6).

Grape seed proanthocyanidins are based on (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-gallate units and partly galloylated procyanidins. B-type procyanidin dimers and trimers, including galloylated derivatives, have been identified in grape.

The major flavonols in grapes are the 3-glucoside forms of quercetin, kaempferol, isorhamnetin and myricetin mainly found in grape skins (Fig. 2.6). Other flavonols include different glucoside derivatives.



Flavan-3-ols	R_1	R_2
(+)-Catechin	H	H
(+)-Gallocatechin	OH	H
(-)-Epicatechin	H	H



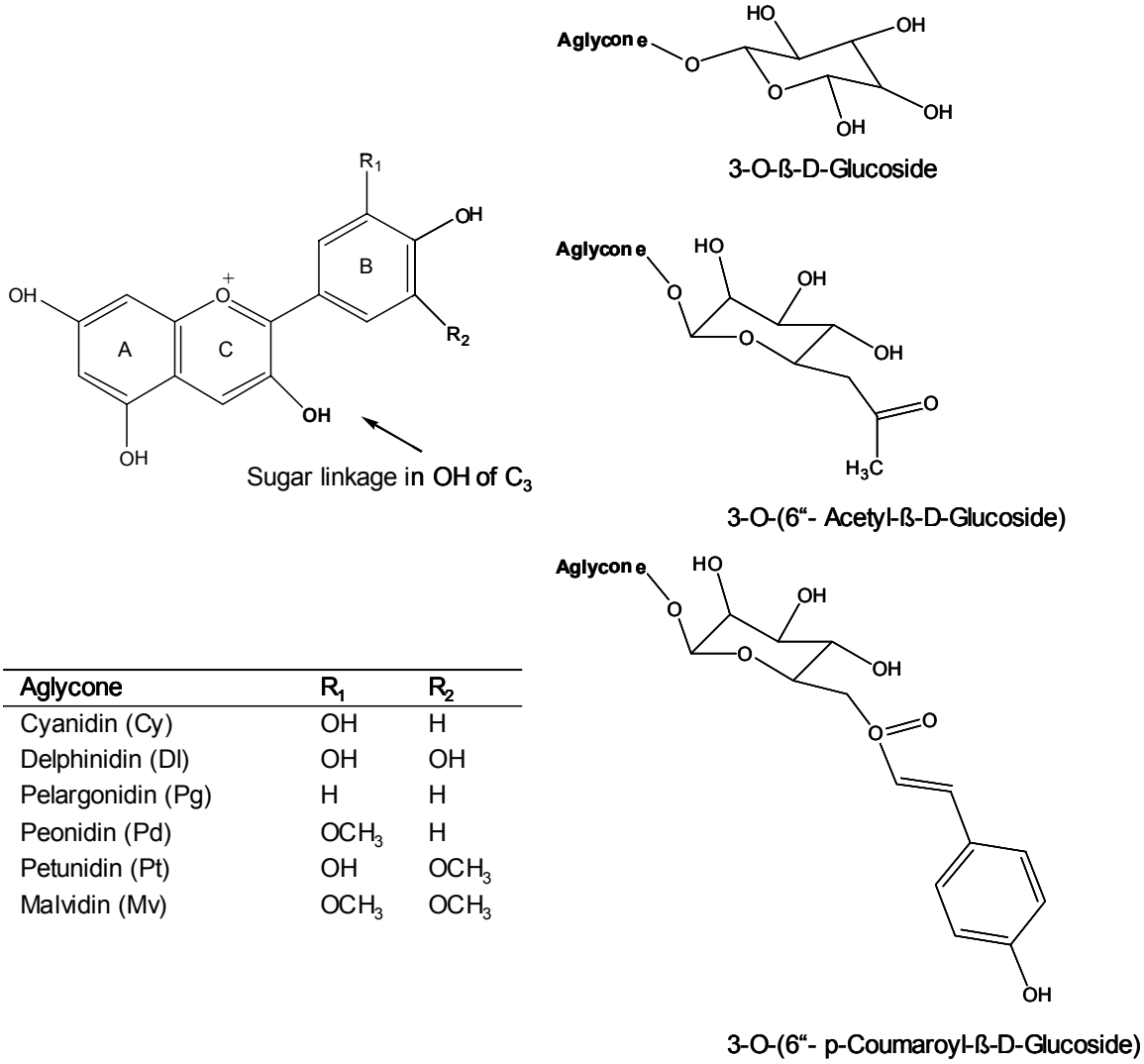
Flavonols	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Myricetin	OH	OH

Figure 2.6: Common flavan-3-ols and flavonols found in grapes.

2.2.2.2 Anthocyanins

Anthocyanin structure is based on a C_{15} skeleton (aglycone) with a chromane ring bearing a second aromatic ring B in position 2. Anthocyanin molecule differs from the other flavonoids in the positive charge on C_5. Anthocyanin structure is complemented by one or more sugar molecules linked at different hydroxylated positions of the basic structure (Delgado-Vargas & Paredes-López, 2000). The most abundant anthocyanins in grapes are the glucoside forms

of cyanidin (Cy), malvidin (Mv), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and pelargonidin (Pg) whose chemical structures are represented in Figure 2.7. Those of *V. vinifera* L. are mostly 3-monoglucosides, whereas non-*vinifera* species also contain substantial amounts of 3,5-diglucosides. The 3-acetylglucoside, 3-p-coumaroylglucoside, and 3-caffeoylglucoside of these anthocyanidins are also present in most grape varieties.



Aglycone	R_1	R_2
Cyanidin (Cy)	OH	H
Delphinidin (Dl)	OH	OH
Pelargonidin (Pg)	H	H
Peonidin (Pd)	OCH_3	H
Petunidin (Pt)	OH	OCH_3
Malvidin (Mv)	OCH_3	OCH_3

Figure 2.7: Structure of main anthocyanins found in *V. vinifera* L. grapes.

2.2.3 Varietal and environmental effects on grape flavonoids

The flavonoid and phenolic content in plants can be influenced by cultivation and harvesting conditions such as growing conditions, degree of ripeness, fruit size and plant variety (Herrmann, 1976). As previously mentioned, flavonol and anthocyanin content in grapes varies within species and cultivars. For instance, Pinot noir and Spätburgunder grapes contain non acylated anthocyanins whereas *non-vinifera* varieties posses a higher content of

3,5-diglucosides. Depending on the species the anthocyanin content ranges from 500 mg kg^{-1} up to 3 g kg^{-1}. Red and white varieties present remarkable differences in flavonol and anthocyanin composition.

Light plays a crucial role in the phenolic metabolism (Macheix, 1990). Light stimulates the synthesis of flavonoids, especially anthocyanins and flavones, since UV light enhances phenylalanine ammonia-lyase activity (Britton, 1983; Macheix, 1990; Dixon & Paiva, 1995).

The synthesis of flavonoids, phenolic acids and other phenylpropanoids is increased in plant tissues following wounding or infection by pathogenic organisms or feeding by herbivores (Britton, 1983; Bennet & Wallsgrove, 1994; Dixon & Paiva, 1995; Strack, 1997). An excessive anthocyanin production has been observed in infected plant tissues (Britton, 1983). Simple phenolic acids, as well as complex tannins on the surface of the plant, are effective deterrents e.g. in plant-bird interactions they interfere their digestion, as well as the astringency of plant tannins make plants less appealing to birds (Strack, 1997; Bennet & Wallsgrove, 1994).

Other stressors such as temperature and nutritional stress can also influence flavonoid synthesis. An increase in flavonoid levels in grapes has been reported under cold and nutritional stress (phosphate limitation). Jones and Hartley (1998) reported that the synthesis of both, proteins and polyphenolics involves the same precursor, phenylalanine. Phenylalanine cannot be used in the synthesis of a phenol if it has been incorporated into a protein and this step is mediated by phenylalanine ammonia-lyase (PAL). Protein synthesis and polyphenolic synthesis create competing demands for phenylalanine. Consequently, when the protein synthesis rate is high, the rate of polyphenolic synthesis is low, and vice versa. In the presence of a stressor, such as an inadequate supply of nitrogen, the balance between the chemical compounds changes; the rate of protein synthesis declines and the synthesis of phenolic compounds increases (Cartelat et al., 2005). Flavonoids and isoflavonoids serve as chemoattractants for nitrogen fixing symbionts (Graham, 1991).

Pesticides may both increase or decrease flavonoid synthesis depending on their chemical nature and plant characteristics (Woese et al., 1997). Some herbicides are reported to reduce carbon fixation in plants decreasing the proportion of carbon necessary for the synthesis of phenolic compounds (Lydon & Duke, 1989), while others are reported to cause oxidative damage in plants promoting PAL synthesis towards flavonoid synthesis (Hoagland, 1996). Some fungicides can also modulate concentrations of secondary compounds. Maneb, benomyl, and nabam for example induce the synthesis of flavonoids in soy (Lydon & Duke, 1993). The studies reported cover only a minor portion of pesticides and plant phenolics and the target modulation effect of pesticides in grapes is still unclear.

2.3 Bioavailability of grape flavonoids

Grape flavonoid metabolism has been a matter of debate and main theme of a large number of studies and publications. The knowledge on the absorption, bioavailability and metabolism of grape flavonoids has been mainly reported for the ingestion of red wine or grape juices and is important to fully evaluate their potential beneficial roles in human health. This chapter considers the mechanisms of absorption and metabolism of anthocyanins, but also of quercetin and catechin, as they are structurally similar to anthocyanins.

2.3.1 Absorption of flavonoids

Flavonoids are mostly present in grapes as glucosides (Hollman & Katan, 1998a; 1998b). Glucoside groups increase flavonoid water solubility but limit passive diffusion across a biological membrane. Only aglycones are considered absorbable, hence an active transport mechanism or hydrolysis of β-glucosides is required (Brown et al., 1998; Gee et al., 2000; Walgren et al., 2000; Day & Williamson, 2001; Manach et al., 2005). The absorption of most flavonoids in humans occurs in the small intestine (Hollman et al., 1995; 1996; Scalbert & Williamson, 2000; Williamson et al., 2000). Two main mechanisms have been reported: Transport of intact flavonoid glucosides by a sodium-glucose co-transporter or the extra-cellular hydrolysis of the glucoside via lactate phlorizin hydrolase at the brush border, followed by passive diffusion of the aglycone (Mizuma et al., 1994; Hollman et al., 1999; Gee et al., 2000; Williamson et al., 2000; Manach et al., 2005). A fraction of flavonoids will escape absorption in the upper small intestine and undergo bacterial metabolism in the lower intestine where the compounds are deglucosylated and the aglycones will be further transported and metabolised (Scalbert & Williamson, 2000; Williamson et al., 2000; Manach et al., 2005). The absorption mechanisms are represented in Figure 2.8.

Once inside the cell, the intact glucoside could either directly cross the basolateral membrane into the portal circulation, or be hydrolysed by cytosolic β-glucosidases before intestinal metabolism and transport (Day et al., 1998; Walle et al., 2000; Williamson et al., 2000). Absorption mechanisms are still a matter of debate, and several works have reported that anthocyanins are not substrates of lactate phlorizin hydrolase and cytosolic β-glucosidases (Mazza et al., 2002; Wu et al., 2002; Galvano et al., 2004; Kay et al., 2004; 2005). However anthocyanin glucosides and glucuronide derivatives have been found in human plasma and serum. Passamonti et al. (2003) and Talavera et al. (2003) have also proposed the stomach as a site of absorption of anthocyanins, although this hypothesis is still under research

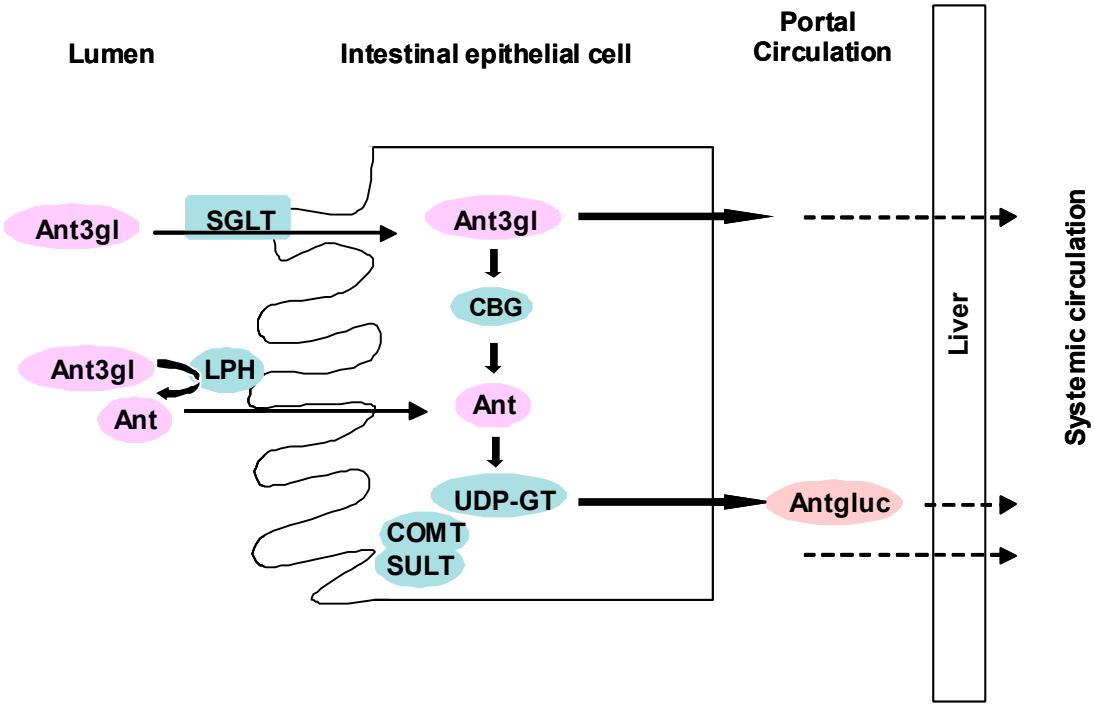


Figure 2.8: Potential mechanisms of anthocyanin (Ant) absorption. SGLT, sodium-glucose co-transporter; Ant3gl, anthocyanin 3-glucoside; CBG, cytosolic ß-glucosidase; LPH, lactate phlorizin hydrolase; UDP-GT, UDP-glucuronosyltransferase; Antgluc, anthocyanin glucuronide; COMT, catechol-O-methyltransferase; SULT, sulfotransferase (Gee et al., 2000; Day et al., 2000).

2.3.2 Metabolism

The majority of flavonoids are found in the circulation and urine as methylated, sulphated, glucuronidated and glycated conjugates (Aziz et al., 1998; Hollman & Katan, 1998a; 1998b; Donovan et al., 2001; Manach et al., 2005). The glucuronation reaction is catalysed by UDP-glucuronosyltransferases which are found in high concentrations in the liver, intestine and kidneys. The principal sites for flavonoid glucuronidation in humans are the intestine and the liver (Mojarraby & Mackenzie, 1998; Strassburg et al., 1998; Crespy et al., 1999; Donovan et al., 2001). Methylation is the second most significant conjugation reaction in flavonoids (Kuhnle et al., 2000; Williamson et al., 2000) and is carried out by methyltransferases found in many tissues including liver and the intestine (Laitinen & Watkins, 1986). The most common methylation reaction in flavonoids is the O-methylation catalysed by catechol-O-methyltransferase. Sulfation and glycylation are also common conjugation reactions in flavonoid metabolism.

2.3.3 Elimination and reabsorption

Little is known about the metabolic fate of flavonoids; however, published data suggest that they are poorly absorbed. Bitsch et al. (2004) found an anthocyanin and anthocyanin metabolites´ content in plasma of 22.7 nmol L^{-1} and 95.5 nmol L^{-1} after 7 hours oral consumption of red grape juice and red wine, respectively. The urinary excretion of anthocyanins in red wines was 0.18% and 0.23% in red grape juice. Lapidot et al. (1998) detected only 1.5 to 5.1% of ingested red wine anthocyanins in the urine of human subjects within 12 h of wine consumption. Later, Bub et al. (2001) found an absorption of 0.03% of malvidin-3-O-glucoside after 6h of red wine ingestion. Colonic bacteria play an important role in the flavonoid metabolism and absorption. They produce glucosidases, glucuronidases and sulphatases that can strip flavonoid conjugates from their sugar moieties, glucuronic acids and sulphates (Scheline, 1973). The subsequent degradation products are evidently absorbed as they were found in the urine (Boulton, 1999; Schneider, 1999; Skibola & Smith, 2000).

2.3.4 Nutritional and pharmacological effect of polyphenols

The nutritional and pharmacological effects of polyphenols depend on their concentration in foods. As Paracelso (1493-1541) once mentioned “the dosis makes the poison”. Thus, antinutritional effects of polyphenols have been demonstrated in animal experiments (Glick & Joslyn, 1970; Rostagno et al., 1973) but also in humans (Stavric & Matula, 1992). Some adverse effects such as growth decrease and inhibition of enzymes have been reported (Rostagno et al., 1973; Mehansho et al., 1985; Oh & Hoff, 1986; Guyot et al., 1996). Some flavan-3-ols are also supposed to interfere pancreatic digestion (Driedger & Hatfield, 1972) as well as to diminish iron bioavailability (Disler et al., 1975; Hallberg & Rossander, 1982; Hurrell, 1988; 1996) or cause allergies (Benezra, 1988).

However, a higher number of studies have reported the beneficial effects associated with raised consumption of polyphenols and some studies suggested the possible protective effect of flavonoids against vascular diseases (Knekt et al., 1996; Keli et al., 1996; Yochum et al., 1999; Hirvonen et al., 2001; Mennen et al., 2004) and certain cancers (Knekt et al., 1997; Garcia-Closas, 2001; Birt et al., 2001; Hirvonen et al., 2001; Arts et al., 2002; Sun et al., 2002). Due to polyphenol antioxidant properties, they prevent from a great number of diseases and damages caused by reaction oxygen species such as arthritis, atherosclerosis, diabetes, inflammation and Parkinson among others.

2.3.5 Structure-activity relationships

Flavonoids from grapes, wine and vine by-products have been demonstrated to inhibit the oxidation of human low-density lipoproteins *in vivo* (Frankel et al., 1995; Teissedre et al., 1996) and possess health promoting properties (Shrikhande, 2000) as previously mentioned. The structure of phenolic compounds is crucial for their radical scavenging and metal chelating activity. In phenolic acids for example, the antioxidant capacity depends on the number and position of the hydroxyl groups in relation to their carboxyl functional group (Rice-Evans et al., 1996; Robards et al., 1999). Monohydroxy benzoic acids with the –OH moiety at the ortho- or para- position to the –COOH show no antioxidant capacity contrarily to –OH groups at the meta- position. The antioxidant capacity of phenolic acids increases with increasing degree of hydroxylation, although methoxyl groups reduce the activity (Rice-Evans et al., 1996; Balasundram et al., 2006).

The structure–activity relationship of flavonoids is related to the substitution patterns on ring B and C (Figure 2.5).

1. The O-dihydroxy structure in the B ring results in a higher antioxidant capacity and higher stability to the aroxyl radical by electron delocalisation (van Acker et al., 1996).
2. The presence of hydroxyl groups at the 3´, 4´ and 5´ positions of ring B enhances the antioxidant capacity of flavonoids compared to those that have a single hydroxyl group (van Acker et al., 1996).
3. A double bond between C_2 and C_3 combined with a 3-OH in ring C also enhances the active radical scavenging capacity of flavonoids (van Acker et al., 1996).
4. Substitution of hydroxyl groups in ring B by methoxyl groups alters the redox potential, which affects the radical scavenging capacity of flavonoids (Pietta, 2000; Seeram & Nair, 2002).

2.4 The biochemistry and stability of anthocyanins

Interest in anthocyanins has increased because of their potential use as natural colorants (E163) and potential beneficial health effects. Regular consumption of anthocyanins and other polyphenols in fruits, wines and jams reduce risks of chronic diseases such as cancer, cardiovascular disease, virus inhibition and Alzheimer´s disease (Middleton et al., 2000; Bohm et al., 1998; Murkovic, 2002; Rice Evans & Parker, 2003).

The colour of anthocyanins is based on the fully conjugated 10-electron A-C ring π-system, with some contribution of the B ring (Figure 2.5). If this structure is disrupted, anthocyanins lose their characteristic colour (Waterhouse, 2002). However, this resonant structure is highly instable and the groups attached to this structure influence its stability substantially (Delgado-Vargas & Paredes-López, 2003). Other factors such as pH, solvents, temperature anthocyanin concentration, oxygen, light, enzymes and other accompanying substances may also influence anthocyanin stability.

2.4.1 Structural effects

Anthocyanin stability depends on the substitution pattern of the aglycone. Glucosyl units and acyl groups attached to the aglycone have significant effect on their stability and reactivity as well as the number and placement of hydroxyl and methoxyl groups that confer more or lesser stability to the molecule. According to Mazza and Brouillard (1987a; 1987b) a higher number of methoxyl and hydroxyl groups weaken anthocyanin stability. Indeed, methoxyl groups are more molecule destabilising than hydroxyl ones. This substitution pattern also influences the colour of anthocyanins. The colour of anthocyanins changes from pink to blue as the number of hydroxyls increase. Glucosilated forms of anthocyanidins are more stable and with a higher occurrence in nature than aglycones (Timberlake & Bridle, 1966a; 1966b). Also different patterns of hydroxylation and glucosylation appear to modulate their antioxidant properties. Hydroxyl groups situated at $C_{3'}$ and $C_{4'}$ clearly increase the antioxidant capacity of the anthocyanins, as well as the glucoside group joined to the flavilium ring affect positively their antioxidant properties; 3-glucosylation in the C ring increased the oxygen radical absorbance capacity (ORAC) for glucose and rhamnoglucose whereas ORAC values decreased for galactose (Wang et al., 1997). Acylation further increases the stability of anthocyanins (Bassa & Francis, 1987). Polyacylated and aromatic acyl substituents are more stable than monoacylated and aliphatic acyls (Asen, 1976; Francis, 1989).

2.4.2 Intrinsic factors

2.4.2.1 PH-value

In aqueous solutions anthocyanins undergo structural transformations which are pH dependent (Brouillard et al., 1982). It has been found that four major anthocyanin forms exist in equilibrium: The red flavilium cation, the blue quinonoidal base, the colourless carbinol pseudobase, and the colourless chalcone. According to pH changes one or other form will

predominate and define the colour of the solution. The flavilium cation is the most stable and the most coloured species. The generalised effect of pH on non-acylated monoglucoside anthocyanin equilibrium is represented in Figure 2.9.

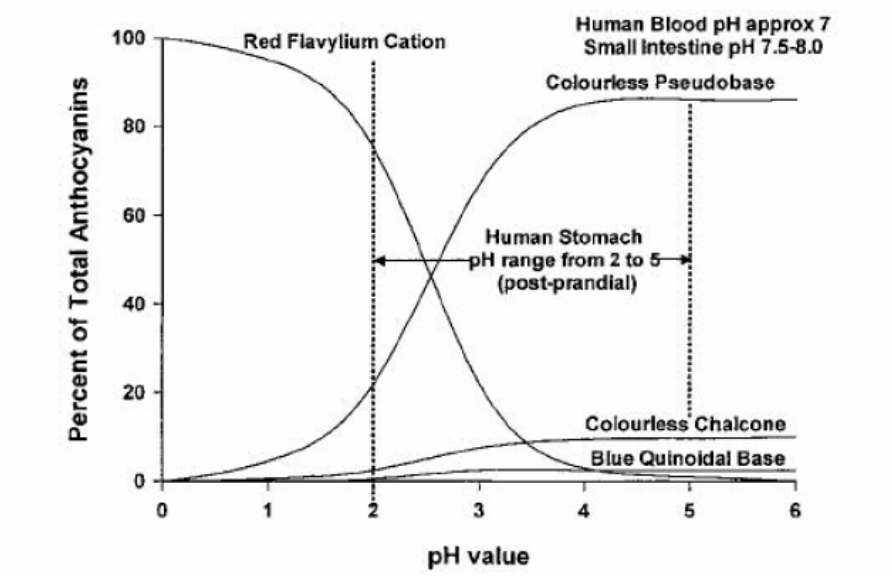


Figure 2.9: Effect of pH on non-acylated monoglucoside anthocyanin equilibria (Brouillard, 1982).

2.4.2.2 Temperature

Processing and storage under low temperature can improve the stability of anthocyanins (Delgado-Vargas & Paredes-Lopez, 2003). Anthocyanins are very sensitive to temperature and temperatures higher than 70°C cause rapid degradation and discoloration of anthocyanins (Cemeroglu et al., 1994; Kirca & Cemeroglu, 2003). Anthocyanin thermal degradation has been reported to follow a first order reaction (Markakis et al., 1957; Keith & Powers, 1965; Tanchev & Ioncheva, 1973; Rhim, 2002; Ahmed et al., 2004; Tseng et al., 2006). Hrazdina (1971) pointed out that the decomposition of anthocyanins upon heating leaded to a chalcone structure which further decomposed to coumarin glucoside derivatives with a loss of the B-ring. Moreover, Adams et al. (1973) reported that the aglycone sugar bonds were highly susceptible to hydrolysis even at acid pH. Later on Simpson (1985) suggested that the thermal degradation of anthocyanins could occur via two mechanisms: hydrolysis of the 3-glucoside linkage to form a labile aglycone and hydrolytic opening of the pyrilium ring to form a substituted chalcone (Furtado et al., 1993); which further degrades into different benzoic acid derivatives (Salidova et al., 2006; Seeram et al., 2001); trihydroxybenzaldehydes have also been identified as an end product of thermal degradation of anthocyanins (Figure 2.10). Anthocyanin degradation is generally correlated to a loss or change in colour.

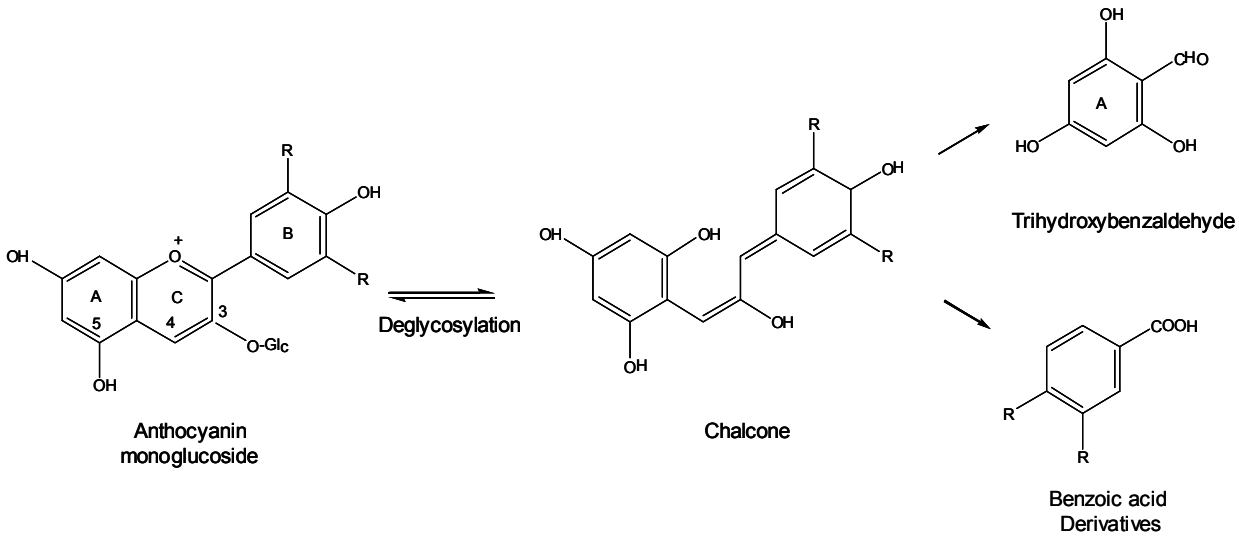


Figure 2.10: Postulated degradation of anthocyanin monoglucoside at pH 3.7 accelerated by heat (Furtado et al., 1993).

2.4.2.3 Light

Anthocyanins colour can be better preserved in dark. The deleterious effect of light was reported on the colour of Concord grape juice in bottles (Tressler & Pederson, 1936). The colour of carbonated grape anthocyanin beverages was maintained in a 70% when beverages were kept in the dark, whereas in beverages exposed in daylight a loss of 50% was found. Light anthocyanin degradation products are reported to be similar to those formed by thermal degradation (Furtado et al., 1993). Again, acylated anthocyanins are less affected by light than the non-acylated ones (Giusti & Wrolstad, 1996b).

2.4.2.4 Oxygen

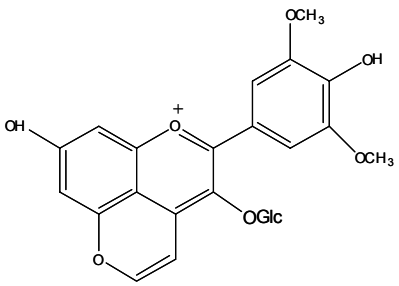
Oxygen and hydrogen peroxide can easily oxidise anthocyanins, and this mechanism can be accelerated by ascorbic acid (Delgado-Vargas & Paredes-López, 2003). Ascorbic acid interacts with oxygen producing H_2O_2 which is responsible for anthocyanin bleaching (Meschter, 1953; Markakis, 1982; Talcott et al., 2003). However, the stability of acylated anthocyanins has been observed to increase in presence of ascorbic acid (Del Pozo-Insfran, 2004). The presence of oxygen, together with elevated temperature, was the most detrimental combination of different factors tested against colour deterioration in isolated and berry juice anthocyanins (Nebesky et al., 1949). Oxygen can oxidise anthocyanins directly or through indirect oxidation, where oxidized media compounds further react with anthocyanins (Jackman et al., 1987). Anthocyanins react also with oxygen radicals, i.e. peroxyradicals protecting in *in vivo* experiments from deleterious effects of oxygen radicals (Matsufuji et al., 2003; Garcia-Alonso et al., 2004; Rossetto et al., 2004).

2.4.3 Formation of pyranoanthocyanins

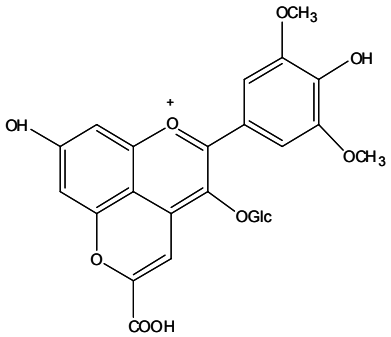
Pyranoanthocyanins are molecules originated by cyclo-addition of pyruvic acid, acetaldehyde, acetone, 4-vinylcatechol, 4-vinylphenol, 4-vinylguaiacol and vinylcatechin with anthocyanins (monoglucosides and their acyl derivatives) (Somers, 1971; Timberlake, 1976; Liao et al., 1992; Santos-Buelga, 1995; Rivas-Gonzalo, 1995; Fulcrand, 1996; Francia-Aricha, 1997; Es-Safi, 1999; Vivar-Quintana, 1999; Remy, 2000).

Pyranoanthocyanins were first reported in wine model solutions being later found in authentic wines and in smaller proportions in black carrot, blood orange juices, strawberries and red onions (Schwartz et al., 2003a; Fossen & Andersen, 2003; Andersen et al., 2004). Pyranoanthocyanins have also been detected in frozen grapes after storage (Revilla et al., 1999) and in grape pomace (Amico et al., 2004).

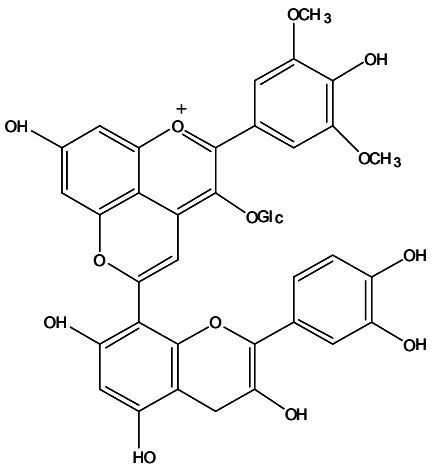
Anthocyanin pyranoanthocyanins and oligomers are responsible for the change of colour in red wines from red to brown-red and are more stable than monomeric ones (Schwartz et al., 2003b). Cyclo-addition reactions cause hypsochromical shift in the visible absorption maxima of the anthocyanins, producing a change in colour towards orange hues (Rivas Gonzalo et al., 1995; Francia-Aricha et al., 1997; Fulcrand, 1998; Hakansson, 2003). Fulcrand et al. (1996), suggested two possible pathways for the formation of pyranoanthocyanins; a cyclo-addition, or an electrophilic addition of a vinylphenol double bond to an anthocyanin nucleus, followed by an oxidation step. This latter was favoured due to the electrophilic site at C_4 and the nucleophilic site of C_5 hydroxy group of the anthocyanins. The substitution at C_4 of the anthocyanin molecule stabilises the pigment molecule as it prevents it from hydratation which leads to a colourless carbinol base. A great number of pyranoanthocyanins has been detected in red wine during maturity and ageing and some of the most common adducts are represented in Figure 2.11 (Alcalde-Eon et al., 2006).



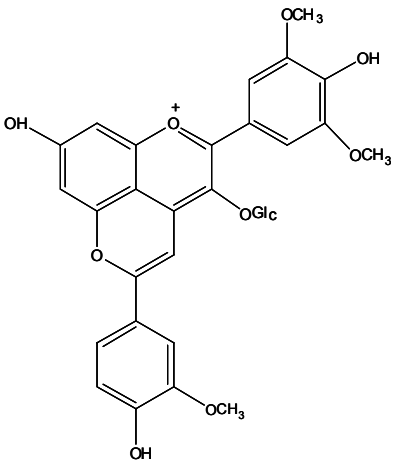
Vitisin B



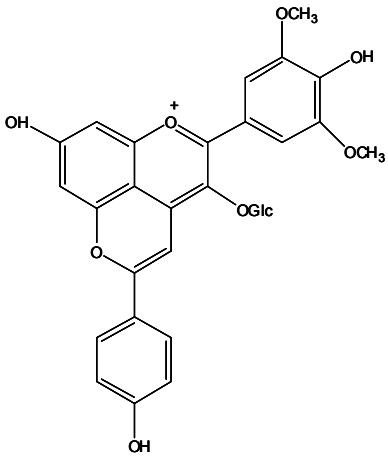
Mv3gl, pyruvic adduct (Vitisin A)



Mv3gl, 4-vynilcatechol adduct



Mv3gl, 4-vinylguaiacol adduct



Mv3gl, 4-vinylphenol adduct

Figure 2.11: Structures of different malvidin-3-O-glucoside (Mv3gl) derived pyranoanthocyanins identified in wines and grape pomace.

2.5 High hydrostatic pressure

The application of high hydrostatic pressure for microbial inactivation and food treatment has been reported since the beginning of the past century. First applications of high hydrostatic pressure for milk preservation were reported by Hite in the 1890s and later extended for the inactivation of microorganisms in fruit and vegetables (Hite, 1914). A pioneering work in the application of high pressure for the coagulation of egg albumin was reported by Bridgman (1914) who received the Physics Nobel Price for the development of a high pressure device in 1946. It then took almost 40 years for Japan to re-discover the application of high-pressure in food processing and launch pressurised products in the market. The feasibility of high hydrostatic pressure to inactivate microorganisms and spoilage catalysing enzymes, whilst retaining colour, flavour and other food quality attributes, has encouraged Japanese and American food companies to introduce high pressure processed foods in the market

(Mermelstein, 1997; Hendricks et al., 1998). Products such as jams, jellies and sauces were first commercialised in the 1990s in Japan. Later on fruit juices especially from apple and orange were commercialised in France and Portugal; guacamole and oysters in the USA and ham and different tapas in Spain and Denmark (Hugas et al., 2002). High hydrostatic pressure is more than a preservation treatment and can also be used to the development of new products or ingredients changing structure and texture (Hayashi, 1990). Over the past two decades, many works have described the influence of high hydrostatic pressure on bacteria (Zobell & Johnson, 1949; Ludwig & Schreck, 1997), proteins (Heremans, 1982), viruses (Ludwig, 1991), enzymes (Kunugi, 1992; Hendricks, 1998), moulds (Eicher, 1998), lipids (Buchheim, 1996) as well as the influence of high hydrostatic pressure in food physical and functional properties (Cheftel, 1992; Tonello, 1994), in food chemical reactions (Tauscher, 1995), or interesting water phase transitions (Kalichevsky et al., 1995; Knorr et al., 1998). The application of high hydrostatic pressure in the food industry can be summarised as follows:

1. It enables food processing at ambient temperature or even lower temperatures.
2. It enables instant transmittance of pressure throughout the system, irrespective of size and geometry.
3. It causes microbial death avoiding heat damage and the use of preservatives improving overall food quality.
4. It can be used to create ingredients with novel functional properties.

2.5.1 Effect of high hydrostatic pressure on chemical reactions

Pressure and temperature can influence thermodynamics and kinetics of chemical reactions. Although the effect of pressure is lower than that of temperature (a ΔP of 100 MPa is equivalent to ΔT of 10°C) it influences media viscosity, melting point, boiling point, density, dielectric constant, solubility and other parameters.
The influence of high hydrostatic pressure on a chemical reaction can be described by the Le Chatelier-Braun principle:

"If a chemical reaction at equilibrium is subjected to a change in conditions that displaces it from equilibrium, then the reaction adjusts toward a new equilibrium state. The reaction proceeds in the direction which counteracts this change in conditions".

A process associated with a decrease in volume is favoured by pressure (Hamann, 1980). Volume changes observed in chemical reactions under pressure are represented in Figure 2.12.

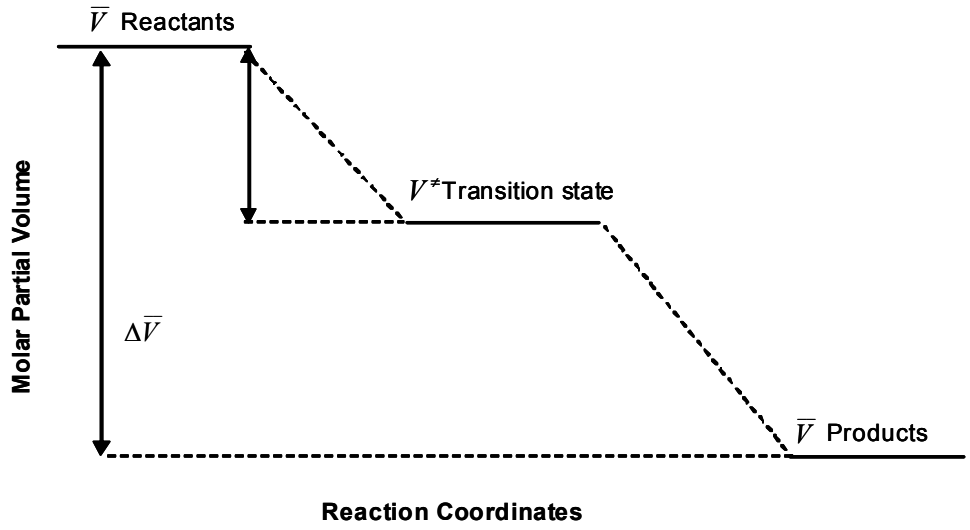


Figure 2.12: Volume profile of a reaction carried out under pressure (Balny et al., 1989).

The partial volume of dissolved components is influenced by 3 factors:

1. Intrinsic share of the dissolved component $\Delta\overline{V}_{\mathrm{int}\,r}$ which is determined by a change in van der Waals radii.
2. Solvation $\Delta\overline{V}_{solv}$ which includes interactions between components and solvent which leads to electrostriction, changes in polarity and dipole interaction.
3. Interaction among all dissolved components normally not considered in diluted samples.

According to mentioned factors, the reaction volume can be defined as follows (Eq. 2.1):

$$\Delta\overline{V} = \Delta\overline{V}_{\mathrm{int}\,r} + \Delta\overline{V}_{solv} \qquad \text{(Eq. 2.1)}$$

The driving force of a reaction is the negative free enthalpy change expressed as $\Delta G = G^{(product)} - G^{(reac\,\mathrm{tan}\,t)}$. It depends on the initial and final concentrations, temperature and pressure and can be expressed in function of the enthalpy and entropy by the fundamental Gibbs-Helmholz (Eq. 2.2):

$$\Delta G = \Delta H - T\Delta S = \Delta E + p\Delta V - T\Delta S \qquad \text{(Eq. 2.2)}$$

where ΔG is the free enthalpy, ΔE the activation energy, p is the pressure, ΔS the entropy and $\Delta\overline{V}$ the reaction volume. Thus, the reaction volume $\Delta\overline{V}$ can be defined by the van´t Hoffs equation (Eq. 2.3):

$$\Delta \overline{V} = V^{(product)} - V^{(reac\tan t)} = \left(\frac{\partial \Delta G}{\partial p}\right)_T = -RT\left(\frac{\partial \ln K}{\partial p}\right)_T \qquad \text{(Eq. 2.3)}$$

which also describes the influence of T temperature on the equilibrium. K is the equilibrium constant and R the general gas constant (R = 8.341 J K^{-1} mol^{-1}). The unit of reaction volume is cm^3 mol^{-1}.

$\Delta \overline{V}$ is decisive to define the state of equilibrium of a chemical reaction under pressure. A negative reaction volume indicates that product formation is favoured under increasing pressure. For example, the dissociation of water has a $\Delta \overline{V}$ = -22 cm^3 mol^{-1} and that of phosphate buffer -18 cm^3 mol^{-1} (Millero et al., 1972; Kitamura & Itoh, 1987).

Pressure also influences the kinetics or speed of a reaction defined by the change in the partial volume from the reactants to the transition state, also called activation volume $\Delta V^{\neq}$ (Eyring, 1935). According to Figure 2.12 the activation volume can be given as Eq. 2.4:

$$\Delta V^{\neq} = \Delta V^{\neq}_{\text{int}\,r} + \Delta V^{\neq}_{solv} \qquad \text{(Eq. 2.4)}$$

which is influenced similarly by the same factors as the reaction volume, and can be expressed as (Eq. 2.5):

$$\Delta V^{\neq} = V^{\neq} - V^{(reac\tan t)} = \left(\frac{\partial \Delta G^{\neq}}{\partial p}\right) = -RT\left(\frac{\partial \ln k}{\partial p}\right)_T \qquad \text{(Eq. 2.5)}$$

where k is the rate constant and $\Delta G^{\neq}$ and $\Delta V^{\neq}$ are the free activation enthalpy and the activation volume of the reaction, respectively.

The amount of contraction under pressure can be calculated by the compressibility factor $\beta^{\neq}$ which is dependent on the intermolecular forces acting within the substance. Compression decreases the average of intermolecular distance and reduces rotational and translational motion. The compressibility coefficient of activation $\Delta\beta^{\neq}$ is shown in Eq. 2.6 (Asano & Le Noble, 1978; van Eldik et al., 1989).

$$\Delta\beta^{\neq} = -\left(\frac{\partial \Delta V^{\neq}}{\partial p}\right)_T \qquad \text{(Eq. 2.6)}$$

Where $\Delta V^{\neq}$ is the activation volume, pressure *p* and *T* is the temperature of the system in Kelvin.

The compressibility of liquids decreases with pressure, since the initial free volume has largely disappeared, and the repulsive potential of molecules is stronger than the attractive under high pressure. On the other hand, compressibility of most liquids increases with rising temperature due to the increase of the internuclear distances (increase in free volume).

2.5.2 Effect of high hydrostatic pressure on food constituents

2.5.2.1 Water

The physical properties of water under pressure are crucial for understanding the influence of high hydrostatic pressure on food, since water represents the major component of most food systems and is often used as pressure transmitting liquid. The properties of water under high hydrostatic pressure vary as a function of the pressure range. The volume contraction of water is approximately 15% at 600 MPa and 22°C (Cheftel, 1992). This volume contraction leads to a density increase which leads to an increase in solute diffusion coefficients (Buckow, 2006). Water adiabatic compression causes an immediate increase of 2-3°C per 100 MPa, this increase will depend on the initial water temperature (Cheftel & Culioli, 1997) and is reversed under decompression. The dielectric constant ε of water changes as a function of changes in pressure and temperature. It decreases following an increase in temperature and pressure influencing ionic reactions and media polarity (Fernández et al., 1997). Self ionization of water to H_3O^+ and OH^- is enhanced by pressurisation, since the ionisation volume is negative (-22 cm^3 mol^{-1} at 25°C). The phase diagram of water as a function of temperature and pressure is represented in Figure 2.13 (Bridgman, 1912).

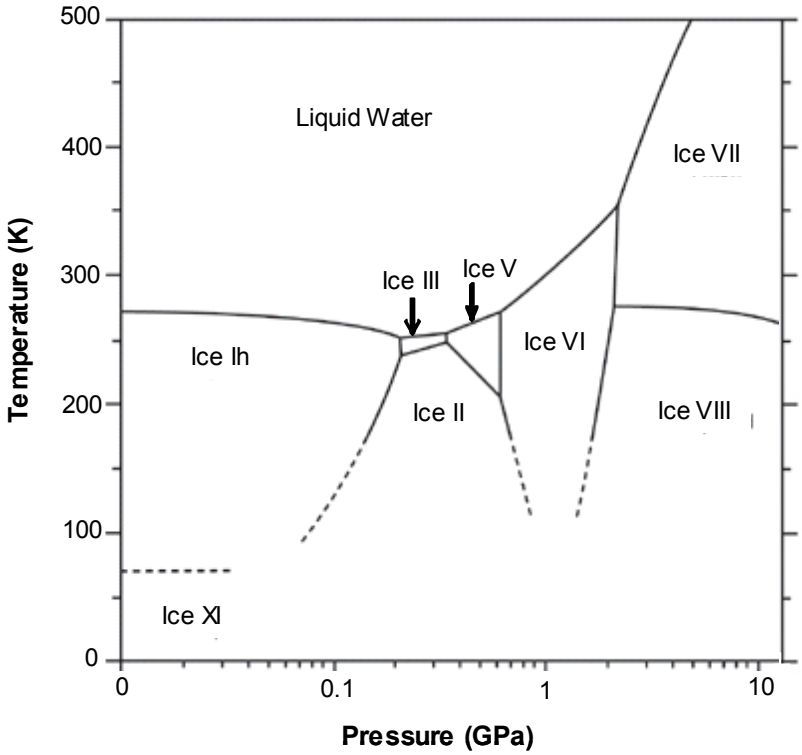


Figure 2.13: Phase diagram of water, illustrating the pressure and temperature conditions under which liquid water and different crystal structures of ice are stable. Ih: Ice formation: II-XI different crystal structures of ice.

The diagram shows that water is liquid in the range of 0.1 MPa up to 1 GPa at 20°C. Besides the liquid state and the ice modification I at normal pressure water exhibits a range of solid phases referred to as forms of "ice". Ice possesses 12 different crystal structures, plus two amorphous states. Ice I has a peculiarity since only this ice modification shows a positive volume change ΔV at the transition from the liquid to the solid state. Besides the depression in the freezing point, a reduction in the enthalpy of crystallization can also be observed ranging from 334 kJ kg^{-1} (at atmospheric pressure) to 193 kJ kg^{-1} (at 210 MPa) (Deuchi & Hayashi, 1992; Cheftel et al., 2000).

2.5.2.2 Lipids

High hydrostatic pressure affects the fusion temperature of lipids especially of triglycerides in approx. 10°C per 100 MPa. Hence, lipids may crystallize under pressure. This will explain the destruction of microorganism cell membranes and cell death through an increase in the cell permeability (Hayert et al., 1997; Cheftel et al., 1995).

High hydrostatic pressure accelerates the oxidation of polyunsaturated fatty acids- e.g. linoleic acid, which might lead to undesirable rancid flavours in foods (Angsupanich &

Ledward, 1998; Butz et al., 1997). Only a combined heat/pressure treatment may stop lipid oxidations without altering food flavour.

2.5.2.3 Polysaccharides

Several works have pointed out that monosaccharides are not affected by high hydrostatic pressure (Cheftel, 1992). However, high pressure reduces the rate of browning reactions (Maillard reaction). This reaction consists of two steps; condensation reaction of amino compounds with carbonyl compounds, and successive formation of melanoidins and other brown polymers. Tamaoka et al. (1991) demonstrated that the development of brown colour is greatly retarded when pressures of 200-400 MPa are applied at 50°C.

2.5.2.4 Proteins

High pressure effects on proteins are related to the rupture and formation of non-covalent interactions within protein molecules and intra- and intermolecular bonds within or between molecules. High hydrostatic pressure may denature proteins depending on the protein type, processing conditions, and applied pressure. High hydrostatic pressure can induce reversible changes in the range of 100-300 MPa and irreversible in pressures over 300 MPa (Thakur & Nelson, 1998). Denaturation is mainly due to the destruction of hydrophobic and ion pair bonds leading to unfolded molecules. At higher pressure, oligomeric proteins tend to dissociate into their subunits becoming vulnerable to proteolysis. Denaturation is a complex process which involves intermediate forms leading to multiple denatured products. Therefore, other factors such as temperature and media composition influence protein denaturation under pressure (Rastogi et al., 2007).

Pressure induces conformational changes distinct from those induced by temperature in protein gels (Heremans, 1999). Studies of Denda and Hayashi (1992) and Yoshika et al. (1992) pointed out that protein gels obtained under pressure were less firm, more elastic, bright and smooth than those obtained by temperature. A better colour and aroma retention in pressurised gels was also described (Cheftel, 1992).

2.5.2.5 Enzymes

Enzymes are a special class of proteins with obvious biological activity arising from their active sites. Changes in active site or protein denaturation can lead to loss of activity, or changes in enzyme functionality. Thus, high hydrostatic pressure can influence enzymatic

reactions enhancing or inhibiting their activity. On the one hand, high hydrostatic pressure can damage cell membranes facilitating enzyme substrate contact (Butz et al., 1994; Gomes & Ledward, 1996). On the other hand, high hydrostatic pressure can affect enzymatic functions partially or totally, reversible or irreversible depending on the enzyme retarding their activity (Morild, 1981; Hara et al., 1990). Other factors such as temperature, media pH or presence of sugars, salts, or other additives also influence enzymatic activity under pressure (Balny & Masson, 1993; Fernández-Garcia et al., 2002).

2.5.2.6 Vitamins

The effect of high hydrostatic pressure on vitamins has been extensively studied (Bognar et al., 1993; Kübel et al., 1997; Taukis et al., 1998). Sancho et al. (1999) pointed out that different high hydrostatic pressure treatments did not affect the stability of vitamin B1 and B6 in model solutions at pH 6.7 subjected at pressures of 200 MPa, 400 MPa and 600 MPa for 30 min. More recently works of Butz et al. (2007) pointed out the stability of vitamin B1 and B2 in meat after different temperature/high pressure treatments. By contrast vitamin C is reported to be pressure labile, since a loss of vitamin C of 13% and 12% after a high pressure process at 200 MPa and 600 MPa for 30 min at room temperature in model solutions was found (Sancho et al., 1999). A loss of 15-26% was also found in pineapple juices processed at 600 MPa, 40°C for 40 min (Taukis, 1998). Lipoproteins such as vitamin A and vitamin E (Tauscher & Butz, 2000) as well as vitamin K and provitamin A (Fernández-García et al., 2001; Sanchez-Moreno, 2003) were also stable under pressure.

2.5.3 Effect of high hydrostatic pressure on cellular systems

2.5.3.1 Plant cells

High hydrostatic pressure enhances cell permeability in plant cells mainly through two mechanisms:

1. Mechanical permeability: Fruits and vegetables with high intercellular air content (e.g. strawberries and apples), are strongly compressed under high pressure, since air is more compressive than water. This compressibility leads to a mechanical destruction of the cell and its membranes.
2. Physical permeability: High hydrostatic pressure can induce phase transitional changes in membrane phospholipids which lead to a decrease in membrane fluidity influencing thereby functions of membrane proteins (Na^+, K^+, ATPase) (Mcdonald, 1992; Heremans & Wytack, 1980; Kato & Hayashi, 1999).

Studies of Butz et al. (1994) pointed out the rupture of intracellular vacuoles and plant cell walls above pressures of 100 MPa independent on treatment duration (Figure 2.14). This breakage enhanced the leakage of intracellular compounds and enzymes which generated multiple enzymatic reactions.

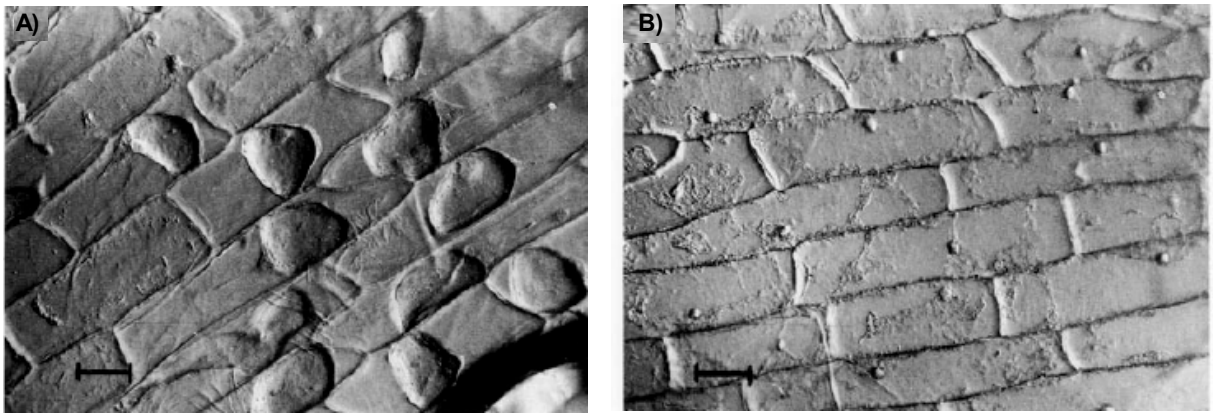


Figure 2.14: Epidermal cells of onions transferred into sucrose solution (100 g L^{-1}) after high pressure treatment at 25°C. A): 30 min, 100 MPa; cell sucrose plasmolysis; B): 30 min, 300 MPa; lack of plasmolysis in treated cells, i.e plant cell walls were previously disrupted by pressure.

Furthermore, studies of Ohmori, 1993 pointed out that pressures from 10-100 MPa caused the destruction of cell membranes and different cell organelles. High hydrostatic pressure enhances the passive ion transport and inhibits the cell membrane active transport. It also causes deprotonation of charged groups responsible of disruption of salt bridges and hydrophobic bonds which lead to conformational changes and denaturation of membrane bound transport proteins making the cellular membranes less and less selective (Barbosa-Canovas et al., 1998).

High hydrostatic pressure (HHP) belongs thus to one of the methodologies able to enhance mass transport phenomena (Rastogi et al., 2003). Higher caffeine extraction yields from coffee and a higher carotenoid release from tomato puree have been demonstrated when extractions were assisted by high hydrostatic pressure (Knorr, 1999; Sanchez-Moreno et al., 2004). More recently, studies of Shouquin et al. (2005) have also reported the aptitude of HHP for the extraction of flavanols from propolis.

2.5.3.2 Microorganisms

Microorganisms as well as tissues of plant and animal origin are cellular systems. High hydrostatic pressure has the advantage of inactivating spoilage catalysing enzymes and microorganisms retaining structure and functionality of tissues when the adequate process

parameters (p, T, t) are selected. The combination of HHP treatments with other parameters such as pH, water activity and temperature can definitively accelerate microbial inactivation:

1. Microorganisms become more susceptible to pressure at lower pH (Linton et al., 1999).
2. A reduction of water activity exerts a protective effect for microorganisms against pressure treatments (Palou et al., 2000).
3. The treatment temperature above or below room temperature tend to increase the inactivation rate of microorganisms (Knorr & Heinz, 1999).

Yeast and moulds are very sensitive to pressure being inactivated by pressures between 200 and 300 MPa (Cheftel, 1995). On the contrary, mould spores are very resistant even under pressures above 1000 MPa. Also, they may germinate under pressures of 50-300 MPa (Sale et al., 1970; Wuytack et al., 1997) which lowers their pressure resistance.

Vegetative forms in growth phase are more susceptible to pressure than vegetative forms in latent form (Mackey et al., 1995). Cells in latent form are small and spherical which reduces the impact of high hydrostatic pressure (Chilton & Isaacs, 1997). Gram-positive bacteria are more resistant to heat and pressure than Gram-negative bacteria (Earnshaw, 1995). Gram-positive bacteria are generally inactivated by pressures of 500-600 MPa at 25°C for 10 min, while Gram-negative required pressures around 300-400 MPa, 20°C for 10 min (Trujillo, 1997). However, Ludwig and Schreck (1997) did not find any relationship between bacteria type and pressure resistance but with the cell morphology; *Bacillus* spp. were more pressure susceptible than *cocci.*

Nonetheless, food matrices are a determinant factor for the level of microorganism inactivation, since protective matrix effects were observed with various species of microorganisms (Styles et al., 1991; Patterson & Kilpatrick, 1998). In addition, the effects of food constituents on pressure resistance are complicated and depend on their stability and function under pressure (Smelt et al., 2002).

3 AIMS

Increasing knowledge about the health promoting impact of antioxidants in everyday foods, combined with the assumption that a number of common synthetic preservatives may have hazardous effects, led to considerate grape pomace as an economical source of demanded compounds. A higher effort to utilise these agricultural wastes must be undertaken since three main limiting factors denote the efficient management of these by-products: the effectiveness of recovery and extraction, the marketability of resulting extracts and the practical suitability for food, cosmetic or pharmaceutical products.

This work exposes and investigates different approaches to overcome previously mentioned limiting factors. In a first part, grape pomace extracts (white and red skins and seeds) from organically and conventionally managed land were characterised and compared to define their ability as a source of bioactive compounds. Their antioxidant and antimicrobial properties were also investigated. In addition, pesticide loads of white grape pomace extracts were determined and their content was compared to the limits stipulated by different regulatory organisms. Pesticide residues in already commercialised grape pomace extracts were analysed and their benefits and drawbacks were discussed.

A second part deals with an efficient application of grape seed extracts (GSE) as potential additives for pea-starch based food packaging films. GSE films and their physico-chemical and antimicrobial properties were estimated. The antimicrobial activity of GSE films was tested *in vitro* in *B. thermosphacta* inoculated meat. Their ability to inhibit bacterial growth, preserve meat quality and extend shelf life was further discussed.

Following these investigations, the extraction of anthocyanins from red grape pomace (predominant compounds) was optimised by an innovative extraction procedure assisted by high hydrostatic pressure. Its feasibility for extraction purposes was compared to extractions carried out by other emerging technologies such as pulsed electric fields and ultrasonics. Moreover, the stability of anthocyanins in model solutions as well as in food matrices (grape pomace extracts and wine) was further studied. The optimal parameters for a safe pasteurisation process of anthocyanin-enriched matrices were determined and the conditions for degradation and synthesis of commercially attractive anthocyanin-derived compounds further investigated. In addition, a time temperature pressure indicator with compounds of natural occurrence was developed for the traceability and safety of pressure sterilisation/pasteurisation processes.

4 MATERIAL AND METHODS

4.1 Material

All reagents and chemicals of analytical grade were purchased by Sigma-Aldrich (Taufkirchen, Germany). The grape cultivars of Riesling and Spätburgunder (*V. vinifera* L.) were grown according to standards for both conventional and organic farming in Germany for the year 2005 and were kindly supplied by Bioland Ökologischer Landbau Weinbau Hoffmann. EG-Kontroll-Nr.: D-RP-006-11985-AB. Bioland-Betriebs-Nr.: 40187 from the Palatinate zone, Germany. Grape pomace was separated into different fractions: skins, stems and seeds. Skins were lyophilised, and milled with a coffee grinder before extraction. Skins were mainly utilised in the experiments and are referred along the work as grape pomace extracts (GPE), to differentiate from grape seed extracts (GSE) (Figure 4.15). Dörnfelder (*V. vinifera* L.) grape pomace was supplied by wine producers from the Palatinate zone, Germany. Red wine Dornfelder, 2004 was provided by Niederkirchener Weinmacher e.G. (Niederkirchen, Germany).

Figure 4.15: Grape pomace red Spätburgunder (left); white Riesling (middle); and Riesling seeds (right) (*V. vinifera* L.)

More than 500 standards for pesticide analysis were provided by Fa. Dr. Ehrenstorfer GmbH, (Augsburg, Austria) and Sigma-Aldrich (Taufkirchen, Germany). HPLC solvents of analytical grade were purchased from Merck (Darmstadt, Germany).

Pea Starch (*Pisum sativum L. Miranda*) 35-45% amylase, was supplied by Nutri-Pea Ltd. (Portage-La-Prairie, Manitoba, Canada). Glycerol was from Sigma Chemicals Co. (St. Louis, Missouri, USA).

Standards used for identification and quantification purposes with HPLC-DAD/ESI-MS were as follows: gallic acid, ferulic acid, 4-hydroxybenzoic acid, sinapic acid, p-coumaric acid, syringic acid (Sigma-Aldrich, Taufkirchen, Germany); protocatechuic acid (Roth, Karlsruhe, Germany); catechin, epicatechin, epicatechingallate, procyanidin B, cyanidin-3-O-glucoside (Cy3gl) and malvidin-3-O-glucoside (Mv3gl) (Extrasynthese, Lyon, France).

Sorbic acid potassium salt (2,4-hexadienoic acid potassium salt), coenzyme Q_0 (2,3 dimethoxy-5-methyl-1,4-parabenzoquinone) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

4.2 General extraction procedure

Bioactive extracts were obtained from 5 g of the milled conventionally and organically grown grape pomace and seeds in Erlenmeyer flasks with 100 mL 60% methanol in water (v/v). The extraction was assisted by an ultrasound generator (Bandelin, Sonorex RK 100H, Walldorf, Germany) for 9 min. After each extraction, samples were centrifuged at 9000 rpm for 10 min, and supernatants were collected and lyophilised. Extract concentrations of 20%, 10%, 5% and 1% (w/v) in distilled water were membrane-filtered (0.45 µm) before analysis.

4.3 Optimization of anthocyanin extraction

4.3.1 Control extraction

All extractions were conducted by a solid/liquid ratio of 1:4.5 where the solvent was a mixture of ethanol and water (50/50, v/v). After each of the treatments the extracts were filtered and the supernatants were collected for analytical analysis. Control extraction was carried out in a water bath incubated at a temperature of 70°C held during 1 h.

4.3.2 Extraction assisted by ultrasonics

Ultrasonic waves (energy generated by sound waves of 20-10^7 kHz) generate gas bubbles in liquid media that produce a high temperature and pressure increase when they immediately burst (Vollmer et al., 1998). Membrane disruption effects of ultrasonics are attributed to intracellular cavitation and micro-mechanical shocks that disrupt cellular structural and functional components up to the point of cell lysis. Depending on wave intensity, exposure time, membrane characteristics and medium type ultrasonics can induce mechanical, thermal and biochemical effects with a large range of applications in the food industry (Figure

4.16) (Zenker, 1998). In this work, ultrasonics was mainly used for extraction purposes using a ultrasonics bath Sonorex Bandelin RK 100H with a frequency of 35 KHz (Schalltec, Mörfelden-Walldorf, Germany), at 70°C held during 1 h.

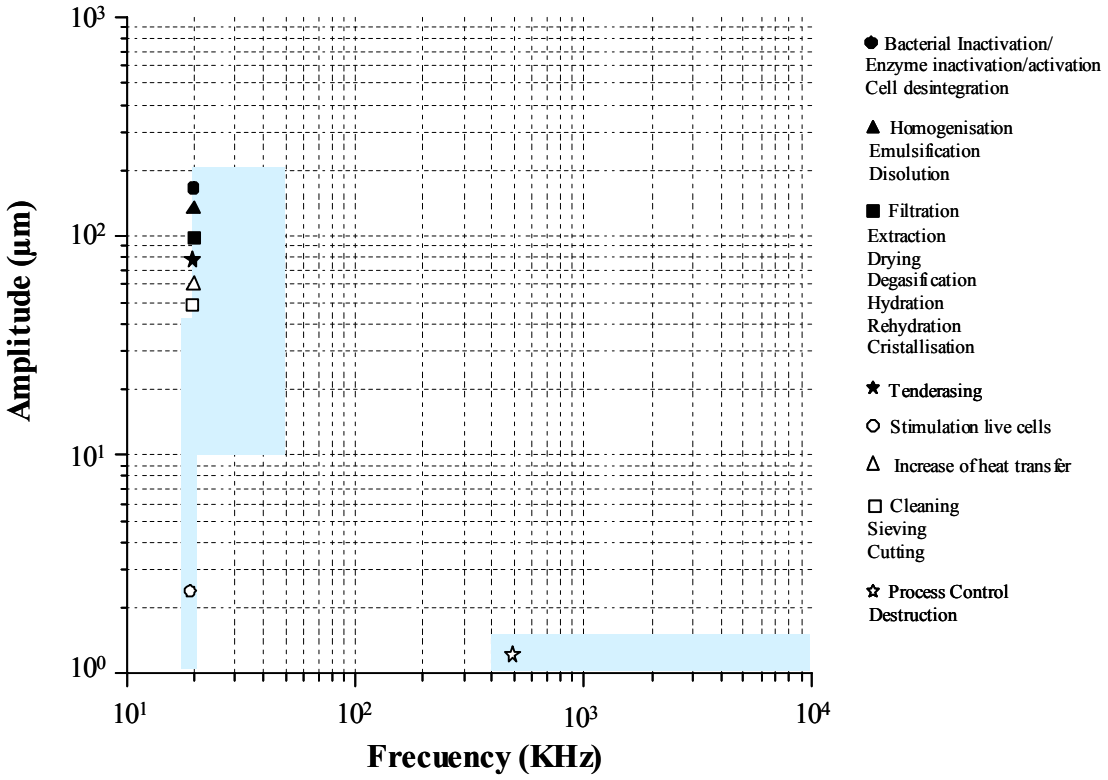


Figure 4.16: Applications of ultrasonics in food science and technology in function of the frequency and amplitude applied.

4.3.3 Extraction assisted by pulsed electric fields

Pulsed electric fields offer a great variety of applications in food as well as biotechnology or medicin based on its impact on biological cell membranes. Dependent on treatment intensity electropermeabilisation of membranes leads to a reversible or irreversible pore formation and cell disintegration. The possible applications of pulsed electric fields and intensities required are represented in Figure 4.17. This work is focused on the improvement of mass transfer from red grape pomace i.e. an irreversible cell electropermeabilisation (0.7-3.0 kV cm^{-1} 1.0-20 kJ kg^{-1}). Electrical membrane breakdown was first studied based on model systems as phospholipid vesicles, planar bilayers as well as microorganisms. Accurate permeability mechanisms have not been yet reported (Zimmermann et al., 1974; Chernomordik et al., 1987; Chang et al., 1992; Ho & Mittal, 1996; Barsotti et al., 1999).

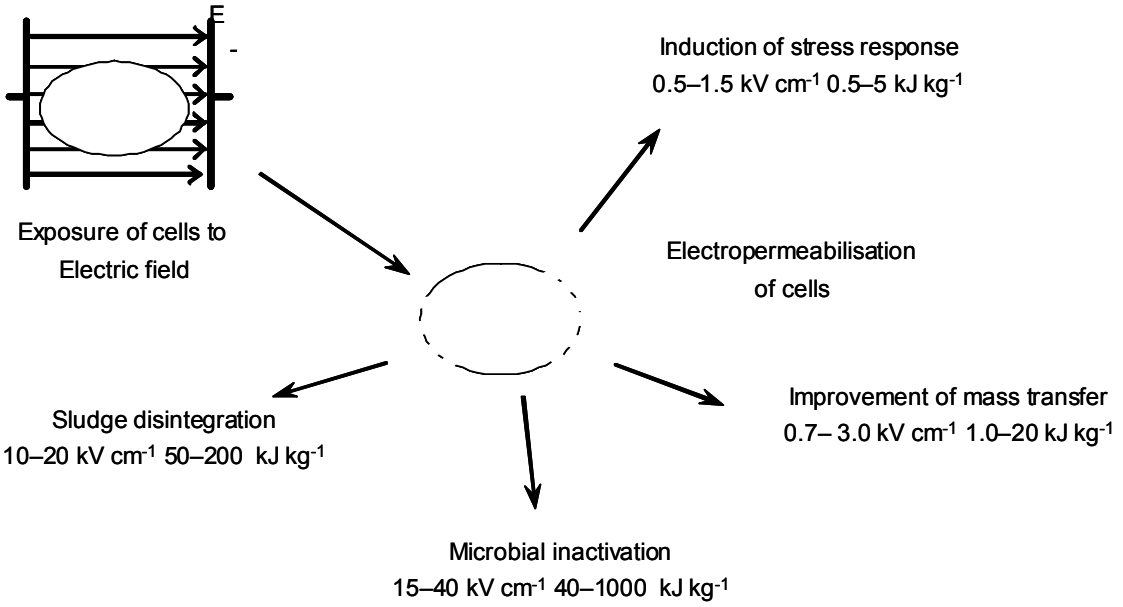


Figure 4.17: Electric field strength and energy input requirements for food, bio and waste water processing.

Pulsed electric fields (PEF) processing involves the application of pulses of high voltage to foods placed between two electrodes. PEF may be applied in form of exponential decay, square wave, bipolar or oscillatory pulses and at ambient or different temperatures for less than 1 s. By the application of an external electrical field the naturally transmembrane potential 10 mV is affected and the new electrical field can be determined by Laplace equation $\nabla^2\varphi = 0$, where φ represents the electrical potential.

The potential difference $\Delta\varphi_M$ in the membrane of a biological cell with spherical shape and a radius R induced by an external electrical field E is defined by Eq. 4.7 which is derived and simplified from Maxwell´s equation (Zimmermann et al., 1974).

$$\Delta\varphi_M = -f(A)\cdot A_F E \qquad \text{(Eq. 4.7)}$$

Where A_F is the distance from the centre in direction of the external electrical field and $f(A)$ is the shape factor in function of the three semi-axis (A_1, A_2, A_3) of elliptical cells (Eq. 4.8).

$$f(A) = \frac{2}{2 - A_1 A_2 A_3 \int_0^{\infty} 1/\left(\left(s + A_F^2\right)\left(\sum_{n=1}^{3}\sqrt{s + A_n^2}\right)\right) ds} \qquad \text{(Eq. 4.8)}$$

When the overall potential exceeds a critical value of 1 V, polarisation and membrane breakdown is induced. Process factors such as electric field intensity, pulse width, treatment time and pulse wave shapes, material type and media (pH, ionic compounds, conductivity and medium ionic strength) play an important role in membrane breakdown (Crowley, 1973; Zimmermann, 1996). Electroporation occurs basically in lipid domains and protein channels since its activity depends on the transmembrane potential.

Following this principle, a pulsed electric field treatment using a PurePulse (PurePulse Technologies, San Diego, USA) exponential decay pulse generator with a maximum voltage of 10 kV and a maximum average power of 8 kW was applied (Figure 4.18). The peak pulse voltage used was 9 kV, resulting in an electric field strength of 3 kV cm^{-1}. A serie of 30 pulses was applied at ambient temperature to obtain a specific energy input of 10 kJ kg^{-1}. The temperature increase after the treatment was less than 3°C. A parallel plate treatment chamber consisting of stainless steel electrodes with an electrode area of 140 cm^2 and a gap of 3 cm was used. The pulse repetition rate was 2 Hz, the total treatment time was 15 s, for filling and unfilling of the sample the time required was 1 min. The subsequent extraction was performed at 70°C and held during 1 h in a shaken Erlenmayer flask.

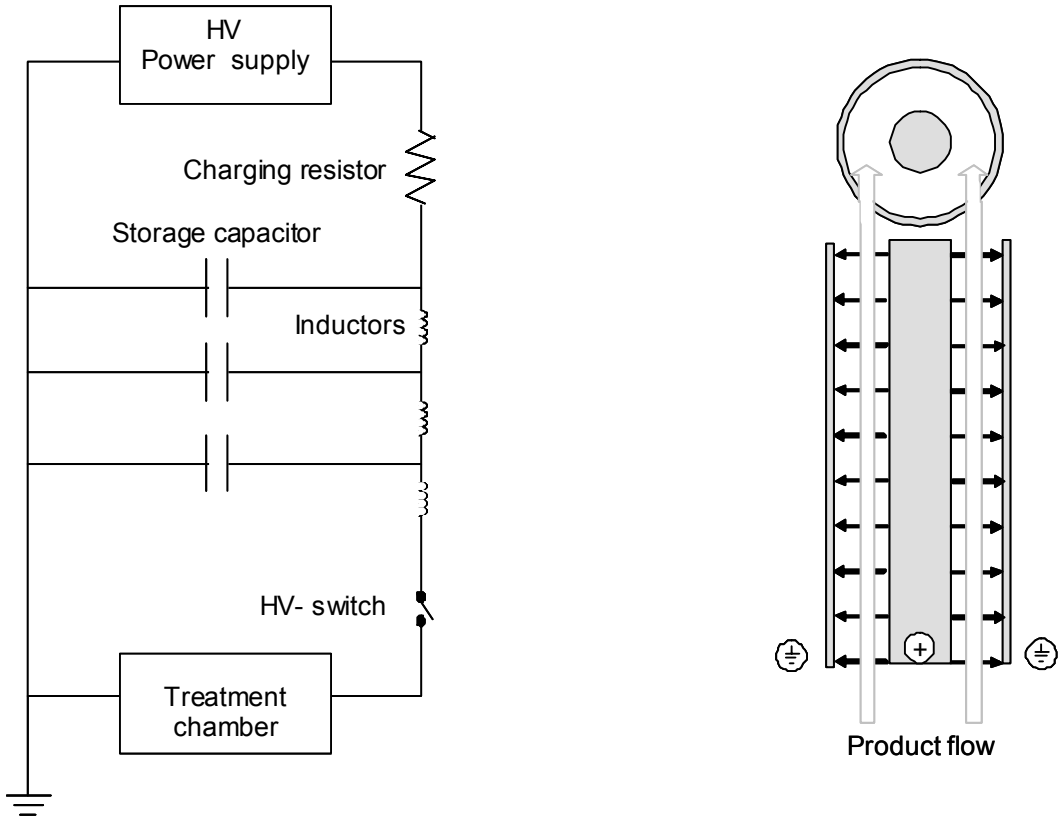


Figure 4.18: Simplified electrical circuits of impulse generation systems for exponential decay (left) and configuration of treatment chambers for continuous PEF-treatment in a parallel plate (right) (Töpfl, S., 2006).

4.3.4 Extraction assisted by high hydrostatic pressure

Experiments were conducted in a high hydrostatic pressure device consisting of a serie of thermostated microautoclaves (i.d. 16 mm, ca. 25 mL) connected by valves (aad GmBH, Frankfurt, Germany) (Figure 4.19). Pressure was generated by a compressed air driven pressure intensifier. The pressure-transmitting medium was water and glycol (80:20, v/v). Due to adiabatic heating there was an initial temperature rise of not more than 4°C on pressure build-up: the initial temperature was usually restored after 1-2 min. The temperature in the vessels was controlled by a thermostat Polystat from Huber (Offenburg, Germany). Samples were pressurised at 600 MPa, 70°C and held during 1 h in teflon tubes (inner/outer diameter 6-8 mm; 2-5 mL) with silicon stoppers.

Experiments at 800 MPa were carried out in a hydraulic press U101 Unipress (Polish Academy of Sciences, Warsaw, Poland) which was manually operated by a twin hydraulic piston. The temperature in the vessels was controlled by a thermostat Polystat from Huber (Offenburg, Germany). Samples were pressurised in heat sealed polyethylene ampoules (250 µL).

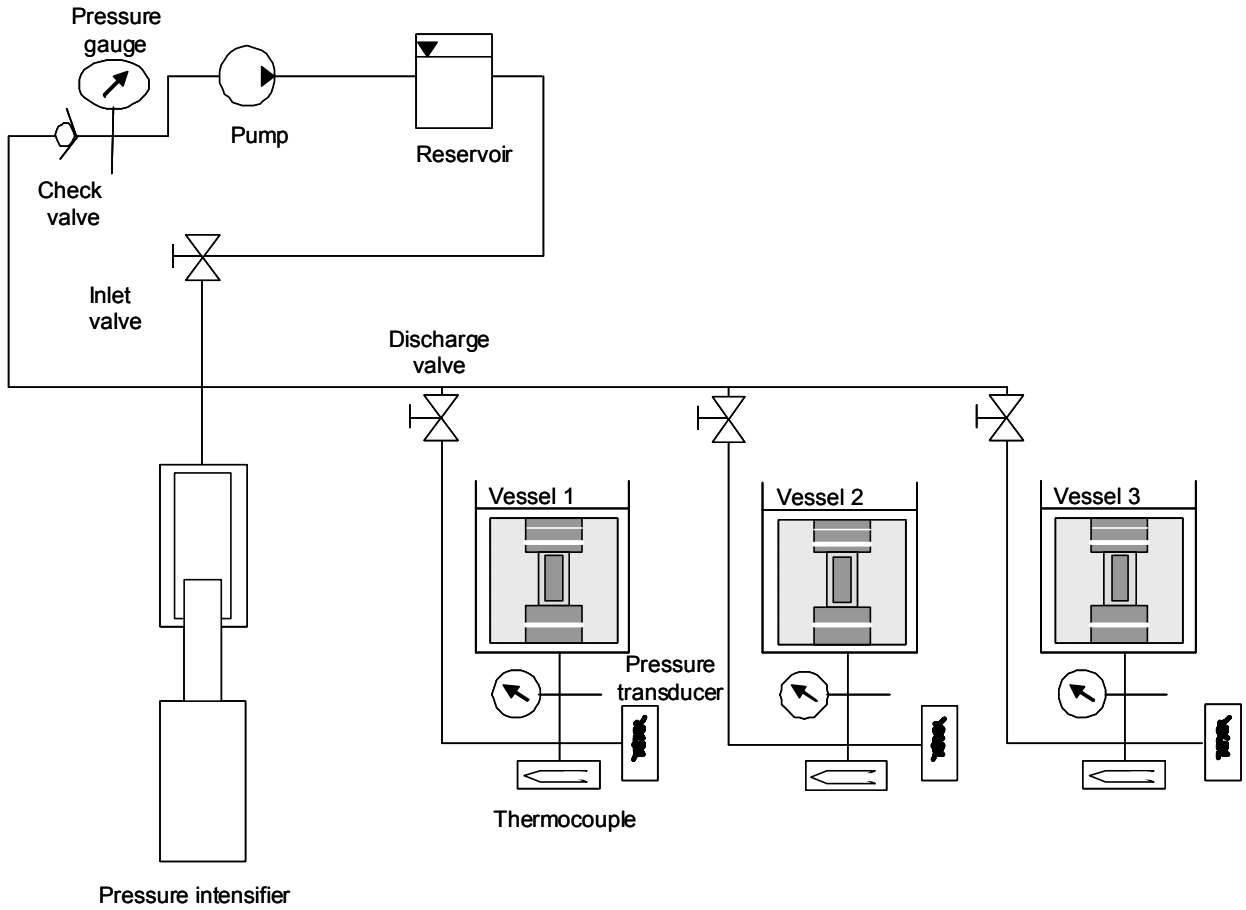


Figure 4.19: Schematic diagram of the high hydrostatic pressure device.

4.3.5 Conventional extraction

A total extraction from grape pomace was carried out in a water bath incubated at a temperature of 70°C held during 3 h. In this case, the solid / liquid ratio was increased to 1:20.

4.4 Analytical methods

4.4.1 Antioxidant capacity

The $ABTS^{+}$ method here used was first reported by Miller et al. (1993) and later improved by Re et al. (1999). In this improved version, a stock solution of 5 mM $ABTS^{+}$ (2,2'-azinobis [3-ethylbenzothiazoline-6-sulphonic acid]) is diluted in water and preincubated for at least 12 h with 140 mM $K_2S_2O_8$ to produce the radical cation $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ solution was then diluted in 5 mM saline phosphate buffer pH 7.4 (0.695 g Na_2HPO_4 x 2 H_2O + 0.159 g NaH_2PO_4 x 2 H_2O + 4.5 g NaCl per L) until absorbance readings reached a value of 1.5 at 735 nm. An aliquot extract of 100 µL was diluted 200-fold in buffer mixed with 2.9 mL $ABTS^{\bullet+}$ and set 15 min at 30°C then absorbance was measured at 735 nm. A calibration curve using TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard was used to calculate the antioxidant activity of the samples, expressed in µmol TROLOX equivalents (TE) g^{-1} dry matter (DM).

4.4.2 Total phenolic content

This method was initially intended for the analysis of proteins taking advantage of the reagent´s activity toward protein tyrosine (containing a phenol group) residue (Folin & Ciocalteau, 1927). Many years later Singleton et al. (1965); (1999) extended this assay to the analysis of total phenols in wine and since then the assay has found many applications.

Folin ciocalteau reagent is obtained by first boiling (for 10 h) the mixture of sodium tungstate (Na_2WO_2 x 2 H_20, 100 g), sodium molybdate (Na_2MoO_4 x 2 H_2O, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL), and water (700 mL). After boiling, lithium sulphate (Li_2SO_4 x 4 H_2O, 150 g) is added to the mixture to give an intense yellow solution. For the total phenolic content determination, an aliquout of 125 µL of the suitable diluted sample was mixed with 625 µL of Folin-Ciocalteau reagent (FCR) (previously diluted 10-fold with distilled water; and incubated at 45°C) and set 3 min at room temperature. An aliquot of 500 µL of sodium carbonate (0.6 M; 105.99 g mol^{-1}; and previously incubated at

45°C) were added to the mixture and incubated at 45°C during 15 min after which the absorbance was measured at 750 nm.

Gallic acid hydrate (Roth, Karlsruhe, Germany) was used as standard for the calibration curve and results were expressed in µmol of gallic acid equ. (GAE) g^{-1} dry matter (DM).

The chemical nature of the Folin-Ciocalteau reagent is not known, but it is believed to contain molybdates which reduced and lead into blue species:

$$Mo(VI) + C \rightarrow Mo(V)$$

Obviously, the FCR is non-specific to phenolic compounds as it can be reduced by many nonphenolic compounds (e.g. vitamin C). Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH 10). Dissociation of phenolic protons leads to a phenolate anion, which is capable of reducing FCR. This supports the notion that the reaction occurs through electron-transfer mechanism. Despite the undefined chemical nature of FCR, the total phenols assay is convenient, simple and reproducible.

4.4.3 Anthocyanin determination by LC-DAD/MS-MS

An aliquot of 1 mL of the extracts was evaporated to dryness in a centrifugal vacuum dryer (Speed Vac SC110 Savant, Germany) coupled to a refrigerated condensor trap (RT 100 Savant, Germany), and the residue was dissolved in 500 µL of acidified water (pH 3) and membrane filtered (0.45 µm) before injection. Anthocyanins were analysed by direct injection of the solutions. The separation was performed with a C_{18} column (Aqua®, Phenomenex, Inc.; 250 x 4.6 mm i.d.; 5 µm), operated at 20°C. Mobile phases contained water/formic acid/acetonitrile where A: 87:10:3 and B: 40:10:50; (v/v/v). The gradient program was: from 10 to 15%B (10 min), 15%B isocratic (3 min), from 15 to 25%B (7 min), from 25 to 55%B (30 min), from 55 to 100%B (1 min), 100%B isocratic (5 min), from 100 to 10%B (0.1 min). The total separation time was 50 min. The injection volume was 20 µL and the flow rate 1.0 mL min^{-1}. The detection was monitored at 320, 370 and 520 nm (Kammerer et al., 2004).

The identity of the individual compounds was assigned based on mass spectroscopy (MS). The mass spectrometer was fitted with an ESI source in positive mode. The column eluate was recorded in the range m/z 50-1000. Nitrogen was used both as drying gas at flow rates of 11.0–12.0 mL min^{-1}, and as nebulising gas at a pressure of 65 psi. The nebuliser temperature was 350°C.

4.4.4 Polyphenol determination by LC-DAD/MS-MS

An aliquot of 5 mL of the original 60% methanol extraction was re-extracted with 100 mL ethylacetate pH 1.5. The extracts were concentrated in a rotary evaporator and dissolved in 10 mL acidified water (pH 3). The extracts were applied to Chromabond C_{18} cartridges (Varian, Frankfurt am Main, Germany) activated with methanol and water. Flavonoids in the cartridge were washed with 10 mL methanol and phenolic acids with 10 mL 0.1% HCl. The supernatants resulting from flavonoids and phenolic acid fraction were evaporated to dryness in a centrifugal vacuum dryer (Speed Vac SC110 Savant, Minesota, USA). For HPLC-analysis the extracts from flavonoids were dissolved in 2 mL methanol, whereas extracts from phenolic acids were in 1 mL 2% acetic acid. Solutions were membrane-filtered (0.45 µm) and were analysed using an Agilent Technologies HPLC/MSD Series 1100 (binary solvent delivery, autosampler, UV-Vis Diode Array Detector (DAD), electrospray ionization (ESI); (Agilent Technologies, Palo Alto, CA). The separation was performed with a C_{18} column (Aqua®, Phenomenex, Inc.; 250 x 4.6 mm i.d.; 5 µm), operated at 20°C. Flavonoids characterisation was followed using the method of Kammerer et al. (2004). Solution A was: 0.5% acetic acid in water (v/v) and B: 0.5% acetic acid, 50% water and 50% acetonitrile in water (v/v/v). Gradient program was: 10-24%B (20 min), 24-30%B (20 min), 30-55%B (20 min), 60-75%B (15 min). Total run was 75 min. Phenolic extracts were analysed following the gradient: from 10-15%B (10 min), 15%B (3 min), 15-25%B (10 min), 25-55%B (30 min) and 55-100%B (6 min). Total run was 59 min. The injection volume was 20 µL and the flow rate 1.0 mL min^{-1}. The detection was monitored at 280, 320, 370 and 520 nm.

The mass spectrometer was fitted with an ESI source in negative mode. The column eluate was recorded in the range *m/z* 50-1000. The mass spectrometer was programmed to scan a MS^2 of the most abundant ion in the full mass. Nitrogen was used both as drying gas at flow rate of 11.0 L min^{-1}, and as nebulising gas at a pressure of 60 psi. The nebuliser temperature was 350°C.

4.4.5 Total anthocyanin content

Individual compounds were quantified using the calibration curve of cyanidin-3-O-glucoside (Cy3gl). Calibration of structurally related substances was determined including a molecular weight correction factor (Chandra et al., 2001). All determinations were performed in triplicate and expressed in $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$.

4.4.6 Pesticide determination by LC/MS-MS and GC/MS-MS

Pesticide residues were measured by the the QuEChERS pesticide multiresidue method, as described by Anastassiades et al. (2003) and EU Standarisation methods (2007) in combination with LC-MS/MS and GC-MS/MS which allows a very sensitive and selective analysis of the pesticide residues.

Recently, the method has been widely accepted by pesticide residue analysts. Many laboratories around the world employ this method in its original form or variations of it. The QuEChERS procedure involves an initial extraction following the addition of acetonitrile to the sample. After addition of a salt-mixture (in this case containing magnesium sulfate, sodium chloride and citrate salts for buffering) to induce phase separation a second extraction/partitioning step is performed followed by centrifugation. Clean-up is performed by dispersive solid phase extraction (D-SPE) which involves the mixing of an extract aliquot with a sorbent and magnesium sulfate as drying agent (in this case a silica-based amino-sorbent). The final extract is contained in acetonitrile and is thus directly amenable to both liquid chromatographic (LC) and gas chromatographic (GC) analysis. The QuEChERS method covers a widely analysis, including highly polar as well as highly acidic and basic pesticides. Additional advantages of the method are its speed, reproducibility and the low solvent requirements.

4.5 Antibacterial assay

Listeria (*L.*) *monocytogenes* Scott A, *L. monocytogenes* ATCC 19115, *Salmonella enterica serovar typhimurium* (*S. typhimurium*) ATCC 14028, *Staphylococcus (S.) aureus* ATCC 25923 and *Escherichia* (*E.*) *coli* ATCC 25922 were cultured in Standard I (Merck) broth at 37°C. *Enterococcus* (*E.*) *faecium* DSM 13590 and *E. faecalis* DSM 20409 were cultured in the Man, Rogosa and Sharpe broth (MRS broth, Merck, Darmstadt, Germany) at 37°C. *Brochothrix (B.) thermosphacta* was culture in Brain Heart Infusion (BHI) broth (Difco, Becton Dickinson and Company, Sparks, Maryland, USA). The agar spot test as described by Uhlmann, et al. (1992) was used for screening the antagonistic activity of the extracts. Standard I or MRS agar (12 g L^{-1}) plates were overlayered with Standard I or MRS soft agar (7.5 g L^{-1}) previously inoculated with ca. 1 x 10^6 colony forming unit (CFU) mL^{-1} of overnight cultures of the corresponding indicator bacterial strain. Wells were done on the soft agar with the back of a sterile Pasteur pipette and 20 µL of each extract and concentration (5%, 10%, 20% w/v) were inoculated in each well. After diffusion, plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the diameter inhibition zones with no bacterial growth in mm.

4.6 Antifungal assay

Penicillium (P.) chrysogenum DSM 844, *P. expansum* DSM 62841, *Aspergillus (A.) niger,* DSM 1988*, A. versicolor* DSM 63292 and *Trichoderma (T.) viridie* DSM 63065 were cultured and grown on malt extract agar plates (12 g L^{-1} malt extract). Two-week-old cultures were used to prepare spore suspensions.

The effect of grape pomace on the conidia germination was carried out according to the method described by Droby et al. (1997). The spore concentration was determined with a Neubauer counting chamber (Optik Labor, Hecht, Germany) and adjusted to 5 x 10^5 spores mL^{-1}. Aliquots of 90 µL of the spore suspension were mixed with 10 µL of GPE in different concentrations: 5%, 10%, 20% (w/v) in a diagnostica multimed microscope slide (Optik Labor, Hecht, Germany) and the conidia germination was determined microscopically after 24 h incubation at 30°C (Figure 4.20)

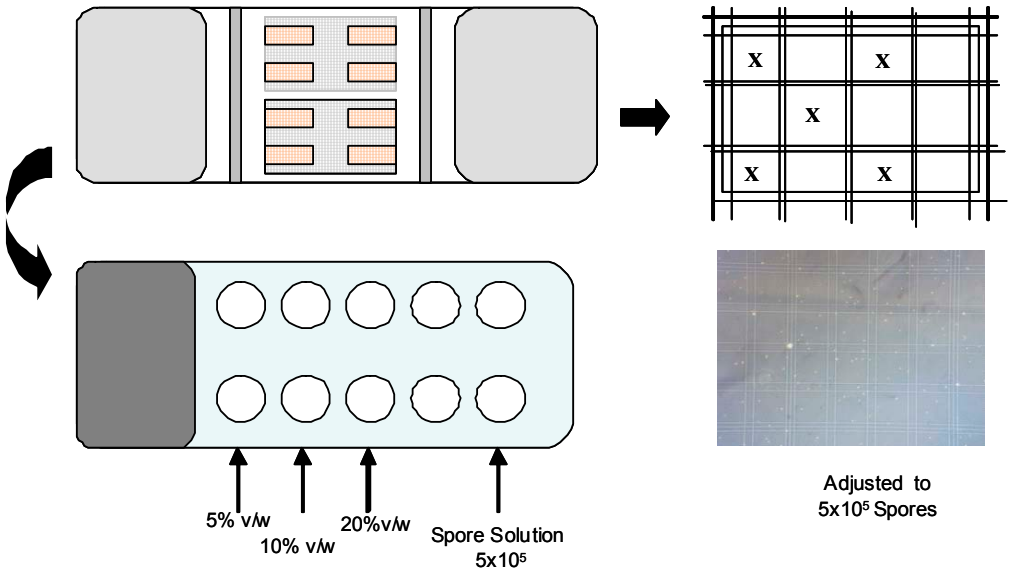


Figure 4.20: Schematic representation of antifungal assay.

4.7 Anthocyanin model solutions under heat/pressure

4.7.1 Anthocyanin ethanolic solutions

The model solutions were prepared with 1.3 mM Cy3gl in 50% ethanol solution. Anthocyanins from Dornfelder (*V. vinifiera L.*) red grape pomace were extracted weighing 10 g of the pomace in 100 mL 50% ethanol solution assisted by ultrasonics (Bandelin, Sonorex RK 100H, Walldorf, Germany).

4.7.2 Anthocyanin condensation reactions

Pure Cy3gl (3.2 mg) and 65.6 mg of sodium pyruvate (~ 100 equ.) were mixed in 10 mL acetate buffer (0.2 M acetic acid, and 0.2 M potassium acetate). Samples subjected to HHP pressure were adjusted to pH 4.4 and heated samples were adjusted to pH 3.8, since the adiabatic pressure increase causes a decrease in the pH of the buffered solutions due to H^+ loss. This decrease in the pH was in a rate of 0.1 per 100 MPa for acetate buffer (Neuman et al., 1973).

4.7.3 Quantification of individual compounds

The loss of Cy3gl and sodium pyruvate was quantified using a calibration curve of the corresponding standard compound. Products of the reaction were estimated by individual compound peak collection and further analytical calibration with HPLC-DAD/ESI-MS under the conditions previously reported (Chapter 4.4.3). Quantification of predominant anthocyanins in wine structurally related to Cy3gl was determined including a molecular weight correction factor (Chandra et al., 2001).

4.7.4 Determination of total monomeric anthocyanins

Monomeric anthocyanins reversibly change colour according to a change in pH. The coloured oxonium form predominates at pH 1.0 whereas the colourless hemiketal form at pH 4.5. By contrast, polymeric or degraded anthocyanins absorb colour at this pH. The difference in the absorbance at 520 nm is proportional to the monomer pigment concentration. Measurements at 700 nm are to correct for haze (Lee et al., 2005). Following this method, an aliquot of the clear extract (1 mL) was placed into a 25 mL volumetric flask and made up to the final volume with pH 1.0 buffer (KCl, 0.025 M). The pH was adjusted with HCl (0.2 N). Another 1 mL of extract was also placed into a 25 mL volumetric flask, made up to a final volume with pH 4.5 buffer (CH_3CO_2Na, 0.4 M). The pH was adjusted with HCl (0.2 N). Absorbance was measured in a UV-1601 Shimadzu spectrophotometer (Shimadzu, Duisburg, Germany) at 510 and 700 nm. Results were calculated using the following Eq. 9 and expressed in $mg_{Cy3gl\ equ.}\ L^{-1}$.

$$\textit{Total anthocyanins (mg L}^{-1}) = \frac{A}{eL} \times MW \times D \times \frac{V}{G} \times 100 \qquad \text{(Eq. 9)}$$

Where:

A is $(A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5.}$

e is cyanidin-3-O-glucoside molar absorbance (26900).

L is cell path length (1cm).

MW is the molecular weight for cyanidin-3-O-glucoside (449.2).

D is a dilution factor

V is the final volume (mL).

G is the sample weight (mg).

4.8 Development of pressure- , temperature- and time- indicators

Two model solutions were prepared with 2 mM potassium sorbate and 1 mM of coenzyme Q_0. The stability under pressure of each individually substance was tested with different molar concentrations. Different concentrations of potassium sorbate and coenzyme Q_0 in model solutions were prepared: (1:1, 2:1, 10:1 mM:mM) and subjected to different heat/pressure treatments.

4.9 Biodegradable/edible films elaboration

Aqueous solutions of 3% (w/w) pea starch and 1.8% glycerol were mixed and heated at 90°C under agitation for 10 min to allow gelatinization. Afterwards the solution was cooled down to 40–50°C and 1% of grape seed extracts was added and homogenized with a ultraturrax during 2 min at 2000 rpm till obtaining a homogeneus mixture. Aliquots of 11 mL of solutions were cast on Petri dishes (φ = 10 cm) and dried at room temperature for 24 h. Thereafter, films were conditioned at 50% relative humidity (RH) for 72 h at 25°C in a sealed chamber containing supersaturated aqueous solution of $Ca(NO_3)_2$. RH was measured by a digital RH-meter (Control Co., Friendswood, Texas, USA) (Figure 4.21).

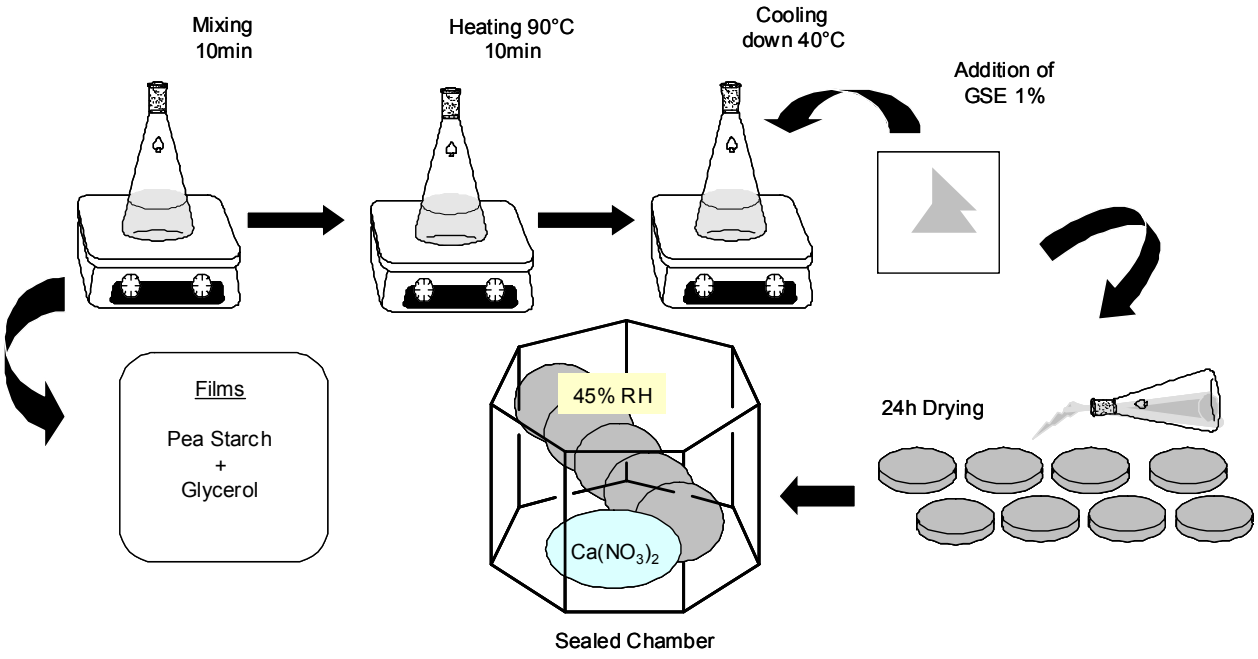


Figure 4.21: Schematic representation of pea starch film elaboration.

4.9.1 Mechanical properties

Thickness of the films previously conditioned at 50% RH was measured with an electronic digital micrometer 0.001 mm sensitivity (B.C. Ames Co., Waltham, USA). Tensile strength (TS) and elongation (E) was determined using a texture analyzing instrument (Texture Analyser, TA-XT2, Texture Technologies, Corp., Scarsdale, New York, USA). The initial grip distance and crosshead speed were 5 cm and 100 mm min^{-1}, respectively. Tensile strength (TS) was calculated by dividing the peak load by the cross sectional area of the film (thickness of film x 1 cm) of the initial film. Elongation (E) was determined by the ratio of difference to the initial length (5 cm).

4.9.2 Colour measurements

Colour of GSE enriched films was determined by a Minolta colorimeter CR-300 (Minolta Camera Co., Ltd., Chou-ku, Osaka, Japan). Colour was recorded using the Minolta Lab uniform colour space. The chromameter was first calibrated with a white tile and recalibrated between every measurement. Films were conditioned for 72 h at 50% RH prior to colour determination. Three film pieces were used for each determination.

4.9.3 Water vapour and oxygen permeability

Water vapour permeability (WVP) was determined by the method of Choi and Han (2001). Films previously conditioned at a RH of 50% were placed on an acrylic cup containing 10 mL distilled water, sealed and weighed. The assembly was placed in a chamber at ~10% RH and 25°C. Weight loss along time was measured periodically to obtain water vapour transmission rate (WVRT) of the films. WVP was calculated using Visual Basic tools programmed by Choi and Han (2001) based on the procedure previously reported by McHugh et al. (1993). Results were expressed in g mm m^{-2} h^{-1} kPa^{-1}.

Oxygen permeability (OP) of films was determined using an oxygen transmission rate test machine (OxTran 6/20, Mocon, Minneapolis, Minnesota, USA). Films previously conditioned at 50% RH were nitrogen flushed for 1 h, thereafter an oxygen flow (100% at 1 atm) was introduced in one side of the films and the oxygen transmission rate (OTR) was measured from the opposite chamber. OP was calculated up to the OTR expressed in cc µm m^{-2} d^{-1} KPa^{-1} multiplied by the film thickness (µm) and divided by the oxygen gradient in the cell of the testing machine that is 1 atm.

4.9.4 Phenolic compounds´ release

Chemical release was estimated following the normative 80/109/EEC (Council Directive, 1989) for testing the migration of constituents of plastic materials and articles intended to come into contact with foodstuffs. To evaluate the migration of phenolic compounds from the film to the food sample, discs of the films of approx. 1 cm diameter were placed into 20 mL of each food simulants: food simulant A: distilled water for aqueous foodstuffs (pH>4.5); food simulant B: 3% acetic acid for acidic foodstuffs and food simulant C: 10% ethanol for alcoholic foodstuffs. Solutions were shaken with film discs along 4 days, at 25°C. In order to study phenolic compound release, aliquots of 0.5 mL were taken after 1, 3, 6, 9, 24, 48 and 72 h, and content of phenolic compounds in solutions was measured by Folin-Ciocalteau method, afore described.

4.9.5 Antibacterial effect on pork loins

Vacuum-packaged boneless pork loin was donated from Maple Leaf Pork (Warman Rd., Winnipeg, Manitoba, Canada). Meat was cut into 100 pieces with equal thickness and diameter (2.5 cm) using a pastry cutter. Pieces were dipped in a *B. thermosphacta* B2 water solution dispersion containing 1 x 10^3 CFU mL^{-1}. After inoculation, both-sides of meat pieces

were covered by previously formed films containing 1% of GSE and sealed in low-O_2 permeable (O_2 transmission <15 cm^3 cm^{-2} day^{-1} atm^{-1} at 23°C) polyvinylediene chloride packaging bags (Winpak, Winnipeg, Manitoba, Canada) and vacuum sealed. Packages were stored at 4°C and incubated for subsequent sampling every 3 d to 12 d after inoculation.

Each pork loin and respective film were homogenized for 1 min in 90 mL of sterile peptone water (0.1% w/v, Difco Laboratories, Ontario, Canada) in a sterile filter stomacher bag (18 x 30.5 cm, filtra-bags, VWR, Mississauga, Ontario, Canada) using a stomacher (Model 400, A.J. Seward, London, UK). From this homogenate, serial dilutions were prepared and spread plated by an Autoplate 4000 equipped with a CASBA-4 automated counting system (Spiral Biotech Inc., Bethesda, Maryland, USA). Petri dishes containing brain heart infusion (BHI) agar, and streptomycin thallous acetate actidione (STAA) agar base with STAA selective supplement (Oxoid Ltd., Basingstoke, Hampshire, UK) were used for the growth of meat bacteria and *B. thermosphacta,* respectively. BHI and STAA plates were incubated for 24 h at 35°C. Results were expressed in log CFU mL^{-1} (Figure 4.22).

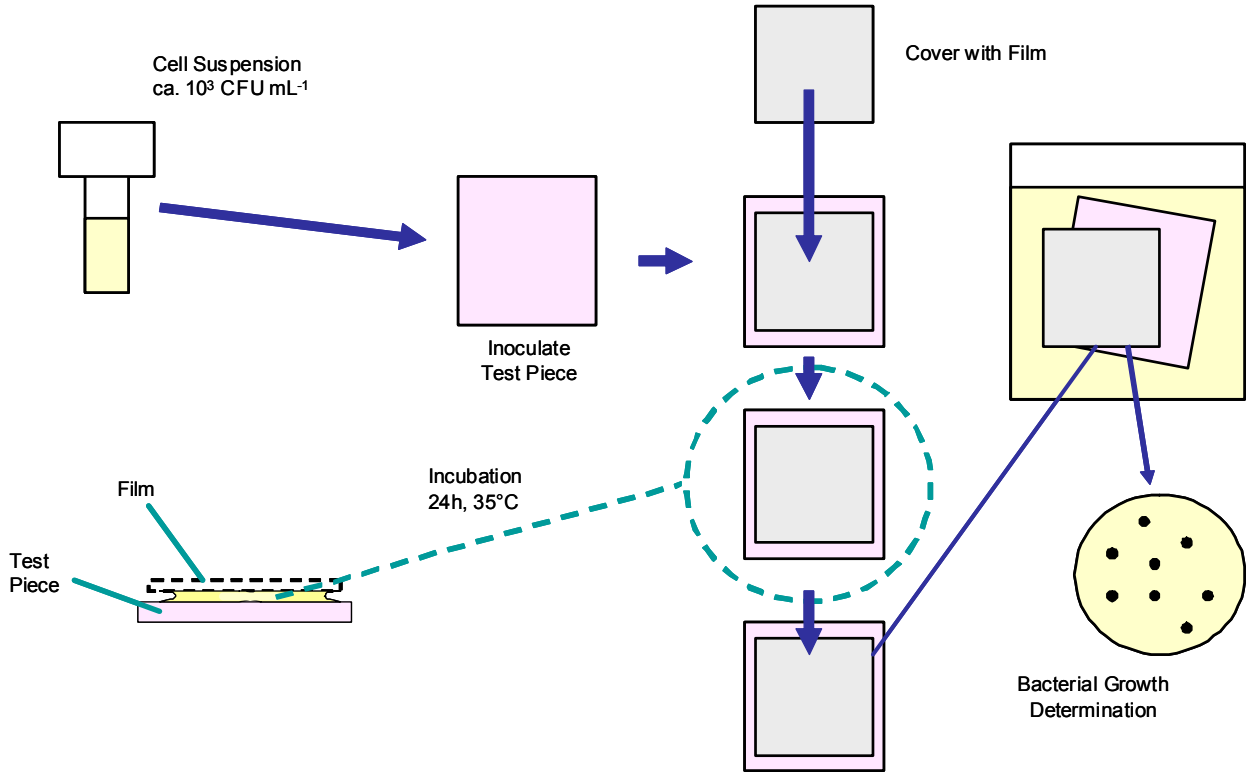


Figure 4.22: Schematic representation of film antibacterial test on inoculated meat (JIS Z 2801:2000. Japanese Test Protocol).

4.10 Statistical analysis

At least 3 replicates of each experiment were carried out and results were tested for statistical significance by *t*-Test for paired samples and ANOVA for greater number of samples. Student-Newman-Keuls test was used for comparison of data. Differences were considered statistically significant at the $P<0.05$ level. Statistical analysis was carried out by SPSS statistical program (Version 11.5).

5 RESULTS AND DISCUSSION

5.1 Organic and conventional grape pomace extracts

According to the increasing consumer demand for natural additives and agricultural wastes disposal problems, grape pomace extracts (GPE) Riesling and Spätburgunder (*V. vinifera* L.) from organically and conventionally managed land were compared for quality and aptitude for industrial commercialization as natural extracts. This study presents the determination of extracts antioxidant capacity, total phenolic content, the characterisation of their individual polyphenolic composition and their antimicrobial activities. In addition, the pesticide loads of the extracts were also determined.

The antioxidant capacity and total phenolic content of organic and conventional grape pomace extracts are represented in Figure 5.23 and Figure 5.24, respectively. The antioxidant capacity expressed in mmol TE $g_{extract}^{-1}$ was significantly different between organic white GPE (2.68 ± 0.07 mmol TE $g_{extract}^{-1}$) and conventional ones (1.59 ± 0.34 mmol TE $g_{extract}^{-1}$) (P<0.05). On the contrary, no significant differences in the antioxidant capacity of extracts from red grape pomace and seeds were found (P>0.05).

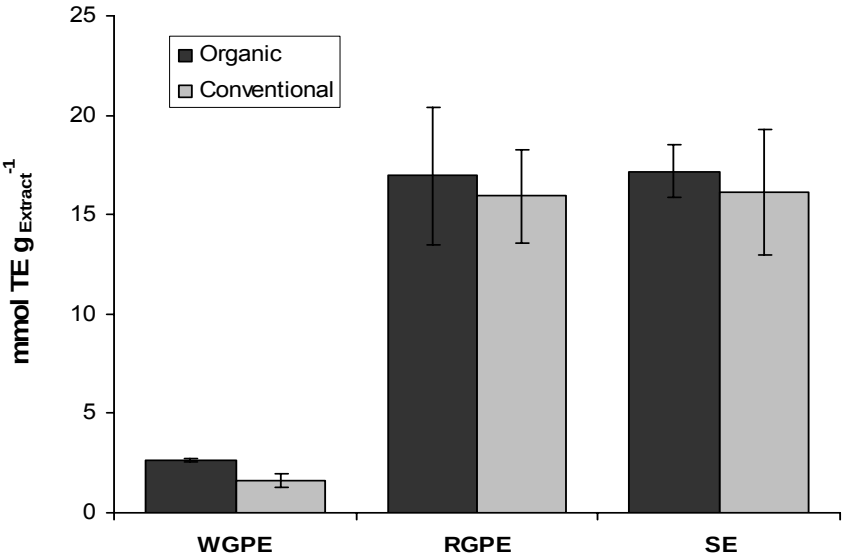


Figure 5.23: Antioxidant capacity of organic and conventional (*V. vinifera* L.) white Riesling (WGPE); red Spätburgunder (RGPE) grape pomace and Riesling seed extracts (SE).

These results are according to studies of Zafrilla et al. (2003) in which a higher antioxidant capacity in organic white wines than in conventional ones was found. Therefore, the antioxidant capacity of organic and conventional red wines was not significantly different

(*P*>0.05). In addition, the total phenolic content of conventional and organic samples in the different grape pomace extracts was not significantly different (*P*>0.05). In agreement with these results, studies of Danner (1986) and Lutz (1990), reported no significant differences between grape must and wine from organic and conventional production in respect of the concentration of desirable ingredients and parameters such as ethanol, sugar, total acids and extract.

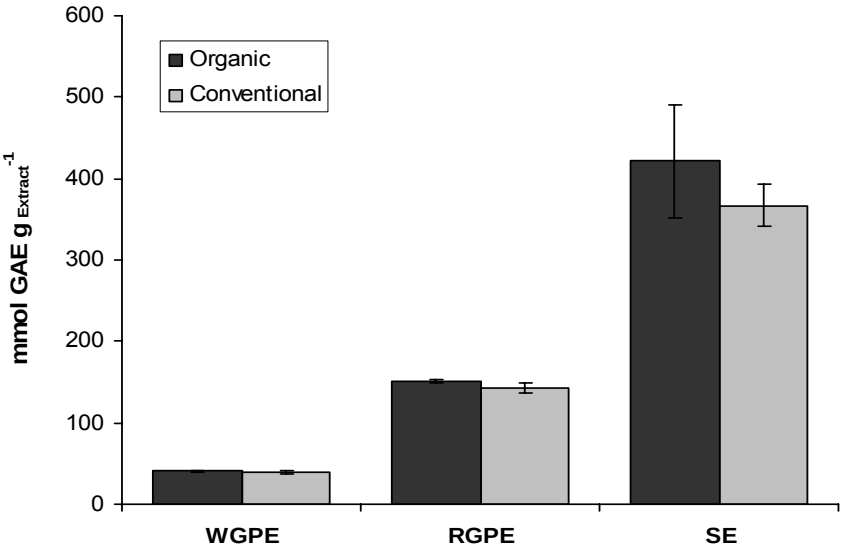


Figure 5.24: Total phenolic content of organic and conventional (*V. vinifera* L.) white Riesling (WGPE); red Spätburgunder (RGPE) grape pomace and Riesling seed extracts (SE).

Extract individual polyphenolic composition was determined and quantified by means of HPLC-DAD/ESI-MS. Peak mass identification was simplified using a combination of retention time, standard peak spectra, mass-to-charge ratio and MS^2 fragmentation. Figure 5.25 and Figure 5.26 illustrate the HPLC Chromatograms from the separation of phenolic acids and flavonoids identified and quantified in GPE.

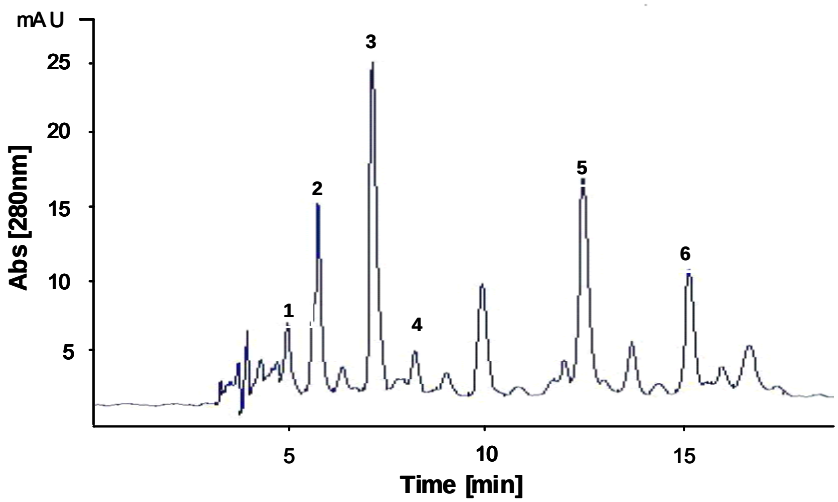


Figure 5.25: HPLC phenolic acid profile of Riesling (*V. vinifera* L.) grape pomace extracts. Compounds: 1. Gallic acid, M^+ = 171; 2. Protocatechuic acid, M^+ = 153; 3. Caftaric acid, M^+ = 311; 4. p-Hydroxybenzoic acid, M^+ = 137; 5. Caffeic acid, M^+ = 179; 6. Syringic acid, M^+ = 223.

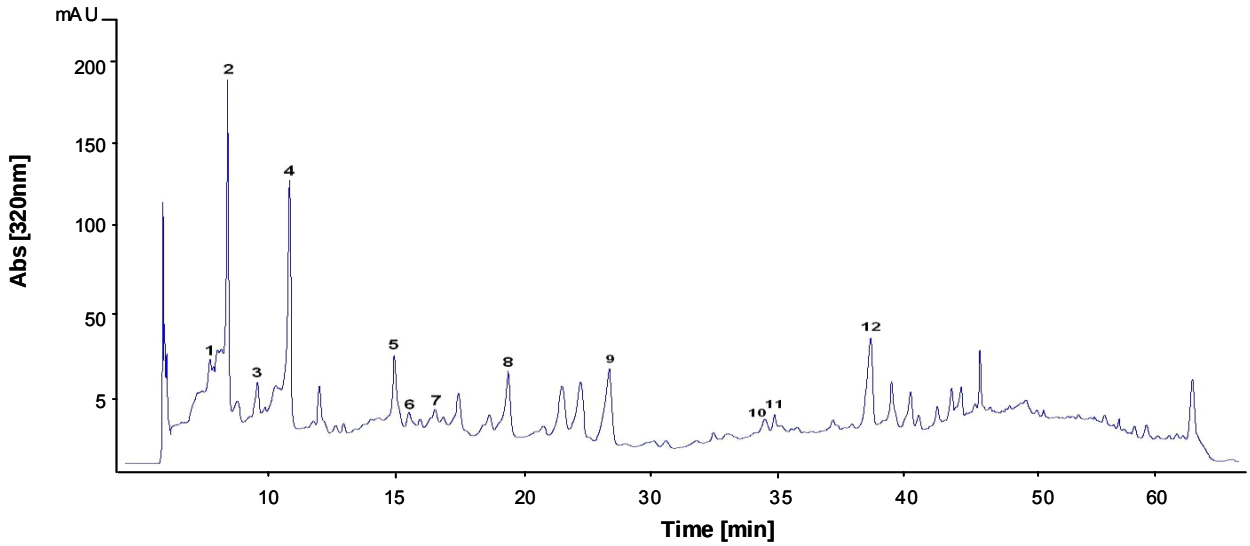


Figure 5.26: HPLC flavonoid profile of Riesling (*V. vinifera* L.) grape pomace extracts. Compounds: 1. Procyanidin B1, M^+ = 577; 2. (+)-Catechin, M^+ = 289; 3. Procyanidin B2, M^+ = 577; 4. (-)-Epicatechin, M^+ = 289; 5. (-)-Epigallocatechin, M^+ = 153; 6. (-)-Epicatechin gallate, M^+ = 441; 7. Quercetin-3-O-glucoside, M^+ = 463; 8. (+)-Catechin dimer, M^+ = 577; 9. (-)-Epicatechingallate trimer, M^+ = 1017; 10. *Trans*-polydatin, M^+ = 389; 11. *Trans*-resveratrol, M^+ = 227; 12. Kaempferol, M^+ = 285.

In white grape pomace extracts main phenolic acids identified were gallic, caftaric and ferulic acids. In case of white GPE, the origin of the samples, i.e. if they were organic or conventional, influenced sample phenolic composition (Figure 5.27). A significant content of caftaric and gallic acid was observed for organic samples when compared to conventional ones (P<0.05), whereas the content of ferulic acid was not significantly different. Main flavan-3-ols identified in white GPE were (+)-catechin, (-)-epicatechin, (-)-epicatechingallate;

flavonols: kaempferol, quercetin-3-O-rhamnoside, quecetin, hyperoside; and proanthocyanidins: procyanidin B1, (+)-catechin dimer and quercetin trimer. The content of (+)-catechin, (-)-epicatechin and procyanidin B1 were significantly higher in organic samples (P<0.05) whereas the content in quercetin and kaempferol was significantly higher in conventional ones (P<0.05) (Figure 5.27). Other flavonoids identified were not significantly different between paired samples (P>0.05). These significantly differences in the total content of polyphenols might explain the higher antioxidant capacity determined in organic white grape pomace extracts as illustrated in Figure 5.23.

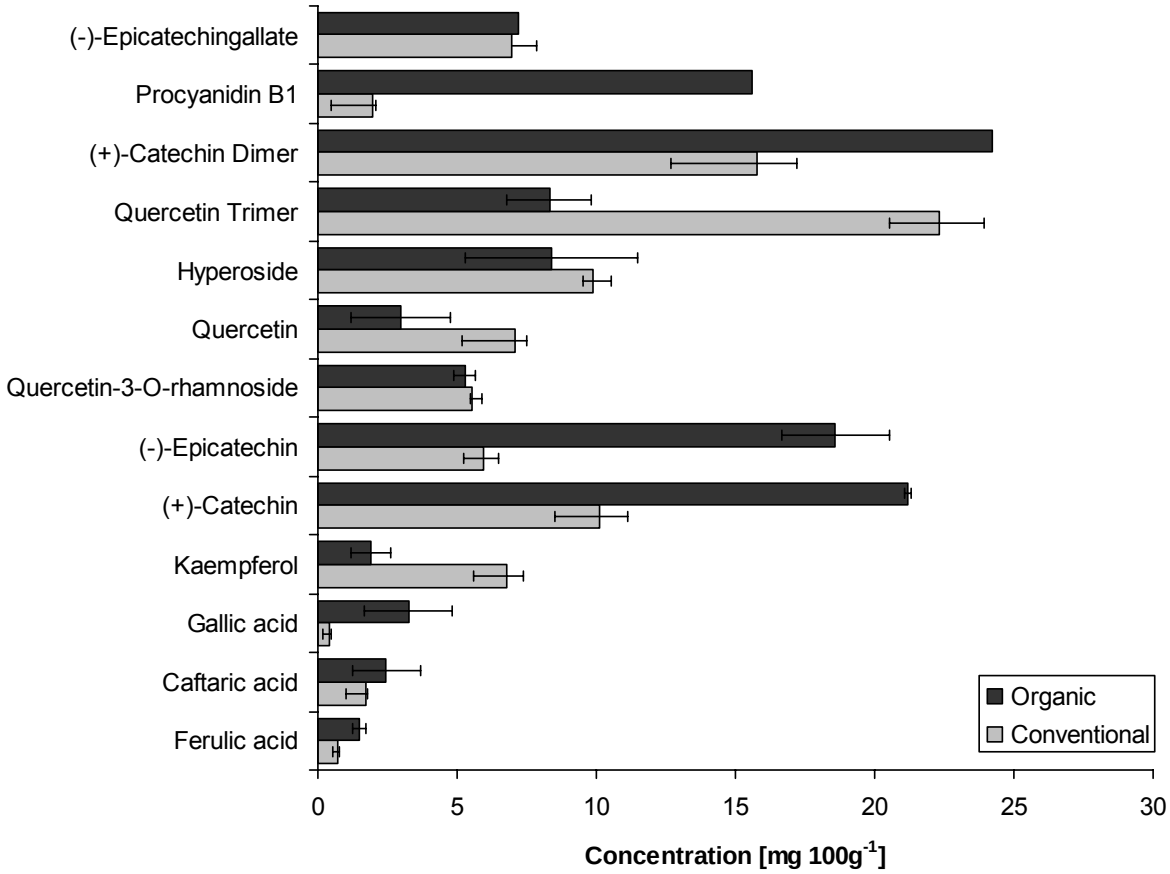


Figure 5.27: Comparison of the polyphenol content of organic and conventional white Riesling GPE (*V. vinifera L.)*.

These results are according to studies of Dani et al., 2007 in which it was pointed out that organic grape juices showed higher values of total polyphenols and resveratrol as compared to conventional ones. The mobility of pesticides in grapes is difficult to discuss and their accurate effect on the polyphenol synthesis is not well understood. They seem to influence the polyphenol content in white grapes as their epidermis is thinner and more vulnerable to external stressors than the red one (Tournas & Katsoudas, 2005). Studies reported cover only a minor portion of pesticides and plant phenolics and the target modulation effect of pesticides must be further investigated. In addition, other intrinsic factors such as light

exposition, nutrients, grape ripening etc. could also have influenced the polyphenolic content estimated. The fact that, the total phenolic content was not significantly different in the samples might be due to the Folin-Ciocalteu test characteristics. Folin Ciocalteau method determines mainly the number of aromatic rings in molecules present in the sample, certainly indicating the content of polyphenols. $ABTS^{\bullet+}$ radical cation assay instead, determines the antioxidant capacity of a great range of compounds not only of polyphenols, vitamins etc., but also the synergistic effects among them which may increase antioxidant capacity. Therefore, antioxidant capacity differences with regard to the polyphenol individual chemical structural features such as; level and position of hydroxylation, double bonds position and methylation is also recognised by $ABTS^{\bullet+}$ radical assay (Rice-Evans et al., 1995; van Acker et al., 1996; Balasundram et al., 2006).

The antioxidant capacity and total phenolic content between organically and conventionally grown red grape pomace extracts were not significantly different (P>0.05). These results might be explained by the non significant differences found in their polyphenol content. Main compounds identified were the anthocyanins; cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, petunidin-3-O-glucoside, malvidin-3-O-glucoside and peonidin-3-O-glucoside and their content was not significantly different between organic and conventional samples (P>0.05) (Figure 5.28 and Figure 5.29). Phenolic acids identified were: protocatechuic acid, vanillic acid, gallic acid, caftaric acid and p-hydroxybenzoic acid. Flavan-3-ols identified: (+)-catechin and (-)-epicatechin and flavonols: kaempferol, quercetin-3-O-rhamnoside, quercetin, hyperoside and isorhamnetin (Figure 5.30), and the content in quecertin-3-O-rhamnoside and quecertin was significantly higher in organic extracts (P<0.05). However, their higher amount did not affect the antioxidant capacity of organic samples. Compared to the anthocyanins, the phenolic acid and flavonoid content in red GPE was considerably lower making them particularly suitable for anthocyanin extraction.

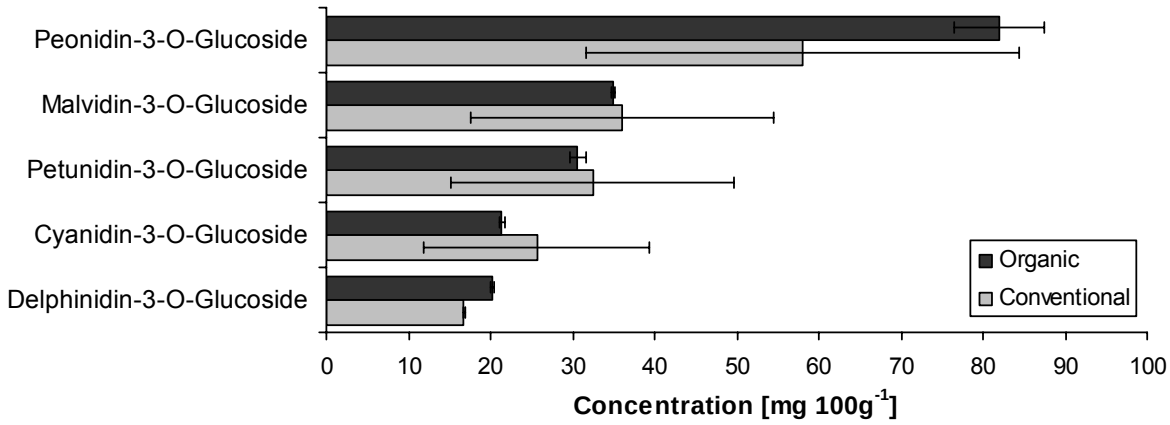


Figure 5.28: Comparison of the anthocyanin content of organic and conventional red Spätburgunder GPE (*V. vinifera* L.*)*.

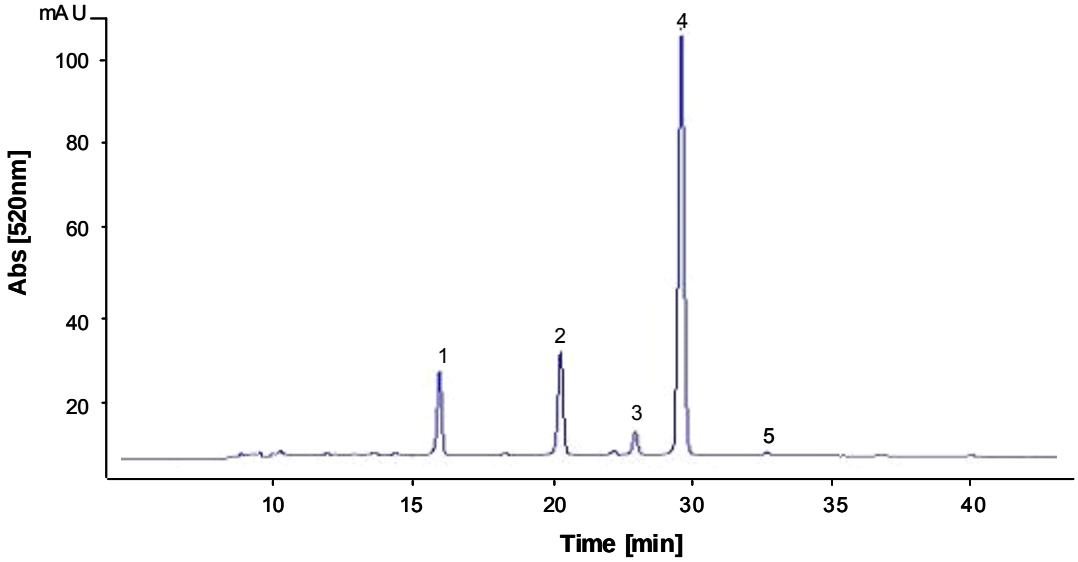


Figure 5.29: HPLC anthocyanin profile of Spätburgunder (*V. vinifera* L.) grape pomace extracts. Compounds: 1. Delphinidin-3-O-glucoside, M^+ = 465; 2. Cyanidin-3-O-glucoside, M^+ = 449; 3. Petunidin-3-O-glucoside, M^+ = 479; 4. Peonidin-3-O-glucoside, M^+ = 463; 5. Malvidin-3-O-glucoside, M^+ = 493.

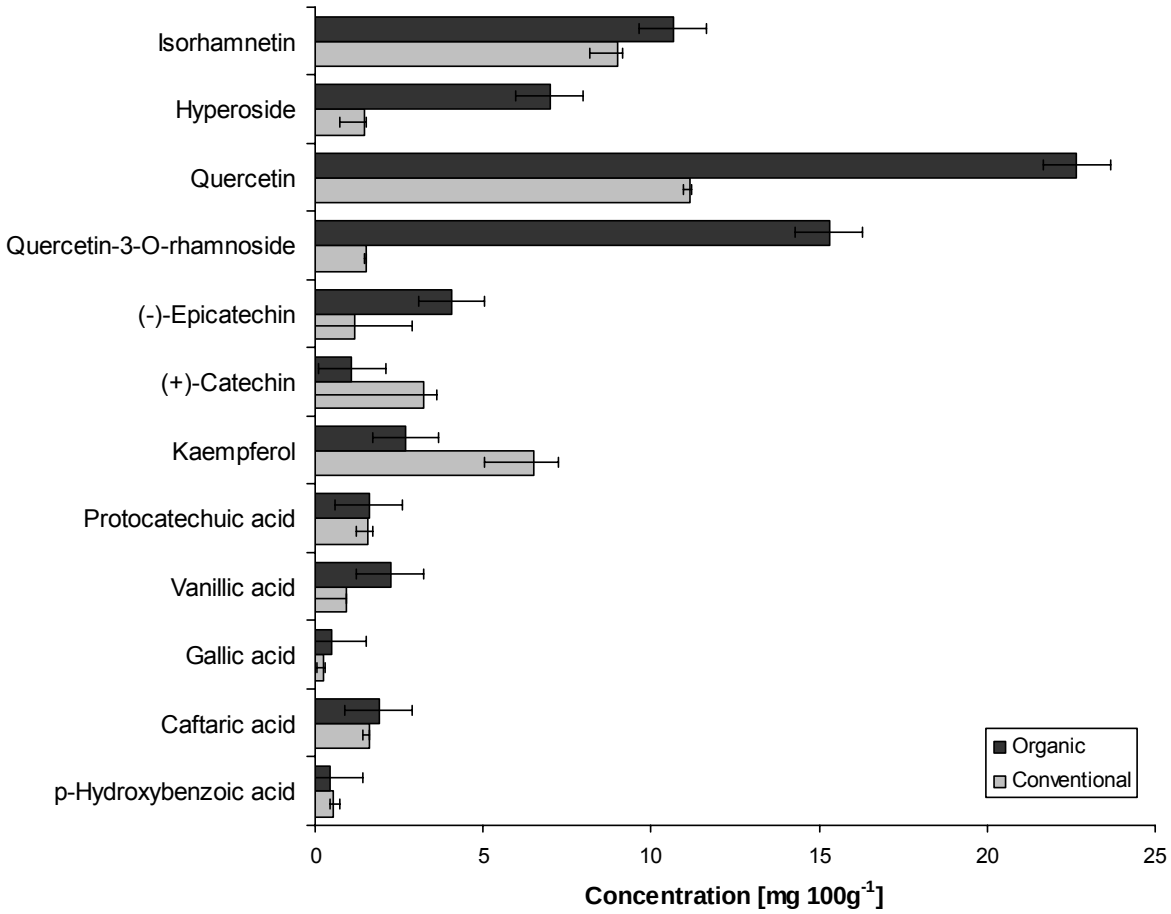


Figure 5.30: Comparison of the polyphenol content of organic and conventional red Spätburgunder GPE (*V. vinifera* L.*)*.

Seeds possessed the highest antioxidant capacity and total phenolic acid content compared to white and red GPE but not significant differences between organic and conventional grape seed extracts were found (P>0.05). The polyphenol profile of the seeds was dominated by flavonoids; flavan-3-ols: *trans*-resveraltrol, (+)-catechin, (-)-epicatechin and *trans*-polydatin; flavonols: quercetin-3-O-rhamnoside and proanthocyanidins: procyanidin B1 and B2, (+)-catechin dimer, gallate dimer and gallate trimer and their content was not significantly different between organic and conventional extracts (P>0.05). Phenolic acids: p-hydroxybenzoic, gallic and protocatechuic (Figure 5.31) were detected in minor amounts and their content in conventional extracts was not significantly different from organic ones (P>0.05). The higher content in proanthocyanidins (flavan-3-ol dimers, trimers and procyanidins) may explain the higher antioxidant capacity determined in GSE (Figure 5.23), as their antioxidant properties have been extensively reported and demonstrated (Ariga & Hamano, 1990; da Silva et al., 1991; Teissedre et al., 1996; Arii et al., 1998; Dauer et al., 1998; Saito et al., 1998).

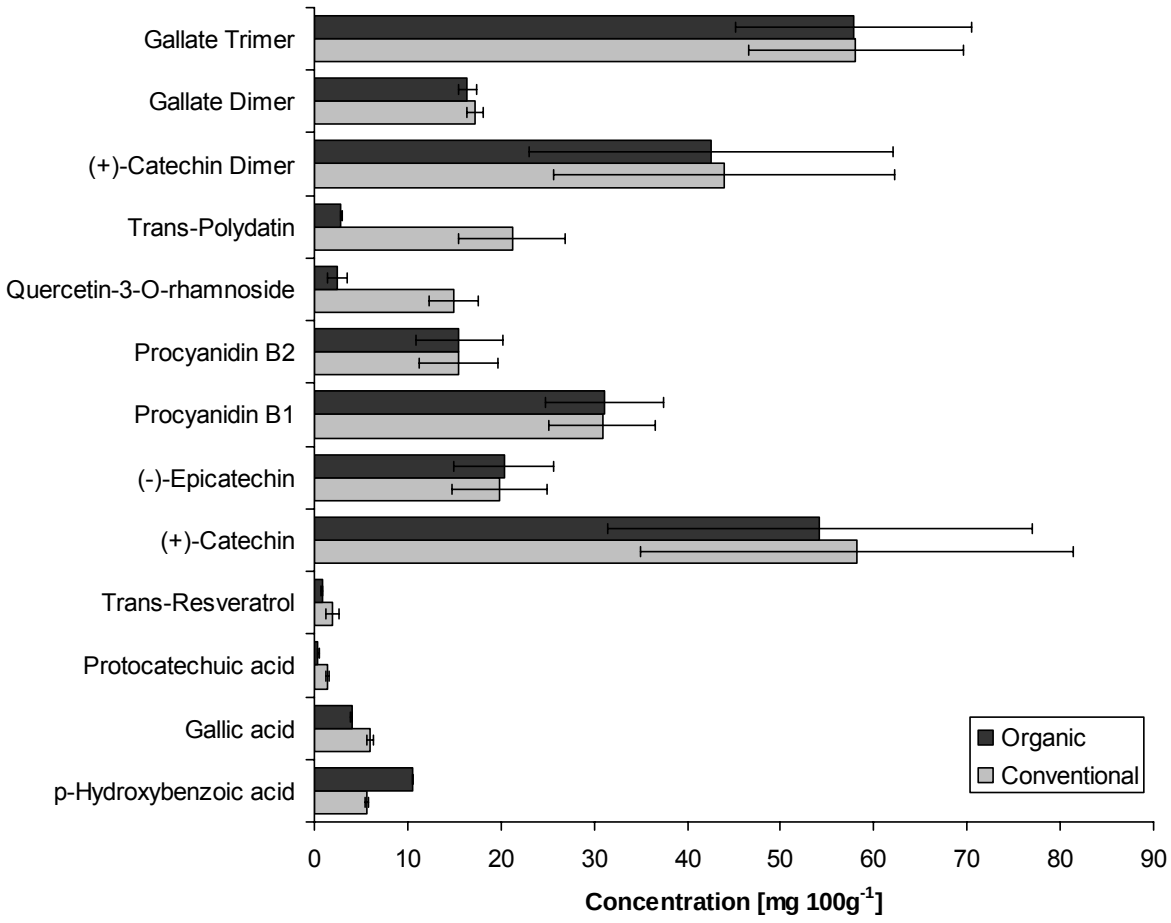


Figure 5.31: Comparison of the polyphenol content of organic and conventional Riesling grape seed extracts (*V. vinifera* L.*)*.

Grape pomace and seed extracts possess a high content in polyphenols independently of the primary raw material: organic or conventional. The antioxidant capacity and total phenolic

content of red GPE and SE was remarkably higher than those estimated for white grape pomace extracts. The high amount of anthocyanins and proanthocyanidins in red GPE and SE, respectively, contributes to this increase in their phenolic content and antioxidant capacity, as their scavenging properties are well understood (Bartolome et al., 2004; Ariga & Hamano, 1990; da Silva et al., 1991). These properties make red GPE and GSE particularly suitable as reservoir of natural antioxidants for industrial purposes.

5.1.1 Antibacterial and antifungal effect of grape pomace extracts

Antibacterial activity of organic and conventional extracts from grape pomace and seed extracts is represented in Table 1 from the appendix. White grape pomace extracts showed a slight antibacterial effect against *S. aureus* which increased according to an increase in the concentration (20% w/v). Extract concentrations of 5% (w/v) and 10% (w/v) instead, showed a similar antibacterial effect and inhibited slightly the growth of *L. monocytogenes, E. faecium and E. faecalis* whereas *S. typhimurium and E. coli* growth was not influenced by white GPE. These results are according to Rhodes et al. (2006) who demonstrated that both bacteria could live in grape juices unaffected by their compounds. The antibacterial effect of organic and conventional white GPE was not significantly different (P>0.05).

Red GPE Spätburgunder *(V. vinifera* L.) possessed a higher antibacterial activity than white grape ones which was certainly related to the higher content of polyphenols previously mentioned (Figure 5.23 and Figure 5.24). Low concentrations (5% w/v) of red GPE were enough to inhibit completely the growth of *L. monocytogenes, S. aureus, E. faecium and E. faecalis.* No significant differences between organically and conventionally grown extracts were found (P>0.05). These studies are complementary to those reported by Baydar et al. (2004) and Özkan et al. (2004) in which it was demonstrated that extracts from red grape pomace inhibited the growth of *A. hydrophila, B. cereus, E. aerogenes, E. faecalis, E. coli, E. coli* O157:H7, *M. smegmatis, P. vulgaris, P. aeruginosa, P. fluorescens, S. enteritidis, S. typhimurium*, *S. aureus* and *Y. enterocolitica*. By contrast extracts from red GPE applied in this study did not inhibit Gram-negative bacteria tested.

Seed extracts showed the largest inhibition zones in all Gram-positive bacteria. Grape seeds contained a higher amount of dimers and trimers of (-)-epicatechin which possess a higher antimicrobial activity than monomer ones (Figure 5.31) (da Silva et al., 1991; Prierur et al., 1994). Indeed, the antimicrobial activity has been acknowledged when the microbial contamination of seedless grapes was around 60% whereas in the seeded ones the contamination was reduced to 20% (Tournas & Katsoudas, 2005). In other studies Baydar et al. (2004); (2006) and Jayaprakasha et al. (2003) pointed out the antibacterial activity of

grape seed extracts against a range of pathogens of food significance. According to these studies, the growth of *L. monocytogenes*, *S. aureus*, *E. faecium* and *E. faecalis* was inhibited. Opposite to these studies, *S. typhimurium* and *E. coli* were not inhibited at the different concentrations applied 20% (w/v), 10% (w/v) and 5% (w/v) (Figure 5.32). The application of a different extraction procedure as the aforementioned authors, different grape variety and different bacterial strains may influence the extract compounds´ characteristics and thus result in the different antimicrobial activities achieved.

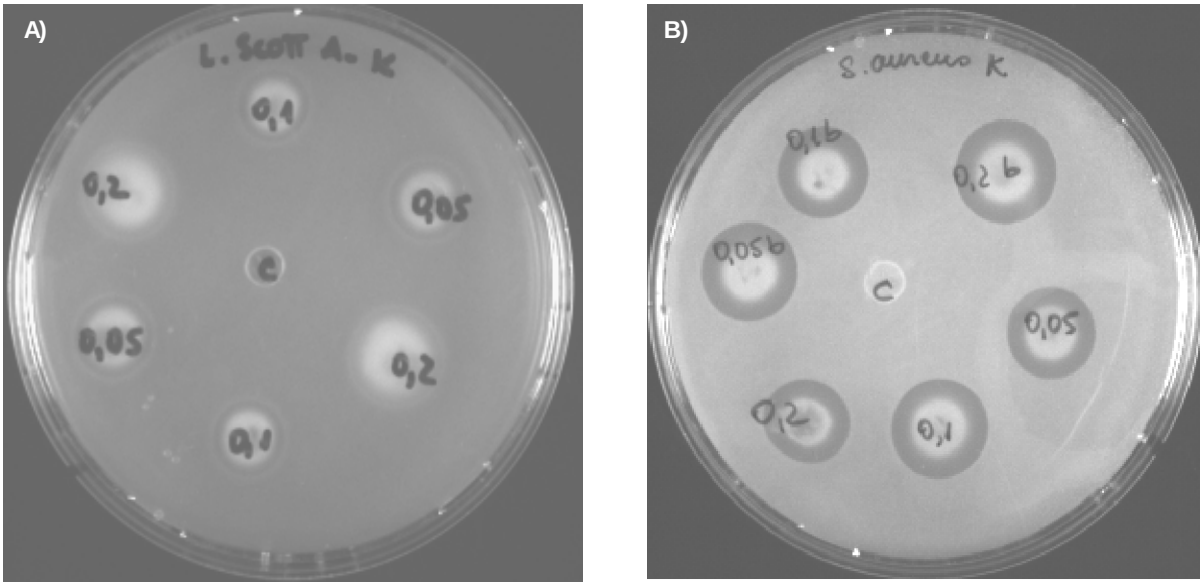


Figure 5.32: Antibacterial effect of GSE against A): *L. monocytogenes* and B): *S. aureus*. On the upper side from organically and lower side conventionally grown seed extracts.

The bacterial inhibition growth caused by grape pomace and seed extracts can be described by several mechanisms of action. Polyphenols can penetrate the semipermeable bacterial membrane where they react with the cytoplasm or cellular proteins. This potential is higher in GPE as phenolic acids are present in undissociated form (Paulus, 1993). Hydroxycinnamic acids and esters e.g. caftaric acid due to their propenoid side chain, are less polar than the corresponding hydroxybenzoic acids, and this property facilitates their transport across the cell membrane (Campos et al., 2003). Scalbert et al. (1991) proposed that the antibacterial activity of tannins could be due to the inhibition of extracellular microbial enzymes. Moreover, the complexing of metal ions from the bacterial growth environment could also be a possible mechanism for their antimicrobial properties. We assume that the lipidic wall of Gram-negative bacteria represented a great barrier for extracted polyphenols to get into the cytoplasm, hence no inhibition was achieved. The antibacterial effect of grape polyphenols in Gram-negatives could be observed if substances that promote lipidic wall hydrolysis such as lactoferrin or lysozyme are added (Pellegrini, 2003; Min et al., 2005).

The polyphenolic composition of a plant tissue determines also the level of susceptibility or tolerance to fungal infections and pests (Hock & Elstner, 1988; Usenik et al., 2004). However, grapes and their pomace possess high levels of sugars, nutrients and water which make them very susceptible to mould contamination, mostly caused by *B. cinerea, Alternaria spp.* and *Cladosporium spp.* and less commonly by *Fusarium, Penicillium, A. carbonarius, A. niger* and *Ulocladium* (Tournas & Katsoudas, 2005). Grape pomace and seed extracts at a concentration of 20% (w/v) were here tested against the growth of different moulds; *P. chrysogenum*, *P. expansum*, *A. niger*, *T. viridie* and *A. versicolor*. Our results showed remarkable differences between the fungistatic effect of organic and conventional GPE but also according to their genuine raw material: grape pomace or seeds.

The fungistatic effect of white GPE from organically and conventionally grown cultivars is represented in Figure 5.33. GPE from conventional cultivars retarded the growth of *P. chrysogenum* up to 50%, and the growth of *P. expansum* and *A. niger* up to 95%. By contrast, the fungistatic effect caused by organic samples was negligible (P<0.05). In the case of *T. viridie* and *A. versicolor*, their growth was similarly affected by both organic and conventional GPE (P>0.05).

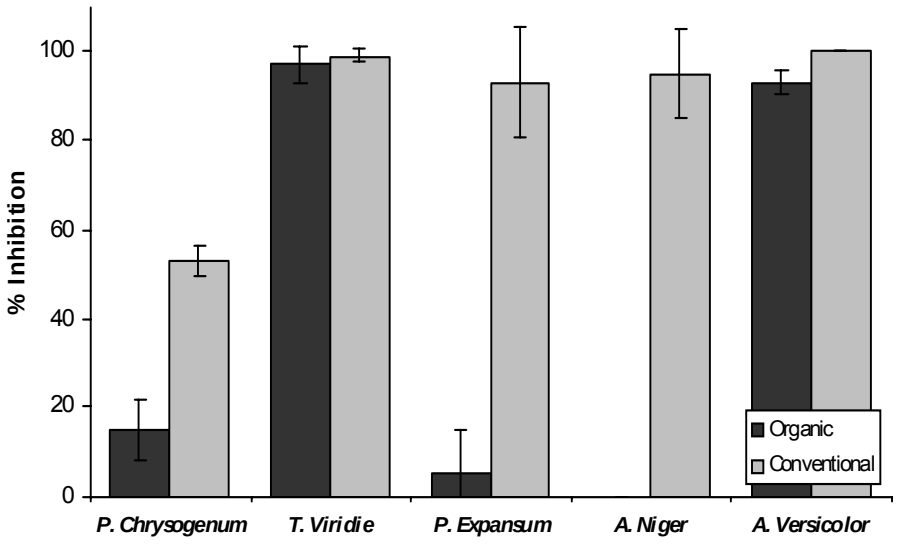


Figure 5.33: Fungistatic effect of organic and conventional white Riesling GPE (*V. vinifera* L.*)*.

Likewise, conventional red GPE retarded the growth of *P. chrysogenum, P. expansum* and *A. niger* up to 95%. By contrast, the fungistatic effect caused by organic samples was negligible (P<0.05). In the case of *T. viridie* and *A. versicolor*, their growth was similarly affected by both organic and conventional GPE (Figure 5.34) (P>0.05). Certain fungistatic effect has been attributed to isolated phenolic acids such as coumaric acid, caffeic acid, ferulic acid and sinapic acid as well as to isolated flavonoids such as (+)–catechin, kaempferol and quercetin (Lattanzio et al., 1994). Hence, the presence of some of these

compounds in the organic extracts is likely to be responsible for the inhibitory effect on *T. viridie* and *A. versicolor*.

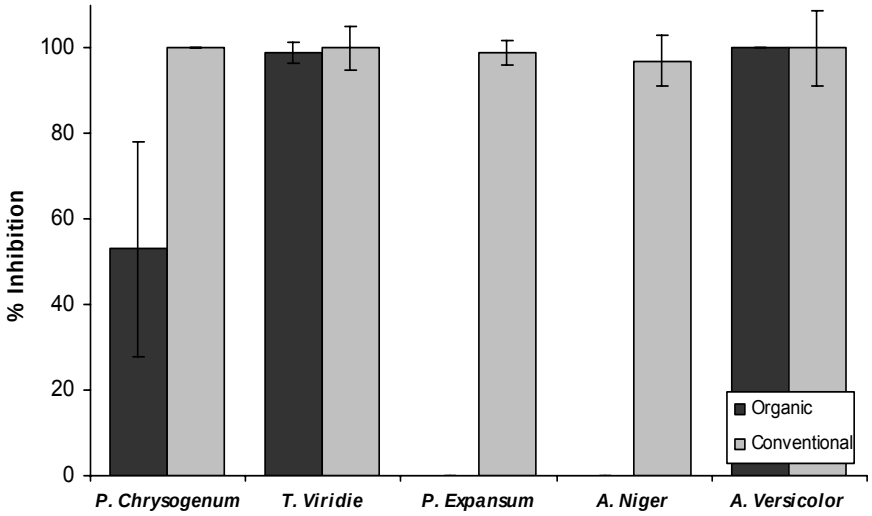


Figure 5.34: Fungistatic effect of organic and conventional red Spätburgunder GPE (*V. vinifera* L.*)*.

Grape seed extracts inhibited the growth of all mould tested apart from *A. niger* (Figure 5.35). The fungistatic effect between organic and conventional seed extracts was not significantly different (P<0.05). These results may indicate that the use of systemic pesticides in vineyards did not penetrate grape seeds during plant development. This may guarantee that the antifungal properties are from seed extract constituents and not pesticide residues.

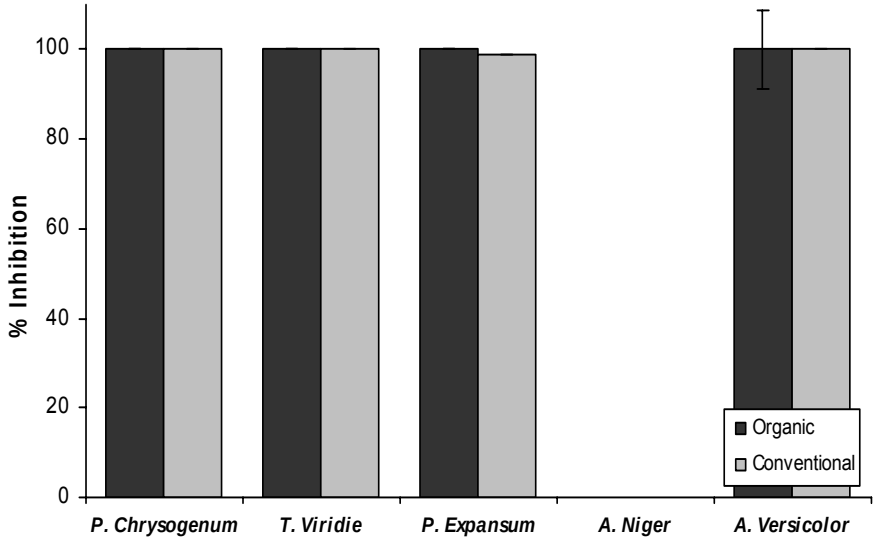


Figure 5.35: Fungistatic effect of organic and conventional Riesling SE (*V. vinifera* L.*)*.

5.1.2 Pesticide content in white grape pomace extracts

A fundamental aspect in food safety is the estimation of pesticide and heavy metal loads of food and derived products. Pesticide concentrations must lie below the limits stipulated by international regulatory organisms (BFR). Grape pomace extracts are an economical source of polyphenols with immeasurable value as antioxidants or antibacterials. For elaboration of grape pomace extracts mainly grape peels and stalks are used. The grape epidermis is usually exposed to pesticides which are still found in the samples after extraction. The presence of pesticides in the extracts could affect extract characteristics and be responsible for the differences found between organic and conventional white GPE. Some of the pesticides can even be concentrated after an extraction procedure and could cause serious undesirable secondary effects of public health significance. The pesticide content in both the genuine grape pomace samples and their extracts was estimated and represented in Table 5.1 and Table 5.2, respectively.

Table 5.1: Pesticide content in organic and conventional genuine white Riesling grape pomace (*V. vinifera* L.*)*.

Pesticide[1]	Conventional GPE mg kg^{-1}	Organic GPE mg kg^{-1}	Maximum Residue Level (MRL) in grapes mg kg^{-1}
Azoxystrobin (Fung.)	0.33	< DL	2.0
Boscalid (Fung.)	0.66	0.09	2.0
Carbendazim (Fung.)	0.006	< DL	0.3
Cyprodinil (Fung.)	0.34	0.04	2.0
Dimethomorph (Fung.)	1.1	< DL	2.0
Fenhexamid (Fung.)	0.16	< DL	5.0
Fludioxinil (Fung.)	0.13	< DL	2.0
Folpet (Fung.)	4.4	0.15	5.0
Indoxacarb (Insec.)	0.19	< DL	2.0
Methoxyfenocide (Fung.)	0.13	< DL	1.0
Myclobutanil (Fung.)	0.04	< DL	1.0
Penconazole (Fung.)	0.06	< DL	0.2
Pyraclostrobin (Fung.)	0.31	0.027	1.0
Spiroxamin (Fung.)	2.4	< DL	1.0
Tebuconazole (Fung.)	0.04	< DL	1.0
Tebufenocide (Insec.)	0.43	< DL	2.0
Trifloxystrobin (Fung.)	0.04	< DL	5.0

DL: Detection limit; Fung: Fungicide, Insec; Insecticide

As expected, traces of fungicides and insecticides were found in grape genuine samples (Table 5.2) which were later on concentrated during a water/methanol extraction. The

[1] The results here expressed have a default expanded uncertainty lower than 50% (corresponding to a 95%) according to the recommendation of the Codex Committee on pesticide residues (EU, validation Method; CCPR 2005, ALINORM 05/28/24)

concentration of boscalid was 6.5-fold, cyprodinil 4-fold, fenhexamid 13.5-fold, fludioxonil 18.4-fold, myclobutanil 30-fold and pyraclostrobin 5-fold higher than from the original raw material. The estimated concentration lied over the Maximum Residue Levels (MRLs) established by regulatory organisms in grapes (Rückstands-Höchsmengenverordnung, BGBI.I.S.1962, 2379, 2007). Mainly pesticides found in the extracts were contact ones, while the content in systemic pesticides such as azoxystrobin was relatively low, since systematic pesticides are mainly found in the pulp (Teixeira et al., 2004).

Pesticide traces were also found in organic genuine samples and extracts (Table 5.1 and Table 5.2). However, levels lied below the values permitted for organic products even after the extraction (EEC Regulation 2092/91). The appearance of pesticides in organic samples must be related to the proximity of conventional managed vineyards, physical or mechanical transport or other pollution pathways. These results indicate the parallel and optimal extraction of pesticides during a methanol/water extraction. The remaining pesticides may also have consequences in extract properties especially of conventional extracts as for example in their higher fungistatic effect (Figure 5.33 and 5.34).

Table 5.2: Pesticide content GPE from organically and conventionally grown grapes Riesling (*V. vinifera* L.*)*.

Pesticide	Conventional GPE mg kg^{-1}	Organic GPE mg kg^{-1}	Maximum Residue Level (MRL) in grapes mg kg^{-1}	ADI (BFR) mg kg^{-1} corporal weight
Boscalid (Fung.)	4.3	< DL	2.0	0.04
Carbendazim (Fung.)	0.004	< DL	0.3	0.02
Cyprodinil (Fung.)	1.4	< DL	2.0	0.03
Diuron (Herb.)	0.01	< DL	0.05	0.007
Dimethomorph (Fung.)	1.3	0.05	2.0	0.05
Fenhexamid (Fung.)	2.8	0.1	5.0	0.2
Fludioxinil (Fung.)	2.4	0.01	2.0	0.37
Fluquinconazol (Fung.)	1.9	< DL	0.05	0.005
Folpet (Fung.)	0.009	0.01	5.0	0.1
Methoxyfenocide (Fung.)	0.17	0.01	1.0	0.1
Myclobutanil (Fung.)	1.2	0.01	1.0	0.025
Penconazole (Fung.)	0.07	0.03	0.2	0.03
Pyraclostrobin (Fung.)	1.6	0.005	1.0	0.03
Tebuconazole (Fung.)	0.08	0.01	1.0	0.03
Tebufenocide (Insec.)	0.19	0.01	2.0	0.02

DL: Detection limit; Fung: Fungicide, Insec; Insecticide

Moreover several fungicide and insecticide traces were found in commercialised GPE (Obipektin GmbH, Swiss) represented in Table 5.3. Pesticide levels in the samples were below the Maximum Residue Levels (MRLs) established by regulatory organisms for grapes

(Rückstands-Höchsmengenverordnung, BGBI.I.S.1962, 2379, 2007). Nonetheless, these results pointed out that commercialised natural extracts are not exempt from pesticides.

Extracts from plant by-products or pomace are a rich source of polyphenols. However, they could also contain high amounts of pesticides, since mainly peels are exposed to them. Pesticides are irremediably extracted and they appear in the final elaborated powder. According to the ADI values established by regulatory organisms (BFR), the risk to suffer health disorders by ingesting these extracts in elaborated products is negligible. Taking into account the low ingestion levels, they are not expected to cause undesirable effects of public health significance. For instance, the no-observed-adverse-effect levels (NOAEL) of GPE may be equal to 18.30 g kg^{-1} body weight day^{-1} in children for myclobutanil (Banasiak et al., 2005). These results lead to the consideration of the efficacy of natural extracts currently commercialised, since their higher or lower antibacterial properties seem to be closely related to the origin of the raw material; conventional or organic. Based on these results, an important theme of debate must be focused on labelling of natural extract products. Labels should honour consumer's right-to-know about each pesticide ingredient and its health and environmental hazards. Such information will enable consumers to make informed decisions about products used in and around the home. Thus, grape pomace extracts product label should not only highlight the potential market benefits; “natural” but also the risks; “non free in pesticides”, if raw material comes from conventionally managed land. Better labelling will also encourage manufacturers to develop and market safer pesticide products.

Table 5.3: Pesticide content determined in commercial GPE (Obipektin GmbH, Swiss).

Pesticide	Commercial GPE mg kg^{-1}	Maximum Residue Level (MRL) in grapes mg kg^{-1}
Carbaryl (Insec.)	0.05	0.0075
Carbendazim (Fung.)	0.04	0.02
Dimethomorph (Fung.)	0.09	0.05
Fenhexamid (Fung.)	0.12	0.2
Fludioxinil (Fung.)	0.02	0.37
Iprodion (Fung.)	0.02	0.06
Iprovalicarb (Fung.)	0.15	0.015
Procymidone (Fung.)	0.82	0.025
Tebufenocide (Insec.)	0.05	0.02
Tebuconazole (Fung.)	0.005	0.03
Thiphanat-methyl (Fung.)	0.02	0.08

Fung: Fungicide, Insec; Insecticide

5.2 Industrial relevance of grape seed extract enriched films

The food industry has at its disposal a wide range of non-edible polypropylene- and polyethylene-based packaging materials able to extend food shelf life. However, they can create serious environmental problems. Only in the EU over 67 million tons of packaging wastes are generated annually, one third of which end up in landfills (Klingbeil, 2000). In response to environmental concerns caused by non biodegradable packaging materials, biopolymers based on starch and natural fibres are the front runners for future bio-based packaging. Taking into account the potential antibacterial and antioxidant properties of grape seed extracts reported in chapter 5.1, these extracts were incorporated into pea starch films. Their physico-chemical properties were investigated as well as their antibacterial effect *in vitro* to inhibit the growth of *B. thermosphacta* in meat. The psychrotrophic microorganism *B. thermosphacta* frequently represents a significant portion of the spoilage microbial flora of meat stored aerobically or vacuum-packed at refrigerated conditions, and it is occasionally the predominant microorganism (Borch et al., 1996; Gill, 1998; Tu & Mustapha, 2002). Thus, an effective antibacterial film able to stop or decrease their growth in meat will ensure food quality and extend shelf life.

5.2.1 Mechanical properties of grape seed extract enriched films

GSE were introduced into pea starch films in a concentration of 1%, since up to this concentration the inhibition of *B. thermosphacta* was achieved. Mechanical properties of the films illustrated in Table 5.4 showed that the thickness of GSE incorporated films increased by 25% compared to control films. The geometrical configuration of glycerol and polyphenols into the film will affect the interaction force with starch molecules significantly resulting in this thickness increase. By contrast the tensile strength of enriched films was half reduced and their elongation increased. The chemical disposition of flavonoids and phenolic acids from GSE with adjacent amylose chains and glycerol molecules loose the intermolecular interactions of starch due to repulsive charges of the GSE phenolic acids which may explain the higher elasticity determined in GSE films.

Table 5.4: Mechanical properties of control and grape seed extract films.

Sample	Thickness (mm)	Moisture (%)	Tensile Strength (MPa)	Elongation (%)
Control film	73.66 ± 4.04^{a}	91.78 ± 0.78^{a}	510.3 ± 5.2^{a}	36.9 ± 7.9^{a}
GSE film	114.33 ± 15.56^{b}	92.60 ± 0.29^{a}	249.5 ± 6.1^{b}	56.10 ± 5.06^{b}

Columns with different letters are significantly different (P<0.05).

Film strength was also tested with different glycerol concentrations. Results showed a glycerol concentration of 60% (w/w) of starch as the most suitable one for film mechanical properties since films containing higher glycerol concentration (80% w/w) resulted difficult to dry, sticky and highly elastic, whereas low glycerol films (30% w/w) broke into pieces during drying (data not shown). These results are according to Arvanitoyannis and Biliaderis (1999) whose starch films tended to become more hydrophilic with an increase in the total plasticizer content. Plasticizers are normally more hygroscopic than starches and glycerol acts as a water holding agent. The higher amount of water molecules in glycerol-plasticized starch films, the higher the plasticizing activity. Despite of the swollen structure of GSE films observed by the thickness increase when GSE was incorporated, the water vapour permeability was not changed significantly (P>0.05) (Table 5.5).

Table 5.5: Water vapour and oxygen permeability of control and GSE pea starch films.

Sample	Water vapour permeability ($g\ mm\ m^{-2}\ h^{-1}\ kPa^{-1}$)	Oxygen Permeability ($cc\ \mu m\ m^{-2}\ d^{-1}\ kPa^{-1}$)
Control film	2.61 ± 0.15^{a}	1.08 ± 1.06^{a}
GSE film	2.40 ± 1.55^{a}	3.11 ± 1.48^{b}

Columns with different letters are significantly different (P<0.05).

On the contrary, the oxygen permeability of GSE enriched films was significantly increased. This result may be explained by the lower polarity of GSE compounds which may accelerate the absorption of oxygen to the film surface (Table 5.5) (Tang et al., 2003). GSE incorporated pea-starch films possessed an orange-brownish colour determined by the Lab colour system. A significant decrease in the lightness values L and an increase in a and b values was determined (Table 5.6).

Table 5.6: Lab values of control and GSE films.

Sample	L	a	b
Control film	24.28 ± 0.94^{a}	-0.10 ± 0.07^{a}	-0.51 ± 0.49^{a}
GSE film	19.56 ± 0.04^{a}	1.70 ± 0.02^{b}	5.17 ± 0.01^{b}

Columns with different letters are significantly different (P<0.05).

The colour change was closely related to the content of polyphenols contained in the GSE (maximal absorbance at 320 nm; orange-brownish) and will determinate consumer acceptance and applicability. Figure 5.36 illustrates the colour characteristics of different grape pomace extracts enriched films.

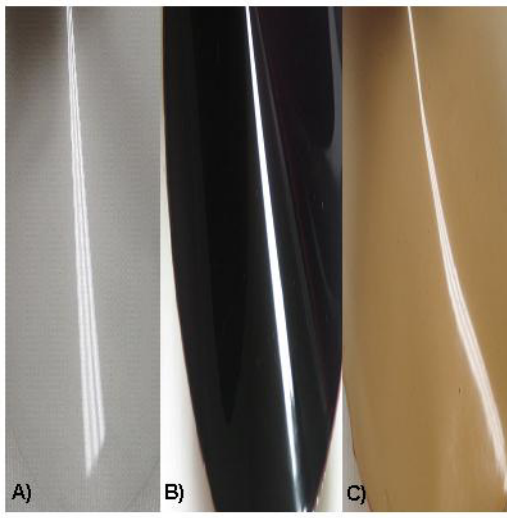


Figure 5.36: Pea starch films: A): control; B): enriched with red grape pomace extracts and C): enriched with grape seed extracts.

5.2.2 Grape seed extract compounds´ release

An active packaging system based on the release of antimicrobial compounds must fulfil an adequate release kinetics adjustable to the specific requirements of the packaged food and the released active compound should maintain a certain antimicrobial efficiency. In this chapter the first aspect of this statement is discussed. Polyphenol particle release from GSE enriched pea starch films into different food simulants: food simulant A; distilled water for aqueous foodstuffs (pH>4.5), food simulant B; 3% acetic acid for acidic foodstuffs and food simulant C; 10% ethanol for alcoholic foodstuffs was studied[2]. After 24 h incubation it could be observed a gradual increase of phenolic compounds into the solvent up to 0.343 ± 0.008 mg GAE mL^{-1} in water, 0.39 ± 0.05 mg GAE mL^{-1} in acetic acid and 0.45 ± 0.01 mg GAE mL^{-1} in ethanol. Figure 5.37 shows that total phenolic release from GSE films reached 0.4-0.5 mg GAE mL^{-1} after 72 h regardless of the immersion solvent types that are food simulants. However, phenolics were released faster to acetic acid than both, water or ethanol at the initial stage of 0-9 h. The solvent pH may affect the release profile significant, while the difference of solvent polarity (e.g. water or ethanol) did not affect the release profile of phenolics. Acidic solvents can cause the hydrolysis of glucoside groups joined to flavonoids, enhancing thus their migration into the solvent. At the equilibrium ~48 h only 8% of the total phenolic content initially present in the films migrated (Figure 5.37). The amount of the active compound released from films has been reported to decrease as the degree of cross-linking of a polymer matrix increased (Buonocore et al., 2004). In addition, thermal amylase and

[2] Chemical release was estimated following the normative 80/109/EEC (Council Directive, 1989) for testing the migration of constituents of plastic materials and articles intended to come into contact with foodstuffs.

amylopectin changes may create a gel network which may entrap or retain polyphenols into the films (Godet et al., 1993). This will also explain the lower particle release into the food simulants.

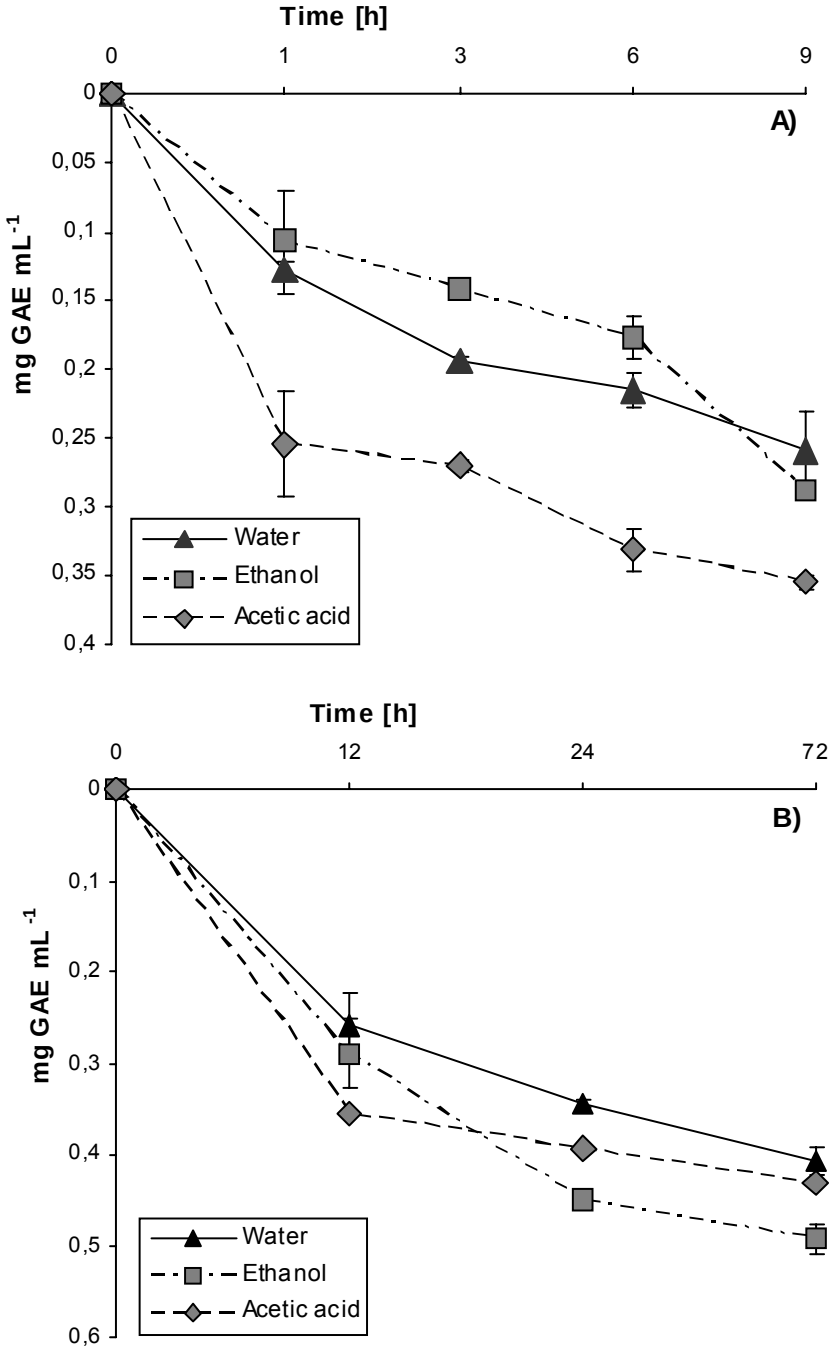


Figure 5.37: Release of polyphenols from GSE films into different food simulants estimated in mg GAE $g_{extract}^{-1}$. A): short term data only until 12 h. B): longterm data until 72 h.

The controlled particle release from swelling polymers has not been extensively studied and only works of Buenocore et al. (2002); (2004), have demonstrated its importance for the antibacterial efficiency of bioactive films. Pea starch films here studied present a lower

compound release than whey protein isolate films according to studies of Buenocore et al., (2004). However, these works were carried out *in vitro* and with a different antimicrobial substance; nisin. This is the first study dealing with the particle release kinetics from pea starch films and it seems to be closely related to the degree of cross-linking of the polymeric matrix. Thus, the use of decompacted substances, cellulose fibers or microcapsules could overcome this problem and control the release kinetics of small molecules from starch matrices.

5.2.3 Antibacterial effect on pork loins against *B. thermosphacta*

The second and important aspect which determinates the feasibility of GSE bioactive films is that it maintains a certain antimicrobial efficiency. Thus, the antibacterial effect of GSE enriched pea starch films was tested against the growth of *B. thermosphacta* in pork loins. *B. thermosphacta* survives at high salt concentrations and low O_2 levels (>0.2%) which complicates its growth inhibition in meat. An effective bioactive film will retard or inhibit their growth increasing thus meat shelf life. Pork loins were inoculated with *B. thermosphacta* and covered from the both sides with GSE enriched pea-starch films and storage during 12 days at 4°C. Pork loin sampling was carried out in different types of agar plates SSTA and BHI to discern among *B. thermosphacta* and *Pseudomonas,* respectively since meat stored aerobically at chill temperatures is also dominated by *Pseudomonas* spp. (Dainty & Mackey, 1992). Results represented in Figure 5.38 highlighted that the growth of *B. thermosphacta* was inhibited in 1.3 log CFU mL^{-1} reductions after 4 days of storage at 4°C. The extract migration from the films into the meat may delimit the antibacterial results here achieved, since only surface bacteria during the initial period were inhibited.

The increase of phenolic compounds availability from the films up to day 4 is probably due to the increase of film hydration obtained in the presence of meat, making easier their extraction. In addition, polyphenols migrated further inside the meat and the surface phenolics concentration co-diluted with contact time which may explain the higher activity during the initial period. In any case, the strong antibacterial activity determined by disc diffusion methods was not observed in meat samples. Similarly, studies of Oussalah et al. (2004) observed antibacterial effect of milk protein-based films enriched in essential oils in meat during the first 7 storage days. The higher or lower bacterial growth inhibition is related to the particle release which directly depends on the nature, structure and features of the film polymers. By higher polyphenol migration, a higher antibacterial activity of GSE enriched films is expected.

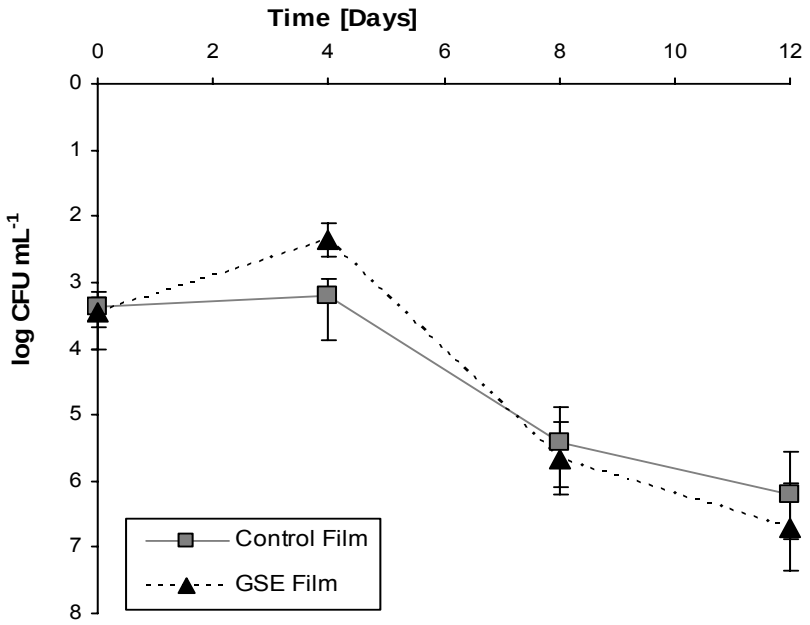


Figure 5.38: Antimicrobial effect of grape seed extract incorporated pea starch films in pork loins inoculated with *B. thermosphacta*.

The concentration of polyphenols in the meat will also determine their beneficial or prejudicial effects on human health. Extract concentrations here applied and determined do not pose any risk to consumer health according to studies of Yamakoshi et al. (2002) in case films would be ingested as an integral food compound e.g. sausages. In these studies, it was reported, that rats which ingested dosages of 2 and 4 g kg^{-1} of GSE did not show evidence of acute oral toxicity. In addition, the administration of GSE as dietary admixture at levels of 0.02, 0.2 and 20% (w/w) to the rats for 90 days did not induce noticeable signs of toxicity. In this study, the no-observed-adverse-effect levels (NOAEL) of GSE were equal to 1410 mg kg^{-1} body weight day^{-1} in males and 1501 mg kg^{-1} body weight day^{-1} in females. As a result, polyphenols applied in edible films which migrate into the meat or could form part of a food product, could not only act as potential antibacterials but also exert positive biological activities on human health if ingested (Ariga & Hamano, 1990; da Silva et al., 1991; Teissedre et al., 1996; Dauer et al., 1998; Saito et al., 1998). Based on the accumulated results, GSE films presented three remarkable advantages for marketability: Firstly, inhibit the growth of undesirable pathogens in meat, improving meat quality and extending its shelf life. Secondly; are totally biodegradable and edible, which will reduce environmental constrains occasioned by polypropylene-based packaging. Finally; may exert beneficial effects on human health if ingested.

5.3 High hydrostatic pressure as grape pomace extraction method

Dornfelder (*V. vinifera* L.*)* pomace was selected for the recovery of anthocyanins and other antioxidants, because of their higher content in anthocyanins compared to other grape varieties grown in the zone of the Palatinate in Germany. Indeed, it is well popular extended the mixture of Dornfelder grapes with other varieties with the aim of improving red colour and flavour of low quality table wines. The extraction of Dornfelder anthocyanins and other antioxidant compounds can be described as a mass transport phenomenum where solids contained in plant structures migrate into the solvent up to equilibrium. Different methods to estimate the antioxidant capacity of complex solutions have been reported, indicating the $ABTS^{+\bullet}$ decoloration assay as one of the most reliable ones (Huang et al., 2005). The $ABTS^{+\bullet}$ radical cation assay provides information about the presence of reducing agents and hydrogen donors in a given sample. They contribute to the reduction of $ABTS^{+\bullet}$ cations which is manifested as a decrease in the absorbance and directly correlated to the water soluble antioxidant capacity of the volume added. As it can be observed in Figure 5.39 the antioxidant capacity of the samples extracted at 200 MPa, 400 MPa, 600 MPa and expressed in TROLOX equivalents (TE) g_{DM}^{-1} were up to three fold-higher than the control samples extracted by agitation. The fact that extraction yields by different pressure intensities, were not significantly different (P>0.05), is certainly due to the similar membrane disruption effect on grape skin cell microstructure (Butz et al., 1994; Fernandez-Garcia, 2001). During high hydrostatic pressurised extraction partially air-filled pores of fruit tissues are responsible for the liquid uptake and sponge effect. Then extraction solvent enters the pores which force the occluded air in the pores to exit causing cell membrane damages of plants and particle release. Additionally, HHP can cause deprotonation of charged groups and disruption of salt bridges and hydrophobic bonds, resulting in conformational changes and denaturation of proteins and carbohydrate structures making cellular membranes less and less selective and compounds more accessible to extraction (Barbosa-Canovas et al., 1998). The results of the present study are contrary to studies of Sanchez-Moreno et al. (2004) where they reported a pressure dependent extractability of carotenoids in tomato puree at moderate pressures on the basis of their chemical features and chromoplast location.

The antioxidant capacity of the extracts gently increased with extraction times being 286.08 ± 52.68, 548.49 ± 39.17 and 716.67 ± 65,4245 µmol TE g_{DM}^{-1} after an extraction assisted by HHP (600 MPa, 50°C) for 30, 60 and 90 min, respectively.

The fact that solids were in contact with the solvent for longer times, may enhance the diffusivity of compounds from the inner to the outer side of plant membranes up to equilibrium. In agreement with these results, Revilla et al. (1998) described a higher phenolic extraction from red grape pomace after 24 h extraction.

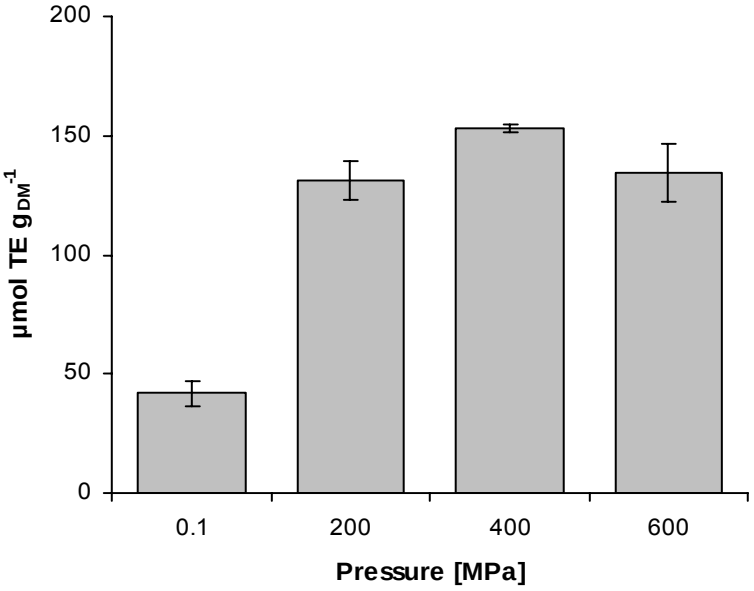


Figure 5.39: Effect of HHP on the antioxidant capacity of Dornfelder (*V. vinifera* L.*)* grape pomace extracts expressed in µmol TE g_{DM}^{-1}. Extractions were carried out at 50°C and 50% ethanol.

5.3.1 Effect of temperature and ethanol on extract antioxidant capacity

Antioxidant capacity of the extracts was higher in heat-pressurised samples than in controls (Figure 5.40). Temperature played an important role in the extraction process since high extraction temperatures increase the solubility of polyphenols and the diffusion coefficient, which reduce extraction times (Lou et al., 1997; Cacace et al., 2003). Extraction yields at 600 MPa and 70°C were two-fold higher than in controls (P<0.05). However, antioxidant capacity yielded at 90°C was remarkably lower than at 70°C. This is probably due to thermal degradation and oxidation of extract constituents such as anthocyanins which are sensitive to high temperatures (Cemeroglu et al., 1994; Kirca & Cemeroglu, 2003). Moreover, chemical transformations affecting the phenolic composition and, as a consequence, antiradical capacity of extracts will therefore be expected to occur. Phenols are known to combine themselves through polymerization reactions under high temperatures thus decreasing the availability of active hydroxyl groups able to scavenge free radicals (Pinelo et al., 2004; Vivar-Quintana et al., 1999).

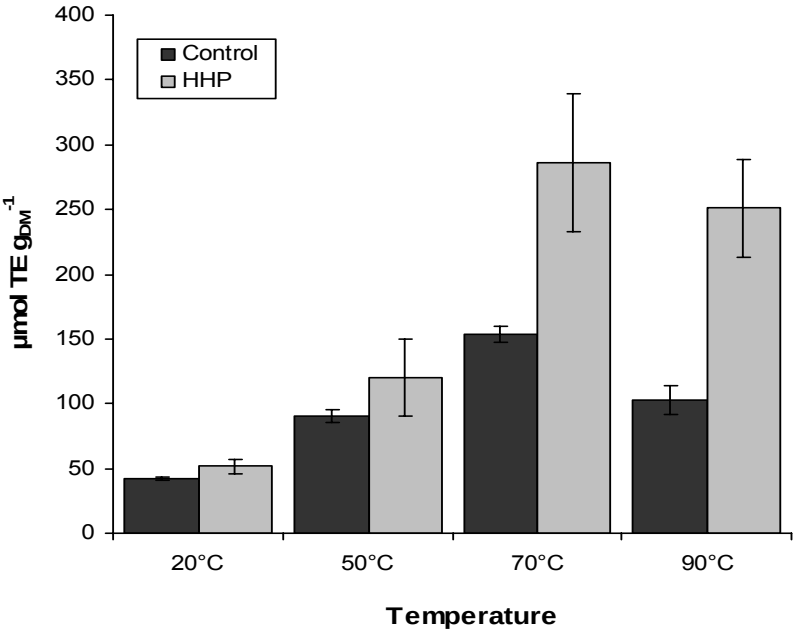


Figure 5.40: Effect of HHP (600 MPa) at different temperatures on the antioxidant capacity of Dornfelder *(V. vinifera* L.*)* grape pomace extracts expressed in µmol TEg_{DM}^{-1}. Extractions were carried out with an ethanol concentration of 50%.

As a result of the higher antioxidant capacity yielded at 70°C and 600 MPa, these conditions were selected for the experiments carried out at different ethanol concentrations. Ethanol was selected as extraction solvent based on previous studies where a mixture of alcohols and water enhanced the extraction of phenolic constituents from grape seeds compared to mono-component systems and due to its environmentally friendly characteristics (Alonso et al., 1991; Spigno et al., 2007). Indeed, the medium plays an important role not only in the extraction but also in the antioxidant capacity of the extracts. Pinelo et al. (2005), demonstrated that phenolic compounds presented a higher antioxidant capacity when they were dissolved in ethanol > methanol > water; ordered from less to high polarity (Lorimer, 1972). Similar results were obtained by van den Berg et al. (1999) who reported that the antioxidant capacity of quercetin in ethanol solution was nearly two-fold higher than that observed in the surfactant Triton X-100. OH- active groups spatial disposition seems to change according to the solvent used being more likely to scavenge radicals when dissolved in ethanol than in other organic solvents. In extractions assisted by HHP the recovery of antioxidants was remarkably higher than in control extractions (P<0.05). In the results represented in Figure 5.41 it is acknowledged that HHP increased the antioxidant recovery up to two-fold higher than control extractions and that the highest recovery was achieved under ethanol concentrations of 50-80% being of 286.08 µmol TE g_{DM}^{-1} and 306.61 µmol TE g_{DM}^{-1}, respectively (P<0.05).

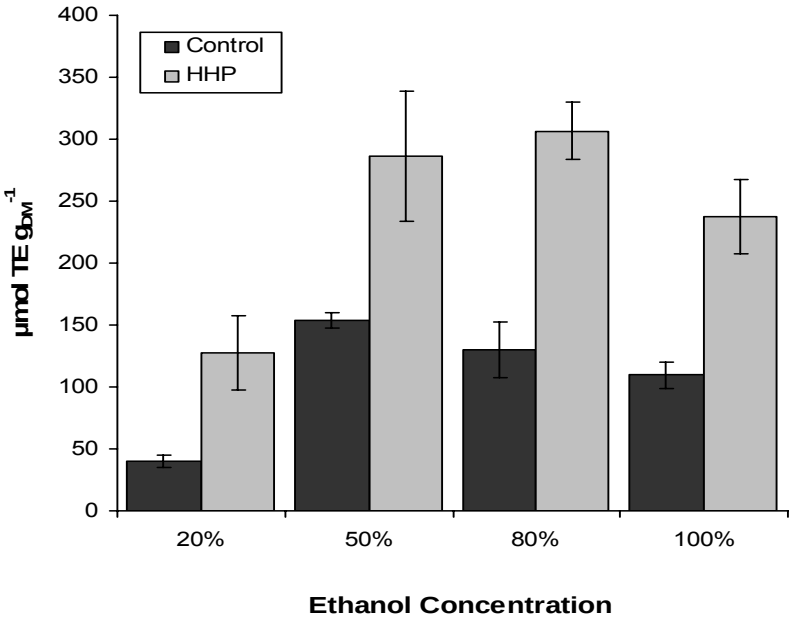


Figure 5.41: Effect of HHP and different ethanol concentrations on the antioxidant capacity of Dornfelder (*V. vinifera* L.*)* grape pomace extracts expressed in µmol TE g_{DM}^{-1}. Extractions were carried out at 70°C.

Besides the efficiency of an ethanol-water mixture, the combined effect of temperature and HHP enhanced the antioxidant recovery as it causes a decrease in the dielectric constant of water ε which leads to a decrease in the polarity. The dielectric constant of water at 20°C (80 ρ Kg^{-1} m^{-3}) is reduced by increasing temperature to 110-120°C (57 and 54 ρ Kg^{-1} m^{-3}, respectively at 10.1 MPa), which comes closer to the dielectric constant of a 60% methanol-water mixture of 50 ρ Kg^{-1} m^{-3} (Fernandez et al., 1997). This feature explains the fact that extraction yields under pressure were 70% of the maximal yields to be obtained through a conventional 60% methanol extraction. This decrease in polarity caused by the application of high hydrostatic pressure likely promotes the extraction of flavonols and anthocyanins from grape pomace, since latter compounds have a lower solubility in water (Metivier et al., 1980).

5.3.2 Effect of temperature and ethanol on anthocyanin extraction

Anthocyanins are the predominant secondary metabolites found in red grape pomace and contributed to the extract higher antioxidant capacity than white varieties (Figure 5.23). Different patterns of hydroxylation and glucosylation appear to modulate their antioxidant properties. -OH groups situated at $C_{3'}$ and $C_{4'}$ clearly increase the antioxidant capacity of the anthocyanins, as well as the glucoside group joined to the flavylium ring affect their antioxidant properties; 3-glucosylation in the C ring increased their oxygen radical absorbance capacity (ORAC) for glucose and rhamnoglucose whereas ORAC values decreased for galactose (Wang et al., 1997) (Figure 2.5). To get a closer insight into the

selective extraction of anthocyanins, their content in the different extracted samples were identified and quantified by means of HPLC-DAD/ESI-MS (Figure 5.42). Peak mass identification was simplified using a combination of retention time, peak spectra, mass-to-charge ratio and MS^2 fragmentation.

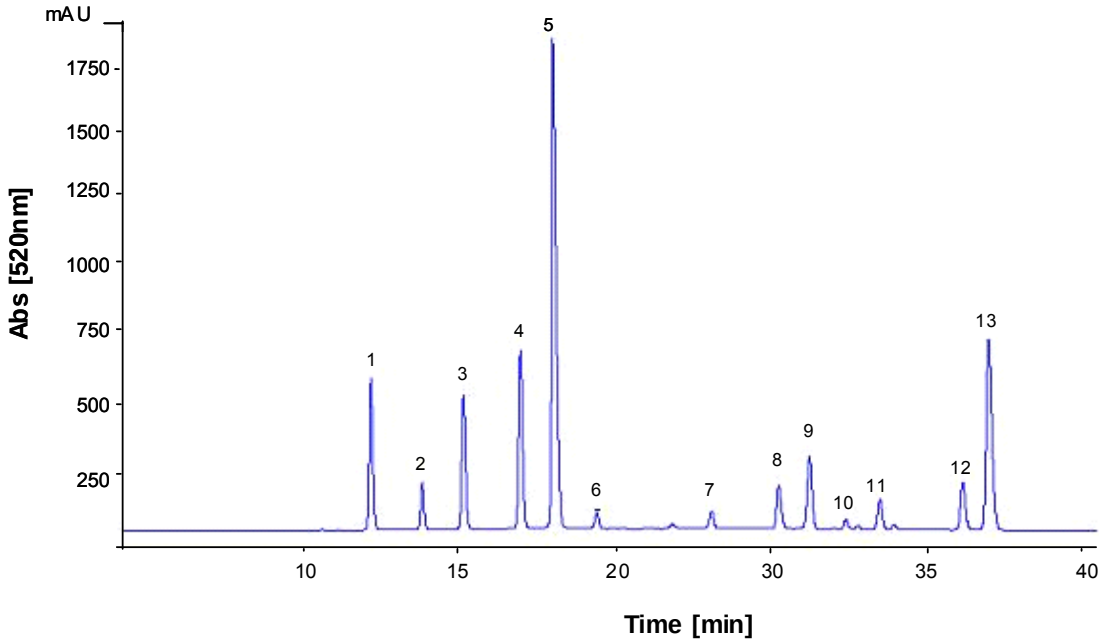


Figure 5.42: HPLC anthocyanin profile of Dornfelder (*V. vinifera* L.*)* extracts. Compounds: 1. Delphinidin-3-O-glucoside (Dl3gl), M^+ = 465; 2. Cyanidin-3-O-glucoside (Cy3gl), M^+ = 449; 3. Petunidin-3-O-glucoside (Pt3gl), M^+ = 479; 4. Peonidin-3-O-glucoside (Pd3gl), M^+ = 463; 5. Malvidin3-O-glucoside (Mv3gl), M^+ = 493; 6. Delphinidin-3-O-acylglucoside (Dl3acgl), M^+ = 507; 7. Petunidin-3-O-acylglucoside (Pt3acgl), M^+ = 521; 8. Peonidin-3-O-acylglucoside (Pd3acgl), M^+ = 505; 9. Malvidin-3-O-acylglucoside (Mv3acgl), M^+ = 535; 10. Cyanidin-3-O-p-coumaroylglucoside (Cy3pcmgl), M^+ = 595; 11. Petunidin-3-O-p-coumaroylglucoside (Pt3pcmgl), M^+ = 625; 12. Peonidin-3-O-p-coumaroylglucoside (Pd3pcmgl), M^+ = 609; 13. Malvidin-3-O-p-coumaroylglucoside (Mv3pcgl), M^+ = 639.

Anthocyanin recovery was overall higher when extractions were assisted by HHP. Extract anthocyanin content was not a good indicator to estimate antioxidant capacity of the samples and vice versa, since both parameters were not linear correlated. Likewise, Landrault et al. (2001) and Beer et al. (2003), did not find any correlation between anthocyanin content and $ABTS^{\bullet+}$ values for French and African wines, respectively.

Anthocyanins were selectively extracted according to pressure intensity (Table 5.7). The highest levels of total anthocyanin monoglucosides (8.91 ± 0.13 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$) were obtained at pressures of 200 MPa whereas pressures of 600 MPa were optimal for the extraction of acylglucosides, (4.27 ± 0.21 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$). Acyl groups increase molecule stability since at the pH of the samples ~3.5 acyl groups protect the pyrylium ring from the nucleophilic addition of water (Brouillard, 1983). Moreover, chemical structure differences

pointed out an anthocyanin selective extraction; p-coumaroylglucosides were better extracted than acylated glucosides and monoglucosides. Likewise, methoxyl and hydroxyl groups seemed to affect polarity and anthocyanin stability, since malvidin was extracted in higher amounts than peonidin > petunidin > delphinidin > cyanidin according to its higher number in methoxyl and hydroxyl groups and content in grapes (Figure 2.7). Extraction time was not a crucial parameter to improve anthocyanin extraction (Table 2 in appendix).

Table 5.7: Effect of HHP on the anthocyanin recovery ($mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$) from Dornfelder (*V. vinifera* L.*)* grape pomace.

Compound	Control	200 MPa	400 MPa	600 MPa
		Anthocyanin monoglucosides		
Dp3gl	0.31 ± 0.01[a]	0.40 ± 0.02[a]	0.34 ± 0.01[a]	0.30 ± 0.03[a]
Cy3gl	0.26 ± 0.01[a]	0.30 ± 0.01[a]	0.28 ± 0.02[a]	0.28 ± 0.01[a]
Pt3gl	0.35 ± 0.02[a]	2.02 ± 0.01[a]	0.39 ± 0.02[a]	0.36 ± 0.05[a]
Pd3gl	0.87 ± 0.18[a]	1.86 ± 0.01[a]	1.27 ± 0.05[a]	1.87 ± 0.41[a]
Mv3gl	2.09 ± 0.61[a]	4.33 ± 0.10[a]	2.99 ± 0.14[a]	3.31 ± 0.40[a]
Total Ant3gl	**3.88 ± 0.82[a]**	**8.91 ± 0.13[a]**	**5.27 ± 0.20[b]**	**6.12 ± 0.92[b]**
		Acylated anthocyanin glucosides		
Dp3acgl	0.27 ± 0.01[a]	0.29 ± 0.01[a]	0.28 ± 0.01[a]	0.28 ± 0.07[a]
Pt3acgl	0.28 ± 0.01[ab]	0.30 ± 0.01[ab]	0.29 ± 0.03[b]	0.29 ± 0.07[a]
Pn3acgl	0.31 ± 0.01[b]	0.34 ± 0.01[b]	0.32 ± 0.01[b]	0.38 ± 0.17[a]
Mv3acgl	0.38 ± 0.02[b]	0.48 ± 0.02[a]	0.40 ± 0.03[b]	0.47 ± 0.04[a]
Cn3pcmgl	0.28 ± 0.01[a]	0.30 ± 0.01[a]	0.29 ± 0.01[a]	0.32 ± 0.00[a]
Pt3pcmgl	0.34 ± 0.01[a]	0.36 ± 0.02[a]	0.35 ± 0.01[a]	0.38 ± 0.01[a]
Pn3pcmgl	0.38 ± 0.01[a]	0.45 ± 0.03[a]	0.41 ± 0.03[a]	0.69 ± 0.02[a]
Mv3pcmgl	0.54 ± 0.04[a]	0.79 ± 0.08[a]	0.54 ± 0.01[a]	1.46 ± 0.09[a]
Total Antacgl	**2.78 ± 0.08[a]**	**3.31 ± 0.02[a]**	**2.88 ± 0.02[a]**	**4.27 ± 0.21[b]**
Total Content	**6.66 ± 0.93[a]**	**12.22 ± 0.15[a]**	**8.15 ± 0.22[a]**	**10.39 ± 1.13[a]**

Values represent mean ± standard deviation, n=3. Different letters in rows indicate significant differences among mean values of treatments (P<0.05).

The optimal temperature for anthocyanin recovery under pressure was 50°C (11.58 ± 2.01 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$), whereas yields obtained at 70°C and 90°C were clearly lower (Table 3 in appendix). Extraction temperatures higher than 70°C cause rapid degradation and discolouration of anthocyanins (Cemeroglu et al., 1994; Kirca & Cemeroglu, 2003) and this thermal degradation has been reported to follow a first order reaction (Markakis et al., 1957; Keith & Powers, 1965; Ioncheva & Tanchev, 1974; Rhim, 2002; Ahmed et al., 2004; Tseng et al., 2006).

When ethanol concentration was varied in samples heated under pressure, the higher the ethanol concentration, the higher was the anthocyanin recovery. The total anthocyanin recovery was 32.8 ± 7.6 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$ at an ethanol concentration of 100% (Table 4 in appendix) which confirms results reported by Spigno et al. (2007) where the advantages of

pure ethanol solutions for anthocyanin extraction were reported. Anthocyanins were extracted thereby in approx. 70% from their original content in grape pomace (extracted by 60% methanol in a ratio 1:20). Yields obtained by HHP extractions were similar to those achieved by Ju and Howard (2003) through a pressurised liquid extraction at 10.1 MPa with a 0.1% HCl aqueous solution and to those obtained with subcritical water at a temperature ranged between 120-150°C (Ju & Howard, 2005). In addition, extractions assisted by HHP were approx. 10 to 12-fold higher than those obtained by a conventional extraction (Revilla et al., 1998). A significant higher recovery was also observed when compared to extractions carried out by enzymatical digestion where only 8.6% from the total anthocyanin content in the original samples was extracted (Kammerer et al., 2005). These comparisons highlight the feasibility of high hydrostatic pressure for anthocyanin extraction. However, an accurate comparison may involve similar grape varieties and solvents.

5.3.3 Advantages of emerging technologies for extraction purposes

High hydrostatic pressure in combination with temperature is an efficient methodology for the extraction of bioactive compounds from grape pomace according to a sustainable development. Besides high hydrostatic pressure (HHP), pulsed electric fields (PEF) and ultrasonics belong to the environmentally friendly and energy efficient technologies being able to enhance mass transfer processes within plant or animal cellular tissues, as the permeability of cytoplasmatic membranes can be affected (Dörnenburg & Knorr, 1993; Töpfl, 2006). In this chapter the feasibility of different emerging technologies such as pulsed electric fields, ultrasonics and high hydrostatic pressure for extraction purposes is compared and discussed.

5.3.4 Extract antioxidant capacity as a function of the methodology

As a result of the previous experiments an ethanol concentration of 50%, a temperature of 70°C and a HHP treatment intensity of 600 MPa were chosen for an optimal extraction (Knorr, 1994; Lebovka et al., 2004). However, extraction times were prolonged to one hour, since antioxidant capacity yields were slightly higher at longer holding times (Chapter 5.3.1). Results represented in Table 5.8 show the higher content in soluble solids and moisture loss when extractions were assisted by ultrasonics, HHP and PEF compared to control extractions.

Table 5.8: Variation of moisture removal and soluble solids uptake of control extractions and those assisted by HHP, PEF and ultrasonics.

Extraction	Moisture (g 100 g^{-1} fresh grape pomace)	Soluble solids (° Brix)
Raw Matter	96.19 ± 0.03[a]	---
Control	80.12 ± 1.81[b]	15.2 ± 1.03[b]
Ultrasonics	78.47 ± 0.23[b]	16.7 ± 2.19[b]
HHP	77.44 ± 1.63[b]	18.3 ± 0.30[b]
PEF	73.65 ± 2.65[b]	17.2 ± 0.03[b]

Values represent mean ± standard deviation, n=3. Different letters in columns indicate significant differences among mean values of treatments (P<0.05).

Extraction yields were expressed in form of the total phenolic content and antioxidant capacity. As a result of the extraction treatments, it is also to be acknowledged that some other substances with antioxidant capacity were extracted and better determined by $ABTS^{\bullet+}$ radical assay. These may include anthocyanin condensation and complexation products, vitamins, aminoacids, minerals and synergistic effects among them. The total phenolic content of the samples extracted with different methods PEF, HHP and ultrasonics was not significantly different (P>0.05) but in comparison to the control samples (Figure 5. 43).

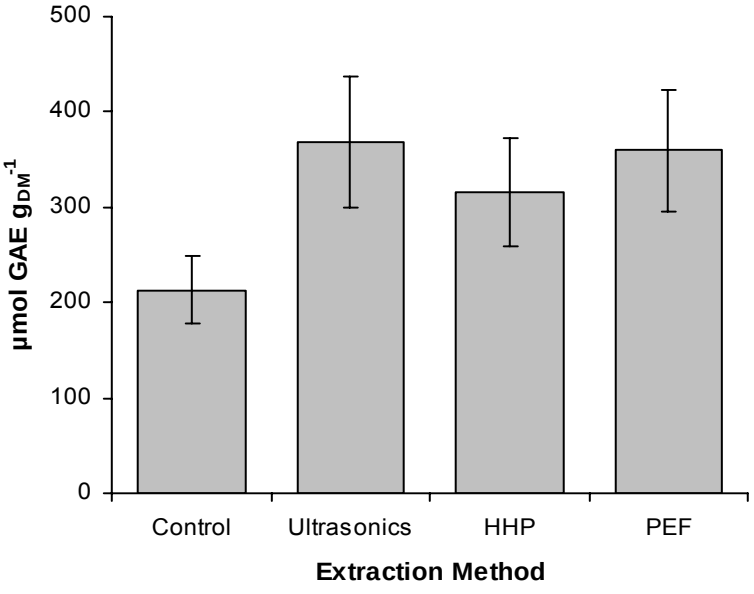


Figure 5.43: Comparison of extract total phenolic content (µmol GAE g_{DM}^{-1}) from Dornfelder (*V. vinifera* L.) grape pomace as a function of the methodology.

Extractions assisted by PEF, HHP and ultrasonics increased phenolic compounds recovery approx. two-fold higher than the control extraction (P<0.05). On the contrary, the results obtained from the antioxidant capacity expressed in µmol TE g_{DM}^{-1} were significantly different among the different treatments used (P<0.05). The highest antioxidant content (784.34 ± 150.41 µmol TE g_{DM}^{-1}) was obtained in the PEF treated samples followed by HHP (548.49 ± 47.97 µmol TE g_{DM}^{-1}), ultrasonics (308.13 ± 46.54 µmol TE g_{DM}^{-1}) and the control (187.13 ±

28.45 µmol TE g_{DM}^{-1}) (Figure 5.44). The maximal extraction attained by PEF was a 75% of the possible total extraction to be obtained.

The use of electrical fields of 3 kV cm^{-1} seems to cause irreversible pore formation in plant membranes increasing the extractability of polyphenols by the release of solutes into the solvent. In addition, PEF provides the possibility of inactivating degrading enzymes which may explain the higher yields in antioxidant capacity compared to the other methods (Figure 5.44). In support to these results, Estiaghi and Knorr (2000) reported that the nutritional content of fruit juices as well as the number of intracellular compounds from grapes during wine production was enhanced when applying an external electrical field in a range of 1–3 kV cm^{-1}.

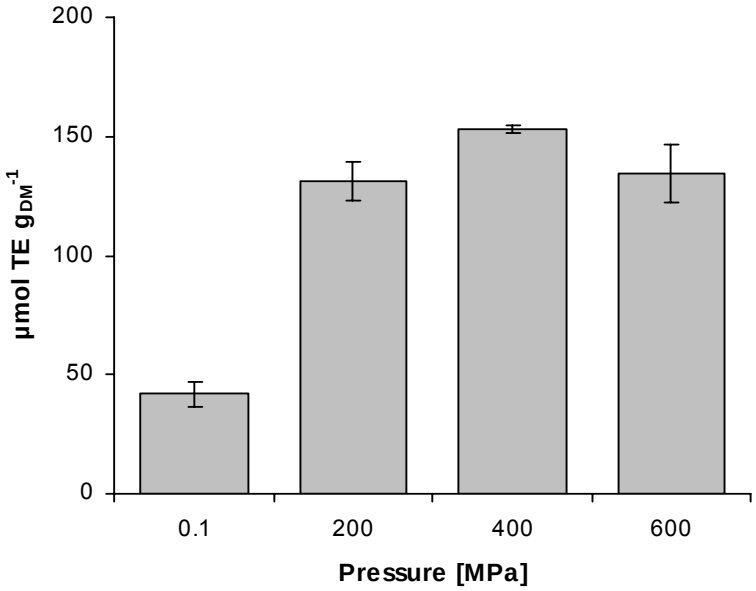


Figure 5.44: Antioxidant capacity (µmol TE g_{DM}^{-1}) of Dornfelder (*V. vinifera* L.) grape pomace extracts as a function of the methodology.

Similarly, HHP increased the extraction yields due to its potential to increase cell permeability as previously reported in the chapter 5.3.1. Ultrasonics extraction also improved the extraction yields compared to the control samples; however, the effect of ultrasonics on antioxidant recovery was less pronounced than for PEF and HHP. The active part of ultrasonics inside the extractor is restricted to a zone located in the vicinity of the ultrasonic emitter. Hence, its lower extraction efficiency in comparison to the other methods studied (Wang & Weller, 2006).

5.3.5 Anthocyanin extraction yields as a function of the methodology

Table 5.9 shows the extraction yields in total anthocyanins, total anthocyanin monoglucosides and acylated glucosides obtained from the differently treated samples. PEF

increased the anthocyanin extraction yields up to 10% in comparison to HHP and up to 17% compared to the conventional extraction whereas the differences between the control samples and those extracted with ultrasonics were not significantly different (P>0.05). These results are in agreement with the studies of Töpfl (2006) in which a higher extraction of anthocyanins from purple fleshed potatoes treated with PEF was described. When a glucose was linked covalently to the OH- at C_3 (anthocyanin monoglucoside), the extraction was 10.25 ± 1.18 $mg_{Cy3glequ.}\ g_{DM}^{-1}$ in extractions assisted by PEF, whereas the control, HHP and ultrasonicated treated samples were 7.4 ± 0.1 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$, 6.05 ± 0.92 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$ and 4.85 ± 0.73 $mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$, respectively.

Table 5.9: Influence of the different extraction methods at 70°C and 50% ethanol on the individual anthocyanin recovery ($mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$) from Dornfelder (*V. vinifera* L.) grape pomace.

Compound	Control	Ultrasonics	HHP	PEF
	Anthocyanins monoglucosides			
Dl3gl	0.40 ± 0.016[a]	0.33 ± 0.008[a]	0.47 ± 0.229[a]	0.43 ± 0.017[a]
Cy3gl	0.30 ± 0.002[a]	0.27 ± 0.003[a]	0.33 ± 0.073[a]	0.37 ± 0.061[a]
Pt3gl	0.48 ± 0.002[a]	0.39 ± 0.017[a]	0.66 ± 0.034[a]	0.95 ± 0.456[a]
Pn3gl	4.22 ± 0.017[a]	1.06 ± 0.125[a]	2.90 ± 0.333[a]	6.08 ± 0.204[a]
Mv3glu	2.06 ± 0.061[ab]	2.79 ± 0.577[b]	1.68 ± 0.260[ab]	2.42 ± 0.447[a]
Total Ant3gl	**7.46 ± 0.098[b]**	**4.85 ± 0.73[b]**	**6.05 ± 0.929[b]**	**10.25 ± 1.185[a]**
	Acylated Anthocyanin monoglucosides			
Dl3acgl	0.28 ± 0.001[a]	0.28 ± 0.002[a]	0.74 ± 0.597[a]	0.30 ± 0.001[a]
Pt3acgl	0.30 ± 0.008[a]	0.29 ± 0.003[a]	0.32 ± 0.032[a]	0.31 ± 0.005[a]
Pn3acgl	0.35 ± 0.002[a]	0.32 ± 0.007[a]	0.39 ± 0.097[a]	0.36 ± 0.017[a]
Mv3acgl	0.50 ± 0.005[a]	0.41 ± 0.018[a]	0.62 ± 0.326[a]	0.54 ± 0.054[a]
Cy3pcmgl	0.30 ± 0.006[a]	0.30 ± 0.009[a]	0.32 ± 0.023[a]	0.31 ± 0.003[a]
Pt3pcmgl	0.38 ± 0.004[a]	0.35 ± 0.004[a]	0.43 ± 0.053[a]	0.38 ± 0.008[a]
Pn3pcmgl	0.53 ± 0.012[a]	0.39 ± 0.044[a]	0.59 ± 0.168[a]	0.51 ± 0. 038[a]
Mv3pcmgl	1.17 ± 0.053[a]	0.58 ± 0.115[a]	1.70 ± 0.222[a]	1.08 ± 0.217[a]
Total Ant3acgl	**0.48 ± 0.091[a]**	**2.91 ± 0.202[a]**	**5.15 ± 1.518[a]**	**3.79 ± 0.343[a]**
Total Content	**7.93 ± 0.189[a]**	**7.76 ± 0.932[a]**	**11.21 ± 2.447[b]**	**14.05 ± 1.528[b]**

Values represent mean ± standard deviation, n=3. Different letters in rows indicate significant differences among mean values of treatments (P<0.05).

PEF remarkably enhanced the extraction of anthocyanin monoglucosides. Acylated glucoside anthocyanins seemed to be physically entrapped within the matrix, or form hydrogen bonds with cell wall polysaccharides and were consequently extracted in less proportion. The highest anthocyanin recovery was obtained by PEF and corresponded to a 60% of the maximal recovery which was obtained by a 60% methanol extraction, solid/liquid ratio of 1:20 and longer holding times.

The improvement in the extractability of individual anthocyanins was according to anthocyanin substitution pattern described in the previous chapter 5.3.2. The higher content in OH- and OCH_3- and acyl groups, the higher was the stability and higher extraction. These

results are according to studies of Bassa and Francis (1987) and Giusti and Wrolstad (1999) where the higher stability of acylated anthocyanins under other intrinsic factors such as temperature and light was reported.

The application of advanced technologies such as HHP, PEF, and ultrasonics offer an extraordinary potential and selectivity for extraction purposes. These technologies enhanced the antioxidant capacity of the samples up to four-fold higher than control extractions at 70°C and extracted anthocyanins selectively. However, the multiplicity of process dimensions when they are combined with conventional process variables such as temperature, time, change in pH, solid/liquid ratios should be further studied in order to optimize the whole process for a future industrial implementation. The combination of effective extraction technologies, low-cost raw materials and environmentally friendly solvents represent a sustainable and economical alternative to conventional extraction methods where large amounts of organic solvents and energy consuming long extraction times are required. The use of novel processing technologies will thereby reduce food processing wastes and facilitate the availability of natural ingredients which guarantee food sustainability and meet consumer demands.

5.4 Impact of high hydrostatic pressure on anthocyanins

High hydrostatic pressure (HHP) has been demonstrated a promising method for extraction purposes in the previous chapter. In addition, high hydrostatic pressure can be used for microbial inactivation avoiding undesirable changes such as vitamin loss and taste or colour modifications (Rastogi et al., 2007). Thus, HHP seems a promising method for wine pasteurisation avoiding or reducing the use of SO_2. SO_2 is usually used as an antiseptic against undesirable microorganisms and as an antioxidant in wines (Amerine et al., 1967). It inactivates grape enzymes such as polyphenoloxidases by reducing their copper cofactor which negatively influences the quality of juices and derivatives. Furthermore, SO_2 can have negative effects on human health (Romano & Suzzi, 1993). However, pressure as a thermodynamic factor influences chemical reactions and is expected to influence also anthocyanin condensation reactions in wine. As a result, pressure/temperature conditions at which anthocyanin degradation occurred, and anthocyanin cyclic derivatives (vitisin A derivative) are formed, were here reported. Moreover, the feasibility of HHP to pasteurise wines was further discussed.

5.4.1 Stability of cyanidin-3-O-glucoside under pressure

For a closer knowledge of the influence of high hydrostatic pressure on anthocyanins; model solutions of cyanidin-3-glucoside (Cy3gl) dissolved in ethanol were subjected to different heat/pressure treatments and their stability was studied. Cy3gl is the predominant anthocyanin found in red raspberries, blackberries and blood orange but is also present in wines and grape pomace extracts (Kirca & Cemeroglu, 2003; Rommel et al., 1992; Amico et al., 2004). Experiments carried out at different high hydrostatic pressure intensities at 20°C and 30°C did not affect the stability of Cy3gl after 0.5 h treatment. Although when temperature was risen up to 50°C a higher degradation of Cy3gl could be observed up to 400 MPa (Figure 5.45) (P<0.05).

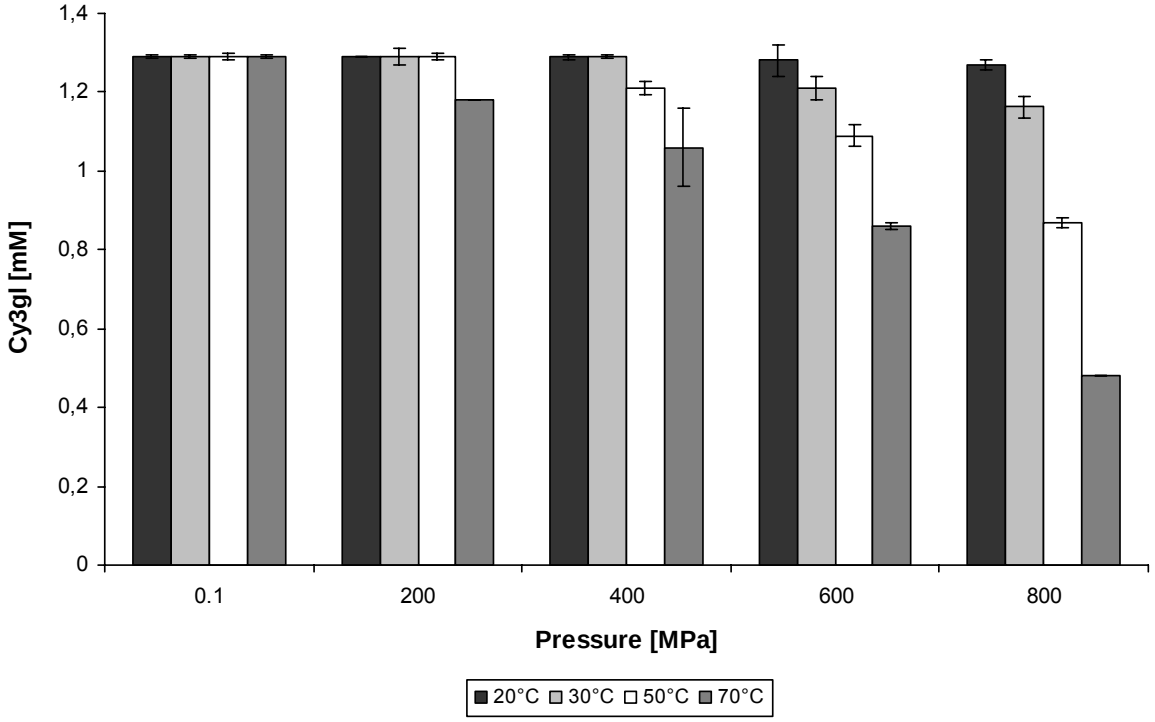


Figure 5.45: Effect of high hydrostatic pressure intensity (MPa) and temperature (°C) on Cy3gl degradation. Treatment holding time: 0.5 h.

The higher the pressure and the temperature, the higher was the degradation of Cy3gl. In addition, longer holding times also increased Cy3gl degradation rate. The degradation of Cy3gl fitted well a first-order reaction of the form:

$$\ln\left(Ct/Co\right) = -kt \qquad \text{(Eq. 5.10)}$$

Where *Ct* was the concentration at *t* time, *Co* initial concentration and *k* reaction constant. The half life could be determined from the slope by the expression:

$$T_{1/2} = -\ln 0.5 / k \qquad \text{(Eq. 5.11)}$$

Cy3gl degradation rate constant in heat-pressurised samples (70°C, 600 MPa) was 0.33 h^{-1} whereas in heated samples (70°C) was only 0.02 h^{-1}. The half life ($T_{1/2}$) in heat-pressurised samples was 0.38 h and 5.77 h for only heated samples. The latter values are within the range determined by Kirca and Cemeroglu (2003) for blood orange juice at 70°C; 2.0 h for 69 °Brix concentrate and 6.3 h for 11.2 °Brix concentrate. The half life of the reaction in heat-pressurised samples was remarkably shorter than in heated ones which indicate a faster Cy3gl degradation.

When ln[*k*/*k*o] was plotted *vs.* different pressures (Figure 5.46) the activation volume could be determined following Eq. 2.3.

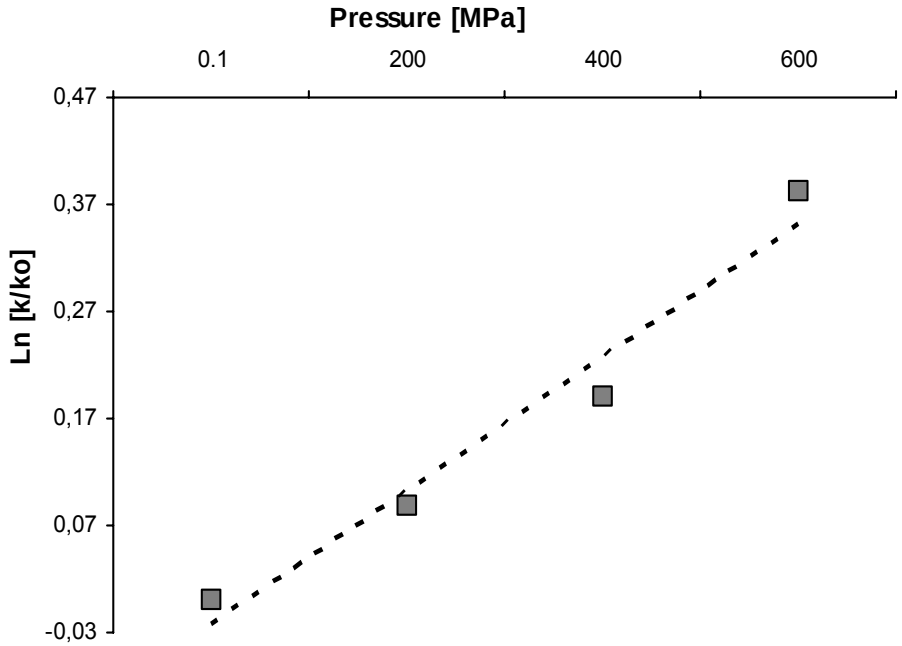


Figure 5.46: Effect of high hydrostatic pressure on the degradation rate of Cy3gl in 50% ethanol model solutions. Treatments carried out at 70°C for 0.5 h.

The activation volume at 70°C was -2.8 cm^3 mol^{-1} which shows the low participation of pressure in this equilibrium. Chemical reactions mainly influenced by high hydrostatic pressure have activation volumes which range from -20 cm^3 mol^{-1} to less values (Millero et al., 1972; Kitamura & Itoh, 1985). Hence, the decisive role of the combination of both parameters; temperature and pressure in the results herein achieved.

According to Cy3gl degradation, some hydrolytic products could be identified by HPLC-DAD/ESI-MS. The concentration of hydrolytic products was remarkably higher when samples were treated at 600 MPa, 70°C for 0.5 h and even higher at 800 MPa, 70°C for 0.5 h when the highest disappearance of Cy3gl was also determined. As a result of the degradation, 9 hydrolytic products could be found and identified by HPLC-DAD/ESI-MS (Figure 5.47).

One of the main components from the degradation had a mass of $[195 + H]^+$ which corresponded to a first deglucosidation of the flavilium moiety yielding a chalcone which further transformed into a coumarin derivative with a loss of the B-ring (Figure 2.10).

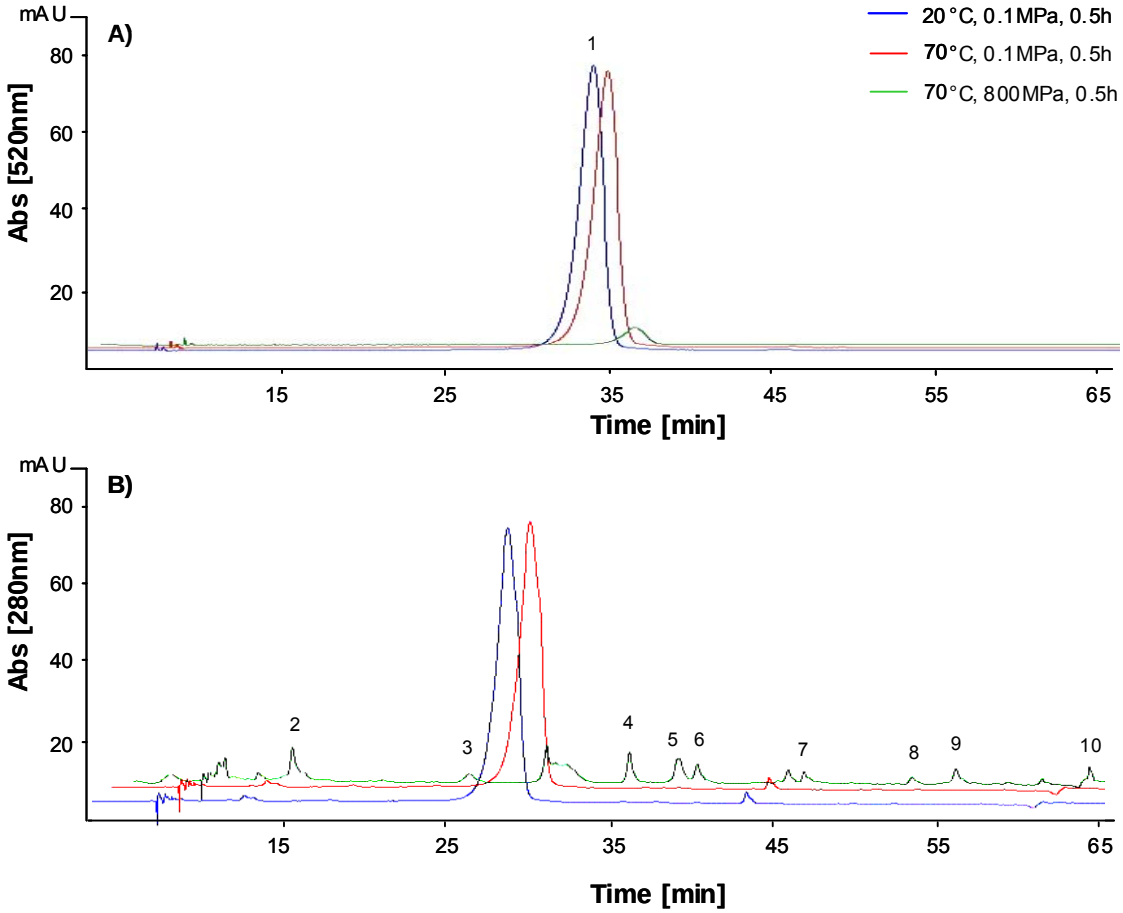


Figure 5.47: A): HPLC-DAD Chromatogram of Cy3gl at different heat/pressure conditions at 520 nm. 1. Cy3gl, M^+ = 449 and B): 280 nm. 2. M^+ = 195; 3. M^+ = 217; 4. M^+ = 319; 5. M^+ = unknown; 6. M^+ = 545; 7. M^+ = 464; 8. M^+ = 301; 9. M^+ = 348; 10. M^+ = unknown.

Quercetin $[301 + H]^+$ was also identified as a results of Cy3gl degradation which is according to studies reported by Tanchev and Ioncheva (1976). Other compounds such as phloroglucinaldehyde and protocatechuic acid also indicated in the literature for anthocyanin thermal degradation were not found. Due to the very low concentration of newly formed peaks in the chromatograms an accurate identification of further products could not be discerned. Results here achieved suggest the formation of other degradation products different from those obtained in heat treatments. Only NMR experiments might indicate the chemical structure and possible degradation pathways of Cy3gl under a heat/pressure treatment.

5.4.2 Synthesis of anthocyanin-derived compounds under pressure

The influence of high hydrostatic pressure on the synthesis of Cy3gl derived products was here investigated. HHP specifity for synthesis depends on negative volume changes, compounds´ concentration and medium characteristics. For this purpose, model solutions containing Cy3gl and pyruvate in excess were subjected to different heat/pressure treatments in acid pH (acetate buffer pH 4.4) since acidic conditions are indispensable for anthocyanin condensation reactions to occur (Es-Safi et al., 1999).

Anthocyanin condensation reactions involve the formation of a new pyran ring by cyclo-addition. Such cyclo-addition reactions cause a hypsochromic shift in the visible absorption maxima of the anthocyanins producing a change in wine colour towards orange hues (Rivas-Gonzalo et al., 1995; Hayasaka & Asenstorfer, 2002). According to these studies, model solutions containing Cy3gl and pyruvate were subjected to different heat and/or pressure processes and the variations in the absorbance spectrum were studied. No changes in the absorbance spectrum of model solutions were found at 25°C nor under pressures of 0.1 MPa, 200 MPa and 600 MPa even after 6 h treatment. However, at 70°C, differences in the absorbance spectrum could be observed. Absorbance differences were remarkable according to increased temperature and holding time. After a heat and/or pressurised treatment of 1.5 h, the main absorbance peak in Cy3gl untreated samples at 520 nm (dark violet), slightly decreased when samples were heated at 70°C. Meanwhile, the absorbance of pressurised samples at 600 MPa and 70°C showed a high decrease in the band at 520 nm and an increase at 350-370 nm (pale-rose) (Figure 5.48).

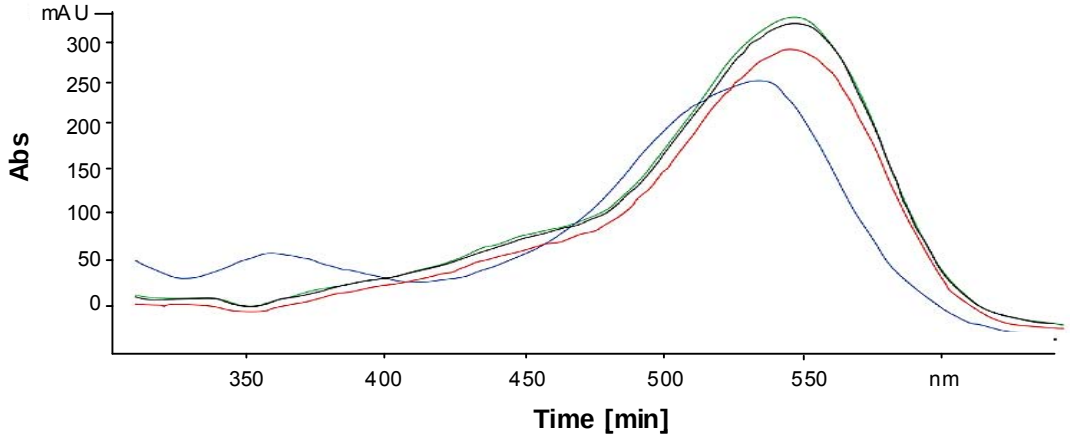


Figure 5.48: UV-Vis spectra of Cy3gl and pyruvate in acetate buffer at 20°C and 0.1 MPa (black); at 70°C and 0.1 MPa (red); at 20°C and 600 MPa (green) and at 70°C and 600 MPa (blue). Treatments holding time 1.5 h.

According to spectra analysis, the analytical experiments carried out by HPLC-DAD/ESI-MS showed the formation of a new peak when samples were pressurised at 600 MPa and 70°C up to 0.5 h whose mass was [517 + H^+] (Figure 5.49). The mass and absorbance here obtained was in agreement with the theoretical mass and absorbance reported for anthocyanin-pyruvic adducts structurally similar to vitisin A. Fragmentation analysis MS^2 showed one major ion [355 + H^+]. This ion corresponded to the anthocyanidin pyruvic adduct by the loss of the glucose.

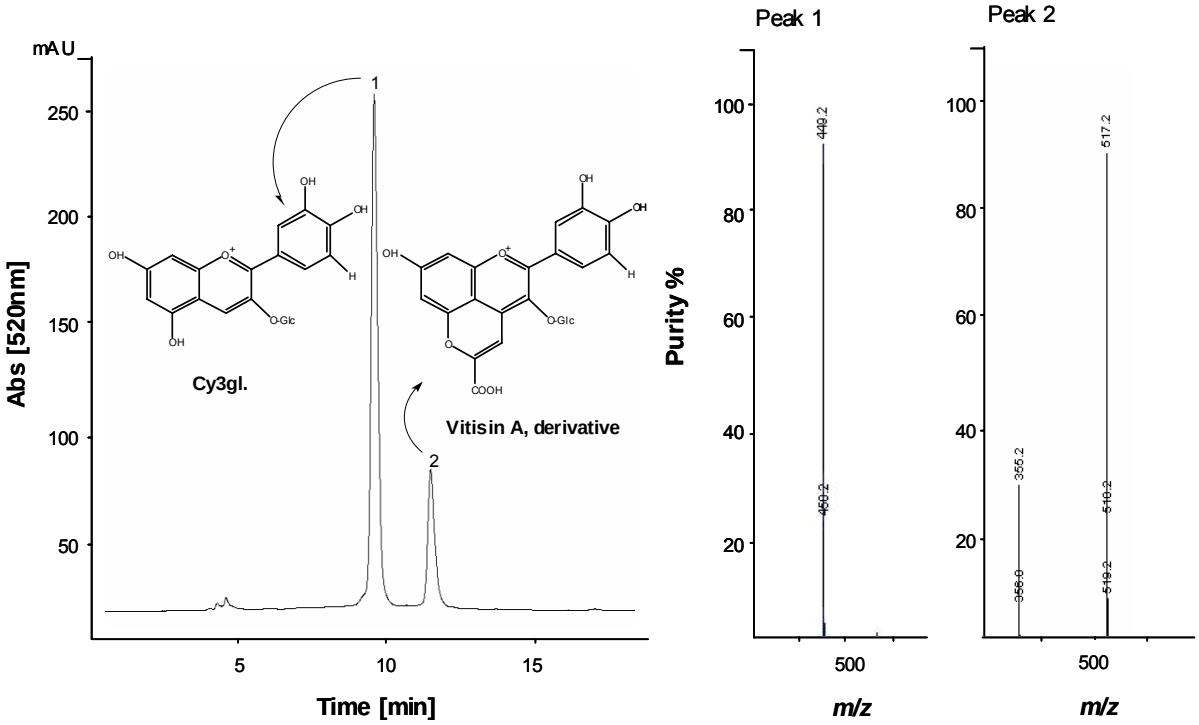


Figure 5.49: HPLC and MS^2 spectra of Cy3gl and pyruvate in acetate buffer at 600 MPa and 70°C held during 1.5 h.

The product obtained matches the features of vitisin A-type derivatives from Rioja wines described by Alcalde-Eon et al. (2006). The cyclo-additon reaction in model solutions involved the reaction of the nucleophilic hydroxyl at C_5 of Cy3gl and its electrophilic C_4 upon the double bond of the enolic form of pyruvate followed by the loss of a water molecule and further oxidation (Figure 5.50) (Schwartz et al., 2003). The appearance of this new peak was directly related to a decrease in the initial concentration of Cy3gl when samples were heated under pressure. No significant spectral changes in samples pressurised at 200 MPa at 70°C after 3 h compared to heated controls were observed (P>0.05).

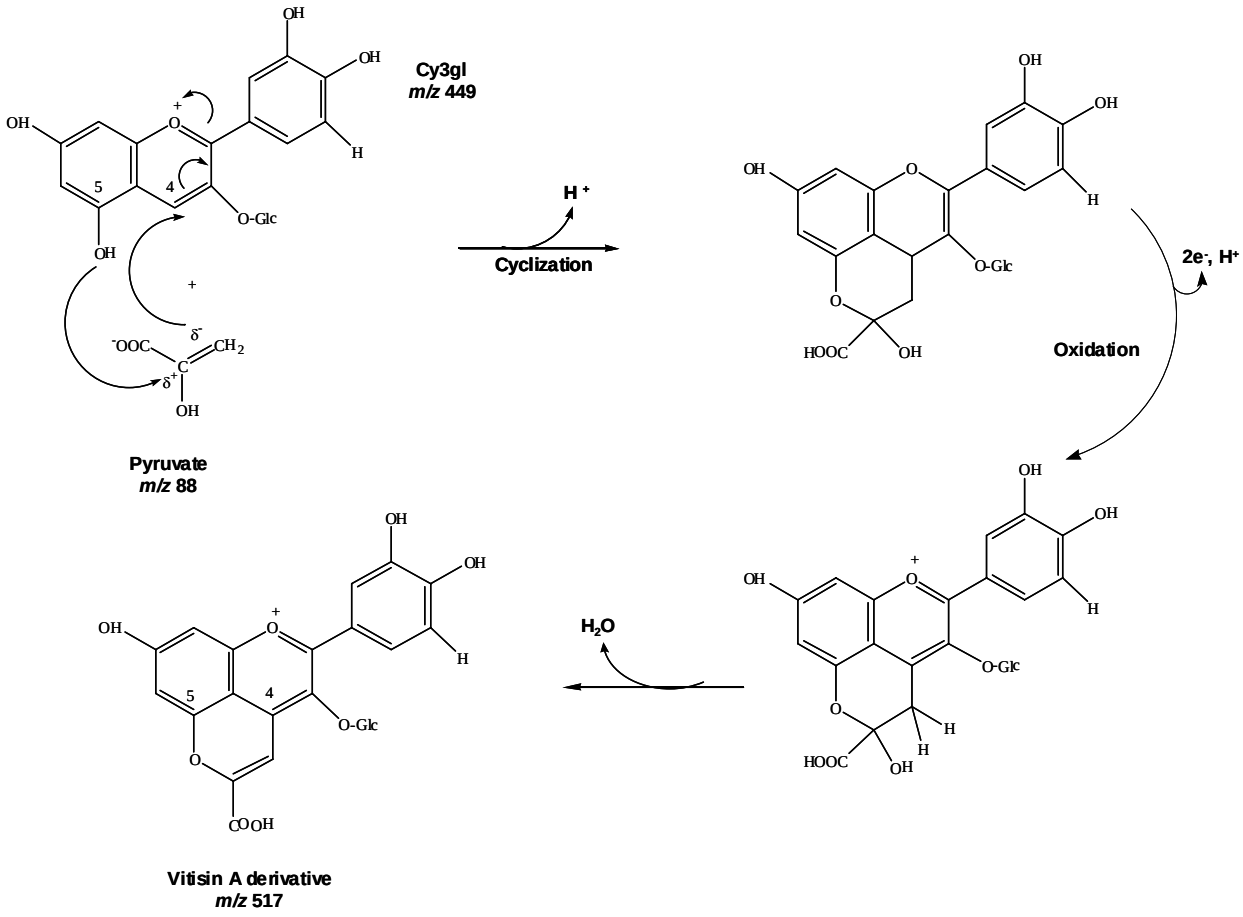


Figure 5.50: Condensation reaction of Cy3gl and pyruvate in heated/pressurised samples.

5.4.2.1 Degradation kinetics of cyanidin-3-O-glucoside under heat/ pressure

According to spectral results, no significant concentration changes were found when samples were subjected to different HHP treatments (200 MPa, 600 MPa) at 25°C. In addition, Cy3gl concentration loss was not significantly different between samples subjected to a HHP process at 200 MPa and 70°C and heated samples throughout the experiment (data not shown). On the contrary, when samples were subjected to a pressure of 600 MPa and a temperature of 70°C, concentration changes were detected as a function of the treatment time and there was a significant degradation of Cy3gl (P<0.05) (Figure 5.51). After subjecting samples to holding times of 0.5 h, a loss of ~25% of Cy3gl was determined. Also, Cy3gl was degraded when samples were heated at 70°C for 0.5 h; however the degradation was only ~5%. The logarithmic representation of Ln[Ct/Co], where C is the concentration of Cy3gl at time t and Co is the cyclic adduct concentration at time 0 *vs.* time (t, in hours), followed a first order kinetic (R^2 = 0.99).

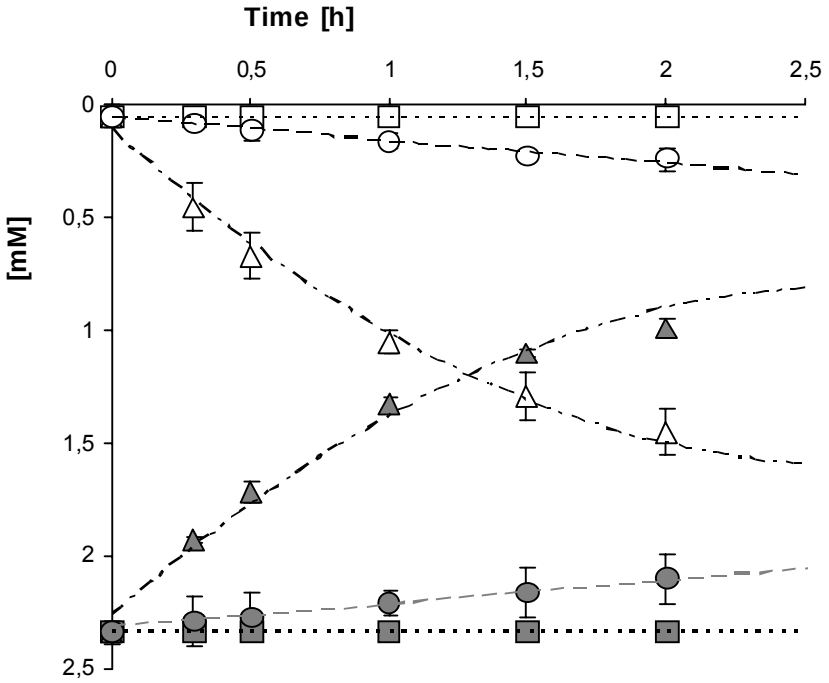


Figure 5.51: Degradation and formation rate of Cy3gl and vitisin A derivative, respectively under different temperature/pressure treatments in acetate buffer. □ Cy3gl, 20°C, 0.1 MPa; ○ Cy3gl, 70°C, 0.1 MPa; ∆ Cy3gl, 70°C, 600 MPa; ■ Vitisin A derivative, 20°C, 0.1 MPa; ● Vitisin A derivative, 70°C, 0.1 MPa; ▲ Vitisin A derivative, 70°C, 600 MPa.

The reaction constant (k) could be calculated from the slope. Results showed that the use of a combined temperature/pressure treatment accelerated anthocyanin degradation being the constant of the reaction k=0.79 h^{-1} clearly higher than in heated samples where the reaction constant was k=0.05 h^{-1}.

These results are according to previous thermal studies carried out by Cemeroglu et al. (1994) and Kirca and Cemeroglu (2003) in which it was pointed out a first-order kinetic thermal degradation of anthocyanins in cherry and blood orange juices. According to statistical analysis, the interaction of HHP and temperature enhanced reaction yields and their influence was significantly different (P<0.05). The influence of temperature and time was also significantly different (P<0.05). On the contrary, the effect of pressure and time did not show any interaction as their combined effect was not significantly different (P>0.05). The effect of a combined temperature/pressure treatment on anthocyanin condensation reactions was remarkable. In this occasion the activation volume of the reaction under pressure at 70°C was -33.3 cm^3 mol^{-1}, which pointed out the significant effect of pressure on anthocyanin condensation reactions. When samples were subjected to HHP and a temperature of 70°C during longer holding times (6 h), a significant decrease in the concentration of Cy3gl was observed. Cy3gl was degraded by nearly 53%. Simultaneously, a predominant peak under HPLC-DAD/ESI-MS was detected. The molecular ion was [594 + H^+], which indicates the

formation of a new compound of higher molecular weight. However, its accurate chemical structure could not be discerned by HPLC-DAD/ESI-MS. Thermal degradation of Cy3gl after 6 h was around 25%.

5.4.2.2 Antioxidant capacity of heated/pressurised samples

The estimation of the antioxidant capacity of the samples subjected to different treatments showed a decrease as a function of treatment holding times (Figure 5.52). The formation of cyclic-adducts led to a loss of an active OH- group in the meta-position of the A ring from the flavilium cation molecule mainly responsible of the loss in the antiradical capacity (Rice-Evans et al., 1996). The 3- and 5- OH groups in A and C rings with 1-oxo function are required for a maximum radical scavenging potential (Figure 2.5).

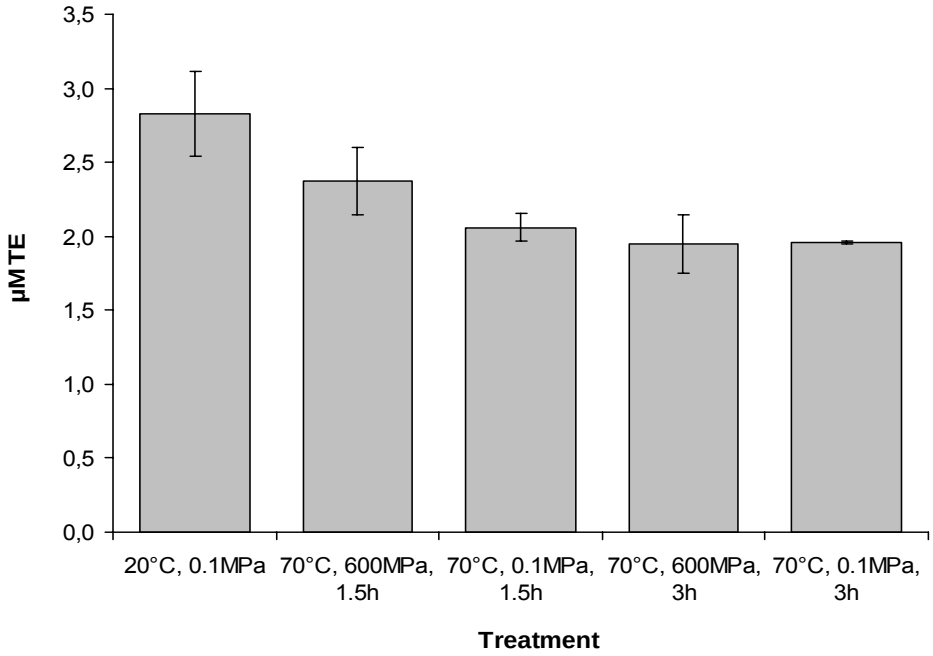


Figure 5.52: Antioxidant capacity of Cy3gl and pyruvate in acetate buffer subjected to different heat/pressure conditions during different holding times.

A heat/pressure combined treatment enhanced the formation of cyclic-adducts, since the loss of Cy3gl was directly correlated to an increase of the formed vitisin A-type derivative. By contrast, when samples were heated for 1.5 h, a stronger loss of antioxidant capacity was determined, but a lower concentration of vitisin A-type derivative was quantified.

This may be explained by the instability of anthocyanins under high temperature. The degradation of anthocyanins has been postulated to firstly deglucosilate forming a chalcone which yields different benzoic acid derivatives (Seeram et al., 2001; Salidova et al., 2006). Also, trihydroxybenzaldehyde has been identified as an end product of the thermal degradation of anthocyanins (Furtado et al., 1993) (Figure 2.10). The formation of these

degradation products causes the rupture of the 3, 4 double bond in conjugation with the 1-oxo function in the C ring which is responsible for electron delocalization from the B ring. Antioxidant capacity is related to structure in terms of electron delocalization of the aromatic system. When anthocyanins react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus (Rice-Evans et al., 1996). This degradation pathway might also explain the small absorbance decrease at 520 nm, as previously observed in Figure 5.48. However, in heated samples other condensation reactions are also likely to occur. Garcia-Alonso et al. (2004) demonstrated that the antioxidant capacity of old wines decreased according to anthocyanin degradation and formation of more condensation products and oligomers.

5.4.3 Effect of heat/pressure on anthocyanin-rich food matrices

For a better understanding of the influence of combined temperature/pressure treatments on food matrices, wine and grape pomace extracts from Dornfelder grape variety, were subjected to a pressure of 600 MPa at 70°C for 1 h. The stability of the predominant anthocyanins in wine was monitored and determined by HPLC-DAD/ESI-MS. Figure 5.53 represent the chromatograms of wine before and after a heat and heat/pressure treatment at a wavelength of 520 nm and 370 nm, respectively. A decrease in the concentration of Mv3gl (peak 3) on pressurised samples was detected, whereas in the heated samples no loss of Mv3gl was observed. Chromatograms taken at a wavelength of 370 nm showed an increase in the concentration of several peaks of higher molecular weight such as; a: [948 + H^+], b: [643 + H^+] and c: [922 + H^+] when samples were heated under pressure. The spectrometric estimation of anthocyanin monomers content in solution tended to decrease when samples were heated and/or pressurised for 1 h. The monomer content was 216.7 ± 1.51 $mg_{Cy3gl\ equ.}$ L^{-1} in control samples, 179.3 ± 0.86 $mg_{Cy3gl\ equ.}$ L^{-1} in samples heated at 70°C, and 158.6 ± 0.78 $mg_{Cy3gl\ equ.}$ L^{-1} in heat-pressurised samples (70°C, 600 MPa). These results confirmed that the degradation of anthocyanin monomers in heat-pressurised wine samples occurred. However, results were not significantly different from untreated and heated samples (P>0.05). In addition a decrease in predominant peaks of Dornfelder grape pomace extracts after a heat-pressurised treatment (70°C, 600 MPa) for 1 h was observed. According to this decrease, an increase of some peaks was monitored at 370 nm (Figure 1 in appendix). Accurate masses could not be determined because of compounds' low purity. In extract samples, the monomer content decreased when heated and heat-pressurised. Monomer content was 224.26 ± 20.53 $mg_{Cy3gl\ equ.}$ L^{-1} in control samples, 207.73 ± 17.21 $mg_{Cy3gl\ equ.}$ L^{-1} in heated samples at 70°C and 162.14 ± 10.62 $mg_{Cy3gl\ equ.}$ L^{-1} in heat-pressurised samples (70°C, 600 MPa).

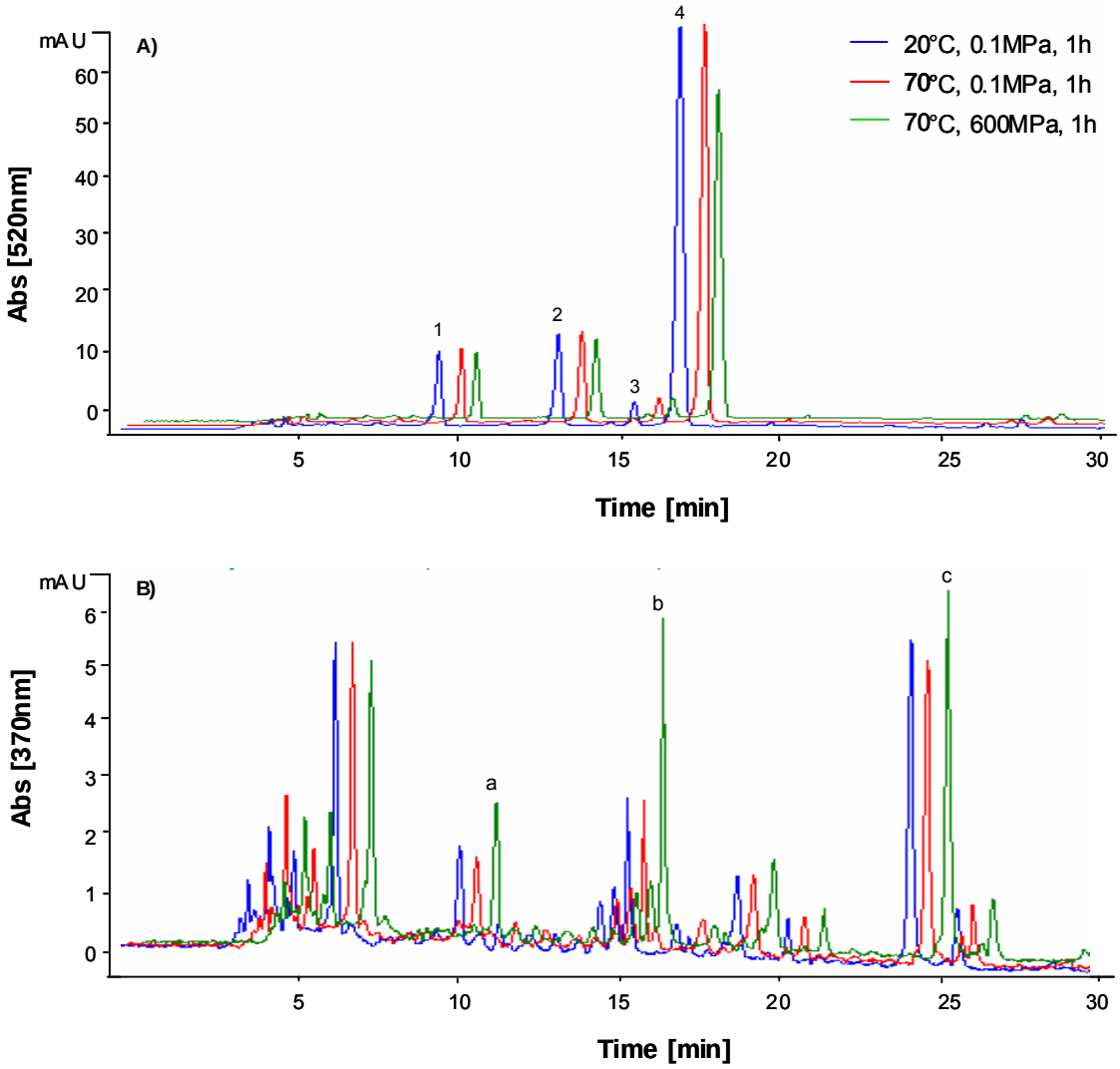


Figure 5.53: A): HPLC-DAD Chromatograms of Dornfelder wine samples at 520 nm and B): 370 nm. Untreated wine samples at 20°C and 0.1 MPa (blue). Samples at 70°C and 0.1 MPa held during 1 h (red); and samples treated at 70°C and 600 MPa held during 1 h (green). Compounds: 1. Dl3gl, M^+ = 465; 2. Cy3gl, M^+=449; 3. Pt3gl, M^+ = 479; 4. Mv3gl, M^+ = 493; Unidentified products: a. M^+ = 948, b. M^+ = 643, c. M^+ = 922.

The degradation of anthocyanin monomers in extracts under HHP significantly occurred (P<0.05) and condensation reactions in the extracts were more likely to occur due to a higher anthocyanin concentration in the samples. The complexity of the matrix offers an infinity of interactions possible to occur among constituents which complicates an accurate identification and quantification of yielded products. Only NMR characterization of heated/pressurised foods might identify the preference in synthesis of one product over another and their influence on product quality.

When wine and extract samples were subjected to a mild pasteurisation conditions (600 MPa, 70°C for 10 min), no significant differences in anthocyanin composition and antioxidant capacity of the samples were found (P>0.05). These results are according to works of Mok et

al. (2006), in which it was demonstrated that physico-chemical characteristics of wines did not change after pasteurisation processes at pressures which ranged from 100 MPa to 400 MPa at 25°C during times between 5-30 min. In addition, after HHP treatments at 350 MPa, 25°C for 20 min the inactivation of aerobic and acid lactic bacteria and yeasts were found. Moreover, studies of Puig et al. (2003), pointed out that a HHP treatment of 500 MPa for 5 min reduced wine bacterial population in 99.99% without altering chemical or organoleptic properties of wine. The induction of chemical reactions in wines by HHP and temperature reported here only occurred after times above 1 h. These reactions are not expected to occur under commercial HHP pasteurisation conditions where pressures range from 400 MPa to 700 MPa, temperatures are not higher than 40-50°C and holding times are not higher than few minutes. Long holding times and high temperatures are critical parameters for anthocyanin condensation reactions to occur under pressure. Pasteurisation conditions studied here ensure the biochemical and microbial stability of wine and represent an alternative to SO_2 normally applied for vinification. As previously mentioned, SO_2 is widely used in winemaking as an antioxidant and bacteriostatic agent. SO_2 behaves as a powerful nucleophile when dissolved and is capable of bonding covalently with electrophiles such as pyruvate and acetaldehyde to form adducts e.g. vitisin A. Vitisin A derivatives contribute to the redness of old wines. However, SO_2 can affect wine fermentation by supporting the development of resistant and undesired microorganisms which affect organoleptic characteristics of wine (Romano & Suzzi, 1993). Thus, through the use of HHP for wine pasteurisation, the addition of SO_2 could be lowered or even avoided which may also be desirable for public health (Lueck, 1980).

5.5 Development of pressure-, temperature- and time- indicators

The need of monitoring parameters conditions during processing, storage and transport are crucial for maintaining process traceability and food quality. Up to now, many time-temperature- indicators (TTIs) have been developed to control critical points related to exposition to heat during the food production chain. Time- and temperature- indicators are markers that mimic the bacterial growth or the behaviour of quality attributes as a function of treatment time and temperature. Several pressure-, temperature-, and time- indicators (PTTIs) have been proposed (van der Plancken et al., 2008), but so far no recommendations or a wide acceptance have been reported. An indicator for a certain food process should be a simple accurate device responding irreversibly to defined conditions. An indicator might be relevant to check aspects related to the safety of food, but could also be a good alternative to monitor quality related characteristics, or process uniformity. Here the heat/pressure

dependent colour degradation of solutions with type Q coenzymes in the presence of potassium sorbate has been investigated for a promising implementation as PTTIs.

5.5.1 Sorbic and coenzyme Q_o reactivity and kinetics

Sorbic acid, and its mineral salts, such as 2,4-hexadienoic acid potassium salt, are natural organic compounds commonly employed as food preservatives, since they prevent the growth of microorganisms, moulds and yeasts. Moreover sorbic acid has a structure able to react as a diene in [4+2] cycloadditions (Diels-Alder reactions), and several reactions with dienophiles involving sorbic acid have been described in the literature (Khandelwal & Wedzicha, 1997). Ubiquinones, or coenzymes of the Q type, are critical components in electron transport systems in the mitochondria. They are of extreme importance to the energy-producing systems of any cell. All ubiquinone types have a common 1,4-benzoquinone group able to act as a dienophyle in [4+2] cyclo-additions (Farina & Valderrama, 1995). As a result, a Diels-Alder reaction between both components is expected and their corresponding Diels-Alder adducts are depicted in Figure 5.54.

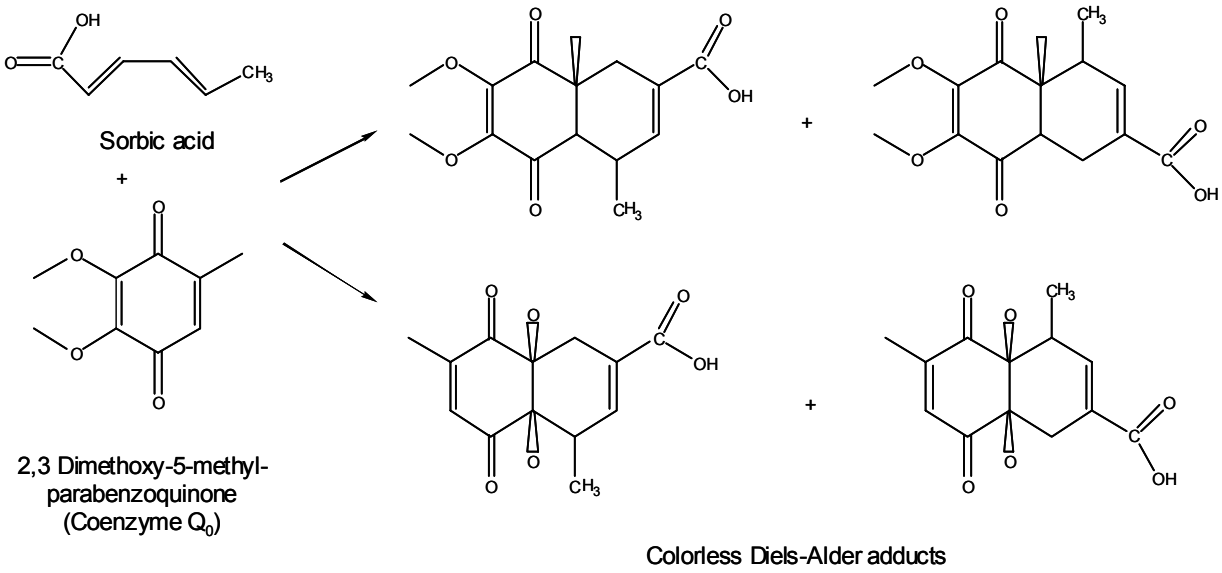


Figure 5.54: Hypothesised reaction pathways for the cyclo-addition between sorbic acid and coenzyme Q_0.

Detailed kinetic studies on the stability of coenzyme Q_0 were carried out over the temperature range 40°C to 80ºC. The linearised Arrehnius equation described the heat dependency of the degradation of coenzyme Q_0 in the presence of sorbate. The heat induced degradation of coenzyme Q_0 followed a first order kinetic (Figure 5.55). Therefore the activation energy could be calculated from the slope of the plot ln[*k*] *vs.* the reciprocal of the absolute temperature, and the value was 3.7 KJ mol^{-1}. The activation energy of the

investigated cyclo-addition was quite low, demonstrating the slight temperature dependency of the reported cyclo-addition and the good perspectives for this indicator to be used in high pressure assisted thermal sterilisation processes, where the sample has to be heated up to 70°C to 90ºC.

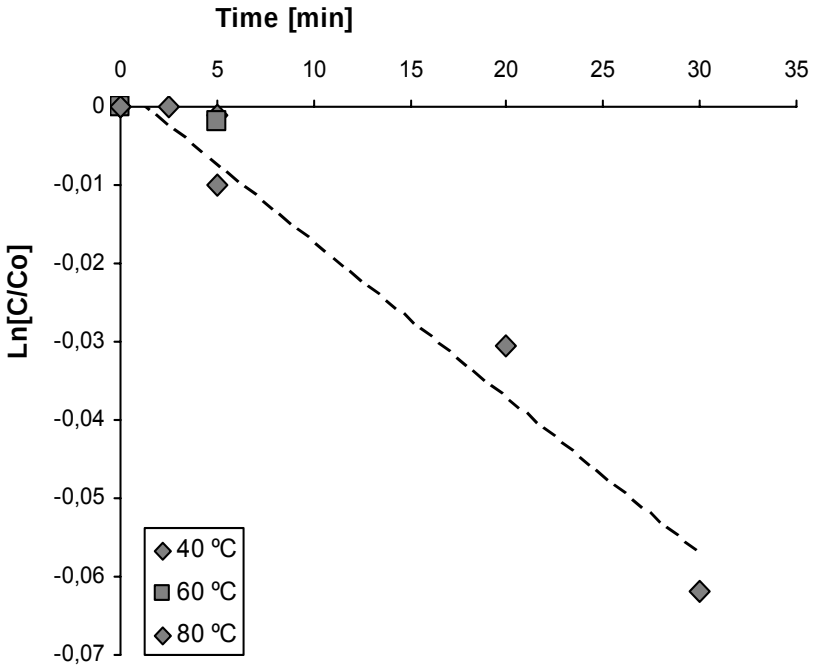


Figure 5.55: Thermal degradation of coenzyme Q_0 at 0.1 MPa (0.5:0.5 mM:mM; coenzyme Q_0:potassium sorbate) in water.

First attempts were performed to prove the pressure and heat stability of coenzyme Q_0 under extreme conditions. After 30 min treatment at 800 MPa and 80ºC, the initial yellow colour of the solution was preserved up to 72%. These results showed that coenzyme Q_0 itself might be degraded up to a certain extent under high pressure. However the colour loss observed was far below than when sorbate was present in the mixture, remarking that the hypothetised cyclo-addition took place. The combination of pressure and heat accelerated notably the Diels-Alder reaction, leading to a loss of colour (Figure 5.56).

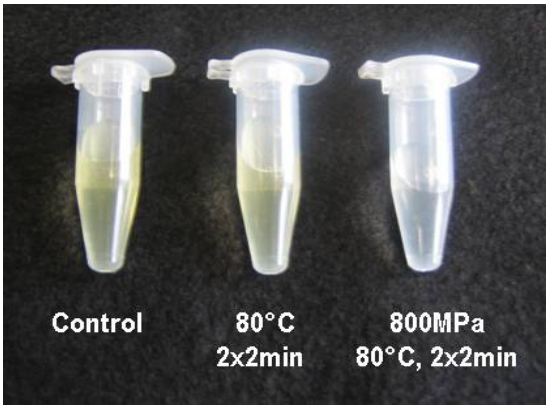


Figure 5.56: Colour change of coenzyme Q_0 and sorbate at heat/pressure conditions (1:2 mM:mM coenzyme Q_0: potassium sorbate) in water.

At a constant temperature, the reaction occurred faster with increasing pressure. The plot of ln [C/Co] *vs*. time represented in Figure 5.57 shows the strong pressure dependence of Diels-Alder reactions. The degradation of coenzyme Q_0 was quantified and could be described by a first order kinetic. The reaction activation volume was ~ -15 cm^3 mol^{-1} which indicates the major impact of pressure to accelerate the degradation of coenzyme Q_0 in the presence of sorbate.

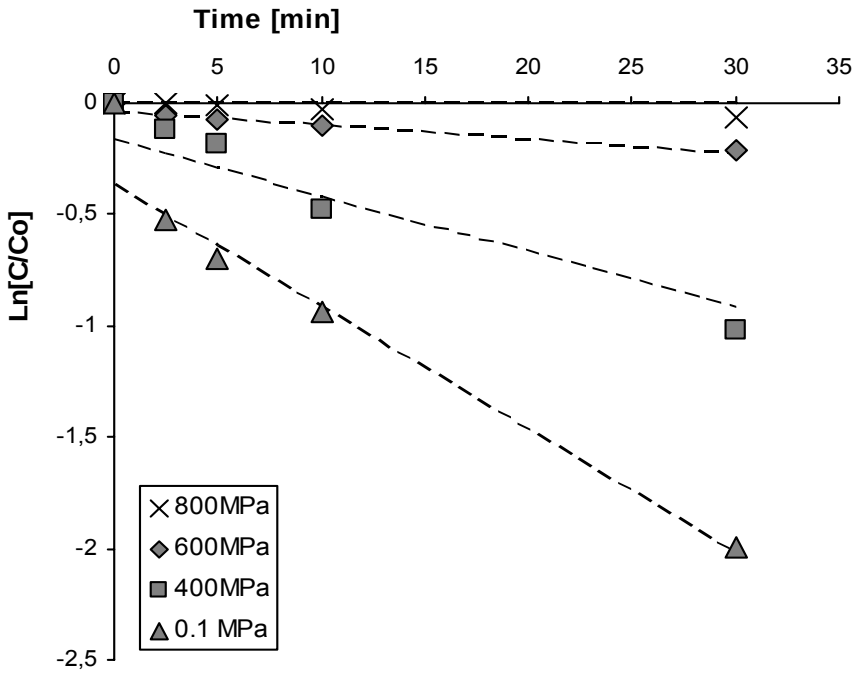


Figure 5.57: Degradation kinetics of coenzyme Q_0 pressurised at 0.1 MPa, 400 MPa, 600 MPa and 800 MPa, respectively, for varying treatment time, at 80°C in water (0.5:0.5 mM:mM; coenzyme Q_0:potassium sorbate).

Increasing pressures and temperatures increased reaction kinetic rates as represented in Table 5.10. However, reaction kinetics could be also modulated by reactants concentration changes.

Table 5.10: Reaction rate constants between sorbate and coenzyme Q_0 under isobaric isothermal conditions, estimated from a first order kinetic model.

	Reaction rate k values (10^{-3} min^{-1})			
	40ºC	**R^2**	**80ºC**	**R^2**
0.1 MPa	1.3	0.99	1.9	0.94
400 MPa	1.0	0.95	6.1	0.98
600 MPa	6.1	0.91	32.2	0.98
800 MPa	14.6	0.97	57.4	0.98

Experiments with 0.5 mM coenzyme Q_0 produced uncoloured samples at higher pressures, even after 2.5 min treatment. Different sorbate concentrations were tested in order to obtain colourless solutions at determined heat/pressure treatments. The higher the concentration of sorbate, the fastest was the reaction rate (colourless solution). Coenzyme Q_0 concentrations tested during this work seemed to limit the reaction rate as a slight deceleration was observed specially at longer pressurisation times.

This work showed that the pressure-induced degradation of coenzyme Q_0 was sensitive to pressure, temperature and treatment time. Hence, an application as an indicator for process uniformity and as an extrinsic PTTI seems feasible. A major benefit of the use of such a coloured indicator is its simplicity. Concentrations of both compounds can be systematically selected to reveal the pressure and temperature achieved in single positions during the pressure assisted thermal pasteurisation/sterilisation processes. Additionally, the response to pressure can be modulated to selected ranges where food safety and quality are crucial. Furthermore, the proposed system contains non-toxic chemicals and could be easily encapsulated within the packaging material, and thus become a simple device for the monitoring of the high pressure processing conditions.

6 CONCLUDING REMARKS AND PERSPECTIVES

Grape pomace extracts (GPE) from conventional and organic farming are an economical source of polyphenols with incalculable value as antioxidants or antibacterials. The content in polyphenols varied according to the genuine raw material (white and red grape pomace or seeds). Red GPE were an important source of anthocyanins (Figure 5.29) while seed extracts (GSE) possessed high amounts of proanthocyanidins (Figure 5.30) and their content did not depend on their organic or conventional farming practices ($P>0.05$). By contrast the content in polyphenols (Figure 5.27) and the antioxidant capacity (Figure 5.23) of white GPE from organic or conventionally managed land were significantly different ($P<0.05$). These results suggested a higher susceptibility of white grape epidermis to external factors, e.g. pesticides which may influence phenolic metabolism. GPE inhibited the growth of pathogenic Gram-positive such as *L. monocytogenes, S. aureus, E. faecium and E. faecalis* (Table 1 in appendix) and moulds such as *P. chrysogenum, P. expansum, A. niger, T. viride* and *A. versicolor* (Figure 5.33, Figure 5.34 and Figure 5.35). A higher inhibition was exerted by conventional GPE. As a result, pesticide loads in grape pomace were estimated (Table 5.1) and their presence in conventional extracts, i.e. extraction was confirmed (Table 5.2). Pesticide traces found in the extracts lied below the permitted levels in grapes. They do not pose a risk for human health but influence extract antibacterial properties. According to the established ADI values, the risk to suffer health disorders by ingesting these extracts in elaborated products is negligible. However, further research into extract ingestion limits and their adverse or beneficial effects on human health must be undertaken.

Based on these studies, extracts from grape seeds, which presented the highest antioxidant and antibacterial activities were incorporated into bioactive pea-starch food packaging films. Tensile strength in GSE films was half reduced, whereas the elasticity and oxygen permeability increased (Table 5.4 and Table 5.5). In addition, they showed a different colour attributed to GSE polyphenols. GSE films were bacteriostatic against *B. thermosphacta* surface loads in meat during the initial four days of storage at 4°C (Figure 5.38). The antibacterial effect observed, was according to a higher polyphenol release observed during the first days. A complete polyphenol migration was not observed. Polyphenols might be physically entrapped or retained among amylose helix of the films which avoided their release and long-term bacterial inhibition. Thus, further research into an increase of particle release and bacterial inhibition must be carried out.

Due to the increasing consumer demand for anthocyanins as natural colorants, antioxidants or nutraceuticals, the extraction of anthocyanins from red grape pomace assisted by HHP

was optimised. Parameters such as time, pressure intensity, ethanol concentration and temperature showed a remarkable effect on extraction yields. The maximal antioxidant activity was achieved when samples were extracted at 70°C, 50% ethanol concentration and pressures up to 200 MPa (Figure 5.40). Moreover, a temperature of 50°C and an ethanol concentration of 100% were the optimal conditions for anthocyanin extraction (Table 3 appendix). Anthocyanins could be selectively extracted according to pressure intensities; monoglucosides yields were higher at 200 MPa whereas acylglucosides were optimal extracted at 600 MPa (Table 5.7). Holding time was not a crucial parameter for anthocyanin extraction efficiency.

Extractions assisted by HHP were compared to other customary advanced technologies such as PEF, and ultrasonics. Extractions carried out by emerging technologies increased the antioxidant activity of the extracts four-fold, three-fold and two-fold higher than the control when extractions were carried out with PEF, HHP and ultrasonics, respectively (Figure 5.44). In addition, the extraction of individual anthocyanins was also studied showing a selective extraction based on the glucose moieties linked to the anthocyanidins; Anthocyanin monoglucosides were better extracted by PEF, whereas the acylated ones were better extracted by HHP. Acylated groups provided stability and protection to anthocyanin molecules under HHP enhancing their extraction (Table 5.9).

The feasibility of PEF and HHP to cause cell disruption and to enhance anthocyanin extraction was demonstrated. The higher yields obtained in extractions carried out by high hydrostatic pressure and pulsed electric fields are of major interest from industrial point of view, since solvent amounts might be reduced and extraction times shortened. Thus, the combination of emerging technologies for extraction purposes and low-cost raw materials represent an economical alternative to traditional extraction methods according to industry demands and a sustainable development. However, each extraction must be further evaluated according to a multitude of process dimensions such as temperature, time, change in pH, solid/liquid ratios to maximise efficiency. An economical evaluation of each choice based on the possible value of the final product, will determine its industrial implementation.

HHP as a thermodynamic factor can influence chemical reactions in foods. The influence of high hydrostatic pressure on anthocyanin model solutions was here for the first time studied. Cyanidin-3-O-glucoside (Cy3gl) dissolved in ethanolic model solutions was sensitive to different heat/pressure treatments. Up to treatments of 400 MPa, temperatures around 70°C and times over 30 min Cy3gl degradation significantly occurs (P<0.05). However, the activation volume was -2.8 cm^3 mol^{-1} which pointed out the minor participation of pressure in anthocyanin degradation reactions. On the contrary, when acetate buffer solutions containing Cy3gl and pyruvate were subjected to different pressure/temperature treatments, a

condensed anthocyanin-pyruvic-adduct was identified (Figure 5.49). The activation volume of the reaction was –33.3 cm^3 mol^{-1} indicating the remarkable influence of HHP to accelerate anthocyanin conden-sation reactions. Anthocyanin-pyruvic-adducts yielded from these reactions presented a different hypsochromical shift from that of genuine anthocyanins towards orange hues. They are also precursors of high polymerised anthocyanins with different colour ranges and functional properties which may be of relevance from an industrial or nutritional point of view.

Taking into account the crucial utilization of HHP as pasteurisation, the stability of anthocyanins in two food matrices: wine and red grape extracts from Dornfelder (*V. vinifera)* after different heat/pressure treatments was also studied. When heat/pressure treatments were applied during long holding times (3-6 h) the degradation of identified anthocyanins was determined as well as a higher concentration in more condensed products was observed (Figure 5.53). The complexity of the matrices enables an infinity of interactions possible to occur among food constituents which complicates an accurate identification and quantification of yielded products. Only NMR characterization of heated/pressurized foods might identify the preference in synthesis of one product over another and their influence on product quality. On the contrary, the effect of high hydrostatic pressure on anthocyanin food matrices was negligible under mild pasteurisation conditions (600 MPa, 70°C, 10 min). From a chemical point of view, the feasibility of HHP for wine pasteurisation was demonstrated. Thus, undesirable effects of sulphur dioxide on wines and on consumer health may be reduced or avoided. However, experiments regarding organoleptic changes of pressure pasteurised wines have to be further investigated.

In addition, for traceability of pasteurisation/sterilisation processes a pressure-, temperature-, time- indicator (PTTI) was developed based on a cyclo-addition reaction which involved sorbate and coenzyme Q_0, both non-toxic substances of natural occurrence. The initial solution possessed a strong yellow colour which changed towards a colourless one after determined heat/pressure treatments. This colour decrease was related to the formation of colourless Diels-Alder adducts from the reaction between coenzyme Q_0 and sorbate. The negative activation volume -15 cm^3 mol^{-1} of the reaction indicated the major participation of pressure (Figure 5.57). Pressure/temperature ideal processing conditions could be monitored by a simple colour change becoming thus a feasible device for monitoring processes assisted by high pressure.

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APPENDIX

Table 1: Inhibition of foodborne pathogens by white Riesling and red Spätburgunder grape pomace and Riesling seed extracts (*V. vinifera* L.).

	Conventional GPE									**Organic GPE**								
	White			**Red**			**Seed**			**White**			**Red**			**Seed**		
Concentration *(w/v)*	20%	10%	5%	20%	10%	5%	20%	10%	5%	20%	10%	5%	20%	10%	5%	20%	10%	5%
L. monocytogenes Scott A	+	+	-	+	-	-	+++	++	++	+	+	-	++	++	+	+++	++	++
L. monocytogenes ATCC 19115	+	+	+	++	++	++	++	++	++	++	+	+	+++	++	++	+++	++	++
S. aureus ATCC 25923	++	+	+	++	+	+	+++	+++	+++	++	+	+	+++	++	++	+++	+++	+++
E. faecium DSM 13590	+	+	-	++	+	+	+++	++	++	+	+	-	+	+	+	+++	++	++
E. faecalis DSM 20409	+	+	-	+	+	+	+++	++	++	+	+	-	+++	++	+	+++	+++	+++
S. Typhimurium ATCC 14028	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Inhibition zone (diameter in mm): 0 (-), 1-3 (+), 4-6 (++), 7-10 (+++).

Table 2: Effect of the extraction time on the anthocyanin recovery ($mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$) from Dornfelder (*V. vinifera* L.*)* grape pomace. Extractions were carried out at 600 MPa, 70°C and 50% ethanol.

Compound	30min	60min	90min
	Anthocyanins monoglucosides		
Dl3gl	0.6 ± 0.2[a]	0.5 ± 0.2[a]	0.316 ± 0.007[b]
Cy3gl	0.36 ± 0.08[a]	0.33 ± 0.07[a]	0.295 ± 0.008[a]
Pt3gl	0.8 ± 0.4[a]	0.66 ± 0.03[ab]	0.36 ± 0.05[b]
Pn3gl	1.6 ± 0.3[a]	1.7 ± 0.3[a]	1.7 ± 1.2[a]
Mv3gl	2.7 ± 0.3[a]	2.9 ± 0.3[a]	3.3 ± 0.6[a]
Total Ant3gl	**6.06 ± 1.28[a]**	**6.09 ± 0.9[a]**	**6.0 ± 1.9[a]**
	Acylated Anthocyanin monoglucosides		
Dl3acgl	0.32 ± 0.04[b]	0.7 ± 0.6[a]	0.310 ± 0.001[b]
Pt3acgl	0.34 ± 0.03[a]	0.32 ± 0.03[a]	0.310 ± 0.007[a]
Pn3acgl	0.43± 0.10[a]	0.4 ± 0.1[a]	0.323 ± 0.008[a]
Mv3acgl	0.75 ± 0.33[a]	0.6 ± 0.3[ab]	0.44 ± 0.02[b]
Cy3pcmgl	0.32 ± 0.02[a]	0.32 ± 0.02[a]	0.34 ± 0.01[a]
Pt3pcmgl	0.40 ± 0.04[a]	0.43 ± 0.05[a]	0.44 ± 0.01[a]
Pn3pcmgl	0.6 ± 0.1[b]	0.6 ± 0.2[b]	0.91 ± 0.06[a]
Mv3pcmgl	2.16 ± 0.02[a]	1.7 ± 0.2[a]	2.04 ± 0.65[a]
Total Ant3acgl	**5.32 ± 0.68[a]**	**5.07 ± 1.50[a]**	**5.11 ± 0.76[a]**
Total Content	**11.38 ± 1.96[a]**	**11. 16 ± 2.4[a]**	**11.11 ± 2.66[a]**

Values represent mean ± standard deviation (n=3). Rows with different letters are significantly different ($P<0.05$).

Table 3: Effect of HHP (600 MPa) at different temperatures on the anthocyanin recovery ($mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$) from Dornfelder (*V. vinifera L.)* grape pomace. Extractions were carried out an ethanol concentration of 50%.

Compound	Control 20°C	HHP 20°C	Control 50°C	HHP 50°C	Control 70°C	HHP 70°C	Control 90°C	HHP 90°C
				Anthocyanin monoglucosides				
Dp3gl	0.32 ± 0.01^{a}	0.30 ± 0.03^{a}	0.34 ± 0.03^{a}	0.38 ± 0.03^{a}	0.317 ± 0.006^{a}	0.6 ± 0.2^{a}	0.32 ± 0.01^{a}	0.37 ± 0.05^{a}
Cy3gl	0.270 ± 0.004^{a}	0.28 ± 0.01^{a}	0.27 ± 0.01^{a}	0.29 ± 0.01^{a}	0.270 ± 0.002^{a}	0.36 ± 0.08^{a}	0.264 ± 0.004^{a}	0.30 ± 0.02^{a}
Pt3gl	0.38 ± 0.01^{a}	0.36 ± 0.01^{a}	0.32 ± 0.04^{a}	0.44 ± 0.04^{a}	0.6± 0.1^{a}	0.8 ± 0.2^{a}	0.364 ± 0.017^{a}	0.43 ± 0.07^{a}
Pd3gl	1.1 ± 0.1^{b}	1.8 ± 0.4^{ab}	1.12 ± 0.56^{b}	1.7 ± 0.3^{ab}	1.1 ± 0.3^{ab}	1.6 ± 0.3^{b}	0.9 ± 0.1^{b}	1.6 ± 0.3^{a}
Mv3gl	3.01 ± 0.72^{a}	3.3 ± 0.4^{a}	2.23 ± 0.52^{a}	4.6 ± 0.9^{a}	2.8 ± 0.6^{a}	2.7 ± 0.3^{a}	2.1 ± 0.4^{a}	4.7 ± 0.7^{a}
Total Ant3gl	**5.1 ± 0.8^{ab}**	**6.0 ± 0.8^{a}**	**4.3 ± 1.2^{ab}**	**7.4 ± 1.3^{a}**	**5.1 ± 1.1^{ab}**	**6.06 ± 1.1^{a}**	**3.9 ± 0.5^{b}**	**7.4 ± 1.2^{a}**
				Acylated anthocyanin glucosides				
Dp3acgl	0.278 ± 0.001^{a}	0.285 ± 0.001^{a}	0.280 ± 0.005^{a}	0.292 ± 0.009^{a}	0.280 ± 0.002^{a}	0.32 ± 0.04^{a}	0.272 ± 0.001^{a}	0.29 ± 0.01^{a}
Pt3acgl	0.289 ± 0.002^{a}	0.296 ± 0.002^{a}	0.293 ± 0.006^{a}	0.309 ± 0.005^{a}	0.282 ± 0.002^{a}	0.34 ± 0.03^{a}	0.287 ± 0.002^{a}	0.30 ± 0.01^{a}
Pn3acgl	0.319 ± 0.009^{a}	0.327 ± 0.02^{a}	0.33 ± 0.01^{a}	0.35 ± 0.01^{a}	0.318 ± 0.005^{a}	0.43 ± 0.10^{a}	0.308 ± 0.005^{a}	0.33 ± 0.02^{a}
Mv3acgl	0.41 ± 0.02^{a}	0.429 ± 0.001^{a}	0.41 ± 0.04^{a}	0.49 ± 0.03^{a}	0.41 ± 0.01^{a}	0.75 ± 0.33^{a}	0.39 ± 0.01^{a}	0.44 ± 0.07^{a}
Cy3pcmgl	0.290 ± 0.002^{a}	0.292 ± 0.009^{a}	0.291 ± 0.005^{a}	0.311 ± 0.005^{a}	0.296 ± 0.007^{a}	0.32 ± 0.02^{a}	0.288 ± 0.001^{a}	0.303 ± 0.007^{a}
Pt3pcmgl	0.347 ± 0.003^{a}	0.35 ± 0.02^{a}	0.351 ± 0.009^{a}	0.38 ± 0.01^{a}	0.347 ± 0.003^{a}	0.40 ± 0.04^{a}	0.346 ± 0.002^{a}	0.36 ± 0.01^{a}
Pn3pcmgl	0.41 ± 0.01^{a}	0.7 ± 0.2^{a}	0.42 ± 0.03^{a}	0.58 ± 0.06^{a}	0.38 ± 0.03^{a}	0.6 ± 0.1^{a}	0.403 ± 0.008^{a}	0.42 ± 0.04^{a}
Mv3pcmgl	0.61 ± 0.04^{b}	1.4 ± 0.5^{b}	0.7 ± 0.1^{b}	1.42 ± 0.49^{ab}	0.54 ± 0.09^{b}	2.16 ± 0.02^{a}	0.60 ± 0.02^{b}	0.7 ± 0.1^{b}
Total Ant3acgl	**2.9 ± 0.1^{b}**	**4.1 ± 0.7^{b}**	**3.04 ± 0.23^{b}**	**4.1 ± 0.6^{ab}**	**2.8 ± 0.2^{b}**	**5.32 ± 0.68^{a}**	**2.9 ± 0.05^{b}**	**3.1 ± 0.3^{b}**
Total Content	**8.09 ± 0.9^{a}**	**10.1 ± 1.5^{a}**	**7.3 ± 1.4^{a}**	**11.58 ± 2.01^{a}**	**7.9 ± 1.3^{a}**	**11.38 ± 1.78^{a}**	**6.8 ± 0.6^{a}**	**10.5 ± 1.5^{a}**

Values represent mean ± standard deviation, n = 3. Rows with different letters are significantly different (P<0.05).

Table 4: Effect of HHP (600 MPa) and different ethanol concentrations on the anthocyanin recovery ($mg_{Cy3gl\ equ.}\ g_{DM}^{-1}$) from Dornfelder (*V. vinifera* L.*)* grape pomace. Extractions were carried out at 70°C.

	Control 20% Ethanol	**HHP 20% Ethanol**	**Control 50% Ethanol**	**HHP 50%Ethanol**	**Control 80% Ethanol**	**HHP 80% Ethanol**	**Control 100% Ethanol**	**HHP 100% Ethanol**
				Anthocyanin monoglucosides				
Dl3gl	0.301 ± 0.005^{b}	0.31 ± 0.01^{b}	0.317 ± 0.006^{b}	0.6 ± 0.2^{a}	0.34 ± 0.02^{b}	0.403 ± 0.009^{ab}	0.36 ± 0.02^{a}	0.50 ± 0.09^{a}
Cy3gl	0.258 ± 0.001^{b}	0.270 ± 0.006^{b}	0.270 ± 0.002^{b}	0.36 ± 0.08^{a}	0.271 ± 0.008^{b}	0.300 ± 0.004^{ab}	0.28 ± 0.007^{a}	0.35 ± 0.04^{a}
Pt3gl	0.333 ± 0.005^{b}	0.35 ± 0.02^{b}	0.6± 0.1^{a}	0.8 ± 0.2^{a}	0.42 ± 0.03^{b}	0.477± 0.004^{b}	0.43 ± 0.03^{b}	0.7 ± 0.2^{a}
Pd3gl	0.70 ± 0.05^{d}	0.9 ± 0.2^{d}	1.1 ± 0.3^{ab}	1.6 ± 0.3^{b}	1.4 ± 0.4^{d}	2.025 ± 0.004^{a}	1.52 ± 0.41^{b}	2.44 ± 0.05^{a}
Mv3gl	1.5± 0.2^{b}	2.4 ± 0.8^{b}	2.8 ± 0.6^{a}	2.7 ± 0.3^{a}	3.8 ± 0.7^{b}	5.59 ± 0.01^{b}	4.52 ± 0.58^{b}	10.9 ± 2.1^{a}
Total Ant3gl	**3.1 ± 0.2^{b}**	**4.23 ± 1.03^{b}**	**5.1 ± 1.1^{ab}**	**6.06 ± 1.1^{a}**	**6.2 ± 1.1^{b}**	**8.8 ± 0.03^{a}**	**7.11 ± 1.05^{ab}**	**14.9 ± 2.5^{a}**
				Acylated anthocyanin glucosides				
Dl3acgl	0.271 ± 0.002^{b}	0.282 ± 0.006^{b}	0.280 ± 0.002^{b}	0.32 ± 0.04^{a}	0.284 ± 0.007^{b}	0.296 ± 0.005^{ab}	0.284 ± 0.009^{b}	0.32 ± 0.02^{a}
Pt3acgl	0.283 ± 0.001^{a}	0.294 ± 0.006^{a}	0.282 ± 0.002^{a}	0.34 ± 0.03^{a}	0.298 ± 0.007^{a}	0.318 ± 0.005^{a}	0.298 ± 0.009^{a}	0.35 ± 0.01^{a}
Pn3acgl	0.301 ± 0.006^{c}	0.31 ± 0.01^{c}	0.318 ± 0.005^{c}	0.43 ± 0.10^{b}	0.36 ± 0.03^{b}	0.364 ± 0.001^{b}	0.7 ± 0.5^{a}	0.47 ± 0.07^{b}
Mv3acgl	0.361 ± 0.001^{b}	0.39 ± 0.04^{b}	0.41 ± 0.01^{b}	0.75 ± 0.33^{a}	0.45 ± 0.05^{b}	0.520 ± 0.002^{b}	0.4 ± 0.1^{b}	0.9 ± 0.2^{a}
Cy3pcmgl	0.284 ± 0.001^{b}	0.309 ± 0.007^{ab}	0.296 ± 0.007^{ab}	0.32 ± 0.02^{ab}	0.299 ± 0.009^{ab}	0.322 ± 0.006^{ab}	0.33 ± 0.04^{ab}	0.38 ± 0.01^{a}
Pt3pcmgl	0.335 ± 0.003^{ab}	0.34 ± 0.01^{ab}	0.347 ± 0.003^{ab}	0.40 ± 0.04^{ab}	0.37 ± 0.01^{ab}	0.413 ± 0.009^{ab}	0.43 ± 0.07^{ab}	0.54 ± 0.05^{a}
Pn3pcmgl	0.365 ± 0.006^{b}	0.39 ± 0.04^{b}	0.38 ± 0.03^{b}	0.6 ± 0.1^{b}	0.51 ± 0.09^{b}	0.69 ± 0.04^{b}	0.53 ± 0.12^{b}	1.5 ± 0.5^{a}
Mv3pcmgl	0.45 ± 0.01^{b}	0.50 ± 0.09^{b}	0.54 ± 0.09^{b}	2.16 ± 0.02^{b}	1.17 ± 0.57^{b}	2.4 ± 0.4^{b}	1.3 ± 0.9^{b}	13.4 ± 4.2^{a}
Total Ant3acgl	**2.65 ± 0.03^{b}**	**2.8 ± 0.2^{b}**	**2.8 ± 0.2^{b}**	**5.32 ± 0.68^{b}**	**3.7 ± 0.8^{b}**	**5.3 ± 0.5^{b}**	**4.3 ± 1.8^{b}**	**17.9 ± 5.1^{a}**
Total Content	**5.7 ± 0.3^{b}**	**7.03 ± 1.23^{b}**	**7.9 ± 1.3^{b}**	**11.38 ± 1.78^{b}**	**9.9 ± 1.9^{b}**	**14.1 ± 0.5^{b}**	**11.4 ± 2.9^{b}**	**32.8 ± 7.6^{a}**

Values represent mean ± standard deviation, n = 3. Rows with different letters are significantly different (P<0.05).

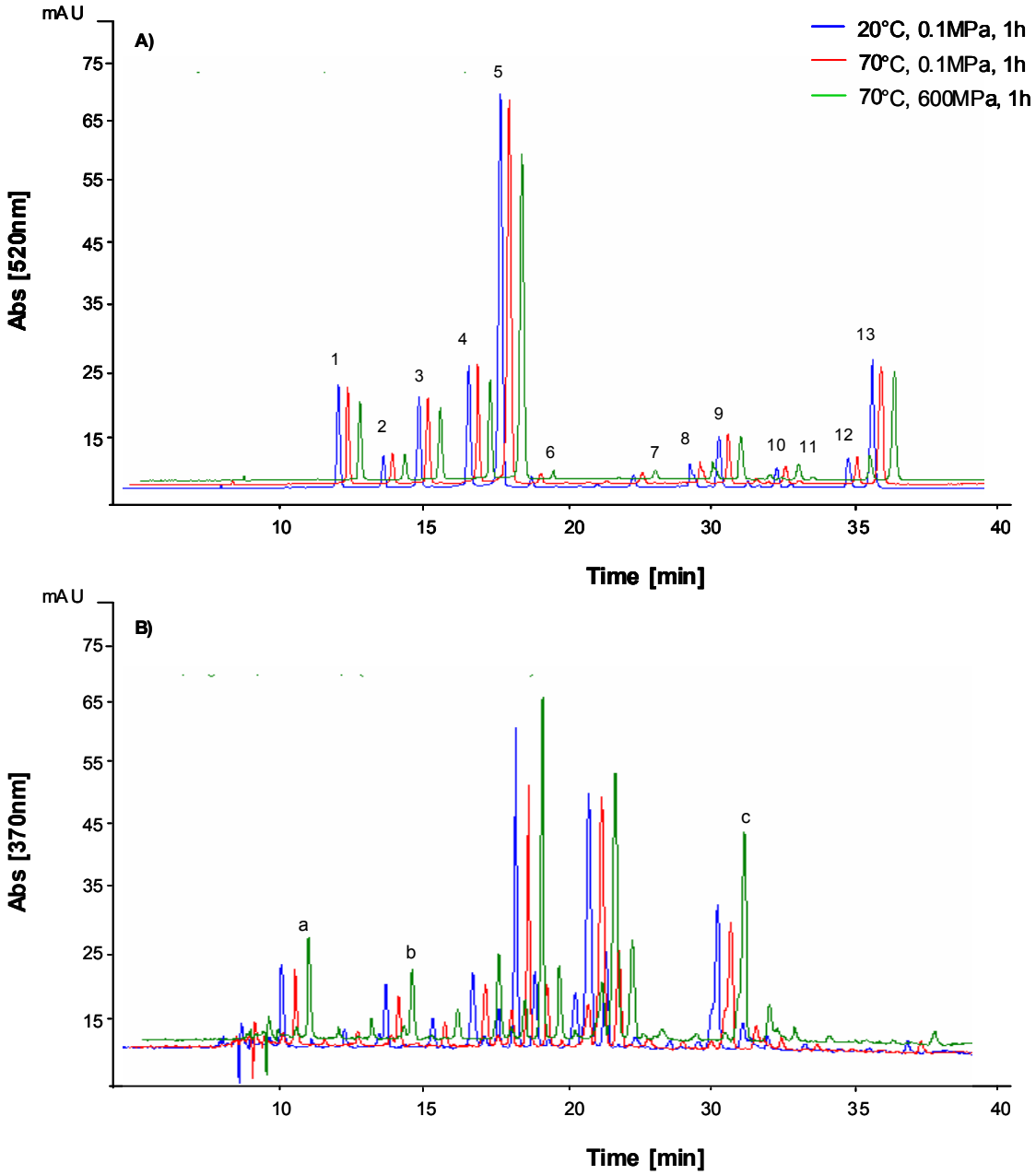

Figure 1: A):HPLC-DAD Chromatograms of Dornfelder (*V. vinifera* L.) extracts at 520 nm and B): 370 nm after different heat/pressure treatments. 1. Dl3gl, M^+=465; 2. Cygl, M^+=449; 3. Pt3gl, M^+=479; 4. Pn3gl, M^+=463; 5. Mv3gl, M^+=493; 6. Dl3acgl, M^+=507; 7. Pt3acgl, M^+=521; 8. Pn3acgl, M^+=505; 9. Mv3acgl, M^+=535; 10. Cy3pcmgl, M^+=595; 11. Pn3pcmgl, M^+=609; 12. Mv3pcmgl, M^+=639. (gl=glucoside; acgl=acetylglucoside; pcmgl=p-coumarylglucoside). a. M^+ = 1650, b. M^+ = 1344; c. M^+ = 730; d. M^+ = 1124

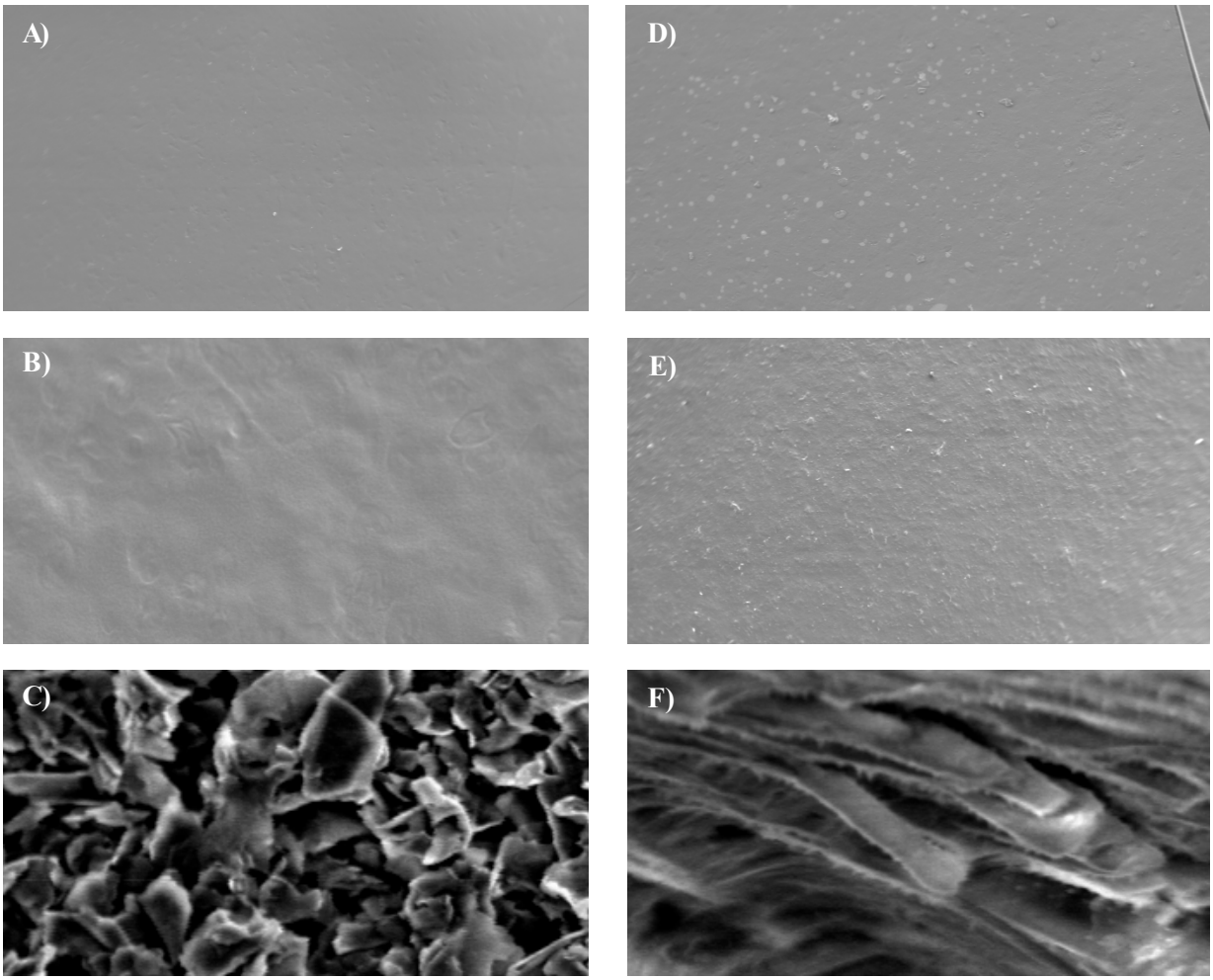

Figure 2: Electronical microscope photos from control pea starch films: A): longitudinal cut; B): parallel and C): transversal and GSE enriched pea starch films; D): longitudinal cut; E): parallel and F): transversal cut.

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LIST OF PUBLICATIONS

International journals

Corrales, M., Butz, P., & Tauscher, B. Extraction of anthocyanins from grape by-products assisted by high hydrostatic pressure. *Journal of Food Engineering. (Submitted)*.

Zhao, X., Corrales, M., Hu, X., Ma, Y., & Tauscher, B. Antioxidant potential and characteristics of selected Chinese purple corn hybrids. *Journal of Crop Science. (Submitted).*

Corrales, M., Vizoso-Pinto, MG., Franz, CMAP., Schuele, E., Butz, P., & Tauscher, B. Toxicological aspects of grape pomace extracts. *International Journal of Food Microbiology. (Submitted).*

Corrales, M., Han, HJ., & Tauscher, B. Antimicrobial and antioxidant properties of grape seed extracts and their application into pea starch films for meat preservation. *International Journal of Food Science & Technology. (Submitted).*

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Corrales, M., Butz, P., & Tauscher, B. (2008). Anthocyanin condensation reactions under high hydrostatic pressure. *Food Chemistry. (Accepted).*

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Corrales, M., Butz, P., & Tauscher, B. (2006). Gewinnung von Anthocyanine aus Dornfelder (*vitis vinifera*), Traubentrester mittels Hochdruckbehandlung. *Lebensmittelchemie*. 60, 146.

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Corrales M.
Active packaging from renewable resources. (2007). National Engineering Research Center for Vegetables, Beijing, China.

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Corrales, M., Butz, P., & Tauscher, B. (2006). Recovery from anthocyanins from Dornfelder (*vitis vinifera spp.)* grape pomace with high hydrostatic pressure. DGCh (German Chemical Society), Karlsruhe, Germany.

Corrales, M., Asmus, L., Ernst.Sieber, R., Thiessenhusen, A., & Weber S. (2005). Oil and gas – what next? BASF. Students´ initiative: Corporate social responsibility in a globalized world. Ludwigshafen-Tarragona, Germany/Spain.

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Belz, M., Corrales, M., Butz, P., & Tauscher, B. (2007). Characterisation of bioactive compounds and antifungal effect of extracts from grapes. GDCh (German Chemical Society), Nürnberg, Germany.

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RESUME

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ACADEMIC DEGREES

06/04-04/08	Federal Research Centre for Nutrition and Food / Karlsruhe University, Karlsruhe, Germany Ph.D.: Optimal extraction and technological revalorisation of bioactive polyphenols from grape pomace Supervisors: Prof. Bernhard Tauscher and Prof. Manfred Metzler
10/02-05/04	Rheinische Friedrich-Wilhelms-University, Bonn, Germany/ Universidad Autónoma de Madrid, Spain M. Sc.: Influence of different production factors on lycopene stability for its use as functional ingredient
10/00-09/02	Universidad Autónoma de Madrid, Spain Bch: Food Science and Technology
10/97-07/00	Universidad Autónoma de Madrid, Spain Pre-graduate in Biology

PREVIOUS EMPLOYMENT

02/05-04/06	Federal Research Centre for Nutrition and Food, Karlsruhe, Germany Research Scientist Project: Influence of a high pressure treatment on chemical changes of special peptides and proteins in foods: Possible formation of hormone-like structures
03/02-06/02	Morella Nuts S.A., Reus, Spain Trainee as a lab assistant in quality control, enzymatical and microbiological analysis of nuts and by-products
09/01-03/02	Universidad Autónoma de Madrid, Madrid, Spain Scientific co-worker at the institute of cell biology. Pollution effects in adrenaline and nor-adrenaline pancreas secretion of pigeons

VISITING POSITIONS

10/07-11/07	National Engineering Research Centre for Vegetables, Beijing, China
10/06-03/07	Department of Food Science, University of Manitoba, Winnipeg, Canada
04/06-05/06	Institute of Food Research and Technology (IRTA), Gerona, Spain