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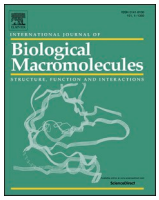

Synergistic effect of washing process and after-treatment parameters on gluten protein composition, rheological, and baking properties

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ABSTRACT

Gluten was extracted from wheat flour with various modifications on lab scale. To assess the effect of process parameters, the washing water was tempered to either 20 °C or 50 °C and 0.1 M NaCl was added to the washing water. In an after-treatment process of the isolated gluten, 3 % of NaCl or 0.5 % of $CaCl_2$ was added. The wet gluten samples were subjected to either drying at 40 °C or freeze-drying. Different combinations of these parameters were used to study the synergistic effect of process and after-treatment parameters on gluten protein composition and functionality. The addition of salt and temperature treatment had synergistic effects on the functionality and composition of gluten proteins. The variability in the functionality of gluten proteins could largely be explained by their protein composition. In addition, gluten treated with salt had a positive effect on specific bread volume resulting in significant ($p < 0.05$) increases to 2.62 ± 0.06 cm^3/g (20 °C, 0.5 % $CaCl_2$, freeze-dried) and 2.67 ± 0.07 cm^3/g (20 °C, 0.1 M NaCl, 3 % NaCl, freeze-dried) compared to the control (2.08 ± 0.06 cm^3/g). Inversely, crumb hardness decreased significantly ($p < 0.05$) to 203.5 ± 38.0 g and 198.0 ± 40.4 g compared to 585.7 ± 73.5 g, respectively. Moreover, the results indicate that changes in the gluten protein composition and functionality due to heat exposure during gluten extraction can be partially reversed by treatment with chloride salts, especially by adding 0.1 M NaCl during washing and 3 % NaCl in the after-treatment.

1. Introduction

Vital gluten (VG) is a by-product of the starch extraction process and widely used in the baking industry to compensate for poor or low protein quality. Nowadays, it is also increasingly used for meat alternatives [1]. The Italian scientist J.B. Beccari was the first one to discover gluten as a component of wheat flour in 1745. Before gluten began to be produced on an industrial scale, only starch was of commercial interest and the by-product gluten was even considered as unusable and discarded. In the 1930s, a New Zealand confectioner began exploiting the potential of gluten as a food ingredient by using gluten in bread, which was patented worldwide as 'Procera' bread. However, it was not until the middle of the 20th century that the benefit of gluten as a versatile food ingredient was discovered. From then on, gluten was more and more in demand by the food industry. Today, gluten is produced industrially on a million ton scale and the process is still based on the same principle used by J.B. Beccari [2].

All gluten extraction processes start with flour, but there are already differences in the various processes when adding water. Basically, a distinction can be made between three main processes: the dough-, the dough-batter- and the batter-process. The processes differ in the amount of water added to the flour, the temperature of the water and the development of the gluten network prior to starch separation, as well as in the yield of gluten and starch. In the dough process, also called Martin process, a firm dough with 40–60 % water content is formed. Starch and the water-extractable fraction are removed by washing in a continuous kneader [3]. In traditional processes, the flour-water-mixture is processed to form an ideally developed gluten network. In modern dough-

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batter- or batter-processes, more water is used to form a suspension rather than a dough and gluten development is only partially promoted [4]. Water temperatures of 40–55 °C are used in the batter process. In an adaption of this process, the Fesca process, the water temperature is lowered to 30 °C to minimize gluten agglomeration [3,5].

Due to the difference in particle size, sieves or rotating drums were used in the past to separate gluten from starch. Modern processes rely on separating starch and gluten based on the higher density of starch, using centrifuges, decanters or hydrocyclones as in the Alfa-Laval/Raisio process. The following drying step is crucial in gluten production as this process parameter significantly determines VG quality. In industry, flash dryers, ring dryers or spray dryers are mainly used. Freeze-drying is the most gentle drying technique, but this method is not economically feasible in an industrial production process [3].

The composition and functionality of gluten, which determine gluten quality, are based on interactions between gluten proteins. Both covalent and non-covalent bonds play a role in this process. Covalent disulphide bonds between cysteine residues link glutenin polymers and form the basic structure of the gluten network. Under the influence of heat, further cross-linking can occur through SH-SS exchange reactions, supplemented by other types of bonds such as lanthionine or isopeptide bonds. Non-covalent interactions such as hydrogen bonds, hydrophobic, ionic and electrostatic interactions are weaker, but contribute significantly to the aggregation of gliadins and glutenins and to the stabilisation of the protein structure. Both types of bonds together determine the structure and functional properties of gluten.

The modification of process parameters of the gluten/starch separation aims to strengthen protein-protein interactions and to weaken protein-starch interactions in order to achieve better separation [3]. The addition of enzymes, such as lipases, cellulases or hemicellulases, during the extraction process can improve the separation [6,7]. It is also known that the addition of NaCl to the dough or washing water improves gluten agglomeration [8–11]. In our previous study, we found that the addition of salt to an existing gluten network can still alter the gluten protein composition and rheological properties [12]. Additionally, many publications have investigated the impact of temperature when washing and drying gluten [13–16].

To date, however, there have been no attempts to examine the combined effect of salt and temperature in the washing process and after-treatment of gluten production. The aim of our study was to systematically investigate the synergistic effect of washing process parameters such as washing temperature (20 °C or 50 °C) and addition of 0.1 M NaCl to the washing water and of after-treatment parameters like the addition of 3 % NaCl and 0.5 % $CaCl_2$ (w/wet gluten weight) in an after-treatment step and the drying temperature (freeze-drying vs. drying at 40 °C) of wet gluten on lab scale.

A thorough insight of how process and after-treatment parameters affect the composition and functionality of gluten proteins offers the potential to mitigate adverse effects associated with the washing and drying process of vital gluten. In a further step, understanding the influence of salt and temperature on vital gluten functionality may enable the targeted modification of its properties.

2. Materials and methods

Commercial German wheat flours (type 550) were obtained from Bavaria Mühle GmbH (Aichach, Germany). Gluten extraction was performed with a flour characterized by a moisture content of 12.43 ± 0.07 %, a crude protein content of 12.08 ± 0.01 %db and a wet gluten content of 26.84 ± 0.35 % (based on 14 % flour moisture). The wheat flour used for the baking trials was characterized by a moisture content of 13.62 ± 0.12 %, a crude protein content of 11.98 ± 0.05 %db and a wet gluten content of 23.45 ± 0.51 % (based on 14 % flour moisture). The dry yeast used for the baking trials was purchased from Casteggio Lieviti S.R.L. (Fermipan red, Casteggio, Italy). All chemicals used were of analytical grade and the salts (NaCl and $CaCl_2$) were purchased from VWR International GmbH (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany) and Acros Organics as part of Thermo Fisher GmbH (Schwerte, Germany).

2.1. Preparation of vital gluten

VG was extracted using the lab-scale washing process reported by Wehrli et al. [17] following the traditional Martin process. To produce wheat dough, 1.0 kg of wheat flour and 550.0 g of distilled water were kneaded in a spiral mixer (SP 12 A-4, Diosna Dierks & Söhne GmbH, Osnabrück, Germany). The kneading protocol involved a premixing step at 100 rpm for 60 s and a mixing step at 200 rpm for 300 s. After kneading, the dough was soaked in distilled water at room temperature for 30 min. Subsequently, gluten extraction was performed as previously described by Wehrli et al. [17]. Briefly, the dough was kneaded (MUM 4405, Robert Bosch GmbH, Stuttgart, Germany) under a flow of distilled water (ca. 20 L/extraction, 20 °C) used as washing water. During the washing process, the resulting starch suspension was drained through the perforated kneading bowl, lined with an 80 µm polyester sieve cloth (PES-80/120, Schwegmann Filtrationstechnik GmbH, Grafschaft-Ringen, Germany). The removal of fibers was conducted twice during the 16 min washing process. After extraction, the gluten samples were frozen at −20 °C until freeze-drying (Beta 1–8 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The freeze-dried samples (FD) were milled at 12000 rpm using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) with a 250 µm sieve. The parameters stated above refer to the production process of the reference VG (20 °C, FD).

To assess the impact of process parameters on the VG functionality, the standard procedure was modified systematically. The process parameters were defined as the temperature of the washing water (20 °C or 50 °C) and additive in the washing water (0.1 M NaCl or no salt), while the term after-treatment describes the combination of additives in the after-treatment step (NaCl or $CaCl_2$) and the drying conditions of wet gluten (40 °C or freeze-drying). Fig. 1 presents an overview of the applied combinations of process and after-treatment parameters. The parameters were set to represent industrial process parameters and the strongest effects on gluten protein composition and rheological properties revealed during previous investigations [12]. On the process parameter side, the effect of using a 0.1 M NaCl solution instead of distilled water as extraction medium was studied. Additionally, the washing water temperature was varied between two levels (20 °C and 50 °C). Regarding the process parameters, the effect of adding 3 % (w/wet gluten weight) NaCl or 0.5 % (w/wet gluten weight) $CaCl_2$ after the extraction process was investigated. Salts were added after centrifugation (4000 rcf, 5 min, 20 °C) of gluten to remove the excess water. The salts were incorporated homogenously by blending the samples with the respective amounts of NaCl and $CaCl_2$ for 15 s in 3 pulses with 5 s delay time. The blending was also carried out for samples without additives to eliminate for the effect of the mechanical stress. Additionally, the impact of the drying process was analyzed by selectively drying by freeze-drying or in a drying chamber at 40 °C for 24 h. In the following, the samples are named according to the applied extraction procedure: 20 °C/50 °C for the washing water temperature, 0.1 M NaCl in case of using a 0.1 M NaCl solution instead of distilled water during the extraction process, 3 % NaCl/0.5 % $CaCl_2$ in case of an after-treatment process with NaCl or $CaCl_2$, respectively, and FD or 40 °C to distinguish between the application of freeze-drying (FD) and drying at 40 °C (Fig. 1).

2.2. Crude protein content

The analysis of the crude protein content of VG samples was carried out in triplicate according to ICC 167 using a Dumatherm N Pro (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). To convert the nitrogen content into protein, a protein conversion factor of N × 5.71

	Process parameter		After-treatment parameter			Sample name
	Temperature washing water	Additive washing water	Additive after-treatment	Drying parameter		
Influence of washing temperature	20 °C			Freeze-drying		20°C, FD
	50 °C			Freeze-drying		50°C, FD
Influence of salt in the after-treatment	20 °C		3% (w/w) NaCl	40 °C drying	Influence of drying temperature	20°C, 3% NaCl, 40°C
	50 °C		3% (w/w) NaCl	40 °C drying		50°C, 3% NaCl, 40°C
	20 °C		3% (w/w) NaCl	Freeze-drying		20°C, 3% NaCl, FD
	50 °C		3% (w/w) NaCl	Freeze-drying		50°C, 3% NaCl, FD
	20 °C		0.5% (w/w) $CaCl_2$	Freeze-drying		20°C, 0.5% $CaCl_2$, FD
	50 °C		0.5% (w/w) $CaCl_2$	Freeze-drying		50°C, 0.5% $CaCl_2$, FD
Influence of salt in washing water	20 °C	0.1 M NaCl		Freeze-drying		20°C, 0.1M NaCl, FD
	50 °C	0.1 M NaCl		Freeze-drying		50°C, 0.1M NaCl, FD
	20 °C	0.1 M NaCl	3% (w/w) NaCl	Freeze-drying	Combined effect of washing temperature, NaCl in washing water, NaCl/$CaCl_2$ in after-treatment and drying temperature	20°C, 0.1M NaCl, 3% NaCl, FD
	50 °C	0.1 M NaCl	3% (w/w) NaCl	Freeze-drying		50°C, 0.1M NaCl, 3% NaCl, FD

Fig. 1. Overview of vital gluten samples with the respective process and after-treatment parameters. By comparing different samples, the influences shown in the left and right columns can be evaluated.

was applied.

2.3. Gluten protein composition

A modified Osborne fractionation according to Wieser et al. [18] was performed [12]. To obtain gliadins, 1.5 mL of 60 % aqueous ethanol (v/v) was added to 20 mg of VG in triplicates. The residue was extracted with 1.5 mL of 50 % (v/v) propan-1-ol, 0.05 mol/L Tris–HCl (pH 7.5), 2 mol/L (w/v) urea and 1 % (w/v) dithiothreitol (DTT) and treated with argon to obtain glutenins. Both extracts were vortex mixed for 2 min, gliadins were stirred for 10 min and glutenins were stirred for 30 min in a water bath at 60 °C. Both extracts were centrifuged for 25 min at 22 °C and 3550 rcf (Z446K, Heraeus, Hanau, Germany). The extraction procedure was repeated twice for gliadins and glutenins and the supernatants were transferred to a 5 mL volumetric flask and filled with extraction solution, respectively. Prior to RP-HPLC analysis, the extracts were filtered through a 0.45 μm membrane filter. RP-HPLC conditions were as described in Hoeller and Scherf [12]. The injection volume was 20 μL for gliadins and 15 μL for glutenins. For quantitation, Prolamin Working Group (PWG)-gliadin (c = 2.5 mg/mL) with a protein content of 93.1 % was used [19]. The integration was done according to Schalk et al. with the software Lab Solutions 5.93 [20].

2.4. Content of SDSS proteins and GMP

The method of Thanhaeuser et al. [21] with modifications was used to determine the content of SDS-soluble (SDSS) proteins and glutenin macropolymer (GMP) [12]. For extraction of SDS-soluble (SDSS) proteins, 20 mg of VG in triplicate was mixed with 1.0 mL of 1 % (w/v) SDS and 0.05 mol/L NaH_2PO_4 (pH 6.9). The residue was mixed with 1.0 mL of 50 % (v/v) propan-1-ol, 0.05 mol/L Na_2HPO_4/KH_2PO_4 (pH 7.5) and 1 % (w/v) DTT buffer to obtain GMP. Both extracts were vortexed for 2 min and mixed for 25 min at 1500 rpm in a thermal shaker at 22 °C for SDSS protein extraction or stirred for 30 min at 60 °C and 1500 rpm in a thermal shaker for GMP extraction. The extracts were then centrifuged for 30 min at 22 °C and 21,300 rcf (Z216MK, Heraeus, Hanau, Germany). Both extraction procedures were repeated twice and the SDSS and GMP extracts were transferred and combined in a 5 mL volumetric flask, respectively. The flasks were filled up with the corresponding extraction solution and filtered through a 0.45 μm membrane filter prior to GP-HPLC analysis. The GP-HPLC conditions were as described in Hoeller and Scherf [12]. The injection volume was 5 μL for SDSS extracts and 15 μL for GMP extracts. For molecular weight assignment of proteins, a molecular size marker consisting of albumin (molecular weight = 66 kDa), carbonic anhydrase (molecular weight = 29 kDa) and cytochrome C (molecular weight = 12.4 kDa) in water was used. For quantitation, PWG-gliadin (c = 1 mg/mL) was used (van Eckert et al., 2006). Integration was performed with the software Lab Solutions 5.106.

2.5. Content of free and bound thiols

The quantification of free and bound thiol groups was carried out following the method described by Schopf and Scherf [22]. For determination of the content of free thiols, 900 μL of 0.05 mol/L Na_2HPO_4/NaH_2PO_4 (pH 6.5), 2 % (w/v) SDS, 3 mol/L urea and 0.001 mol/L EDTA was added to 10 mg of VG. The samples were incubated in a thermal shaker for 60 min at 22 °C and 500 rpm. Then, a 0.1 % (w/v) solution of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was added to the samples and shaken for 45 min at 22 °C and 500 rpm. Subsequently, the samples were centrifuged at 22 °C and 11,000 rcf for 5 min and 200 μL of the resulting supernatants were transferred into a 96-well plate. The absorbance of the samples was immediately measured at 412 nm at 22 °C using a photometer (Infinite M Nano+ Multiplate Reader, Tecan AG, Männedorf, Switzerland). For quantitation, a calibration curve with glutathione standard (GSH) with concentrations from 10 μmol/L to 209 μmol/L was prepared and treated in the same way as the samples. All measurements were performed in triplicate.

For bound thiols, 2 mg of VG were mixed with 200 μL of 2.5 % $NaBH_4$ and incubated for 60 min at 50 °C in a thermal shaker. The samples were then treated with 100 μL of 1 mol/L HCl. The subsequent extraction procedure was carried out as described for the determination of free thiols. Oxidized glutathione standard (GSSG) ranging from 3.8 μmol/L to 152.09 μmol/L was used for calibration. The work-up of the

calibration points was in accordance with the sample preparation protocol. The content of bound thiols was calculated by subtracting the content of free thiols from the total thiols.

2.6. Water retention capacity

The water retention capacity of the VG was quantified using the AACC International method 56–11.01 with water as solvent. Briefly, 5.00 g of VG was hydrated with 25.0 mL of distilled water. For 20 min, the hydrated samples were vortexed at intervals of 5 min. Afterward, the samples were centrifuged at 1000 rcf for 15 min at 20 °C. The resulting pellets were allowed to drain for 10 min and subsequently weighed. The water retention capacity was calculated as the retained water and dry sample weight ratio. The pellets were frozen at −18 °C for rheological testing.

2.7. Shear rheometry

The rheological characterization was conducted using a Modular Compact Rheometer (MRC 502, Anton Paar Germany GmbH, Ostfildern, Germany) equipped with a convection oven (CTD 180 HR chamber, Anton Paar GmbH, Graz, Austria) connected to a modular humidity generator (MHG 100, ProUmid GmbH & Co. KG, Ulm, Germany). The tests were conducted using a cross-hatched parallel plate geometry (plate diameter = 25 mm) at a 2 mm measurement gap at 25 °C. After 20 min equilibration, the samples were trimmed and the cutting edges were covered with paraffin oil to prevent dehydration. Subsequently, a frequency sweep was conducted with frequencies ranging from 0.1 Hz to 20 Hz at a strain of 0.1 %. The parameters were chosen based on the results of an amplitude sweep, indicating the limit of the linear viscoelastic range of the samples to above 0.1 % strain. The frequency dependency of storage modulus G' was fitted using the power law model (Eq. 1),

$$G'(\omega) = G'_0 \omega^{n'} \quad (1)$$

with ω being the angular frequency, G'_0 referring to storage modulus at $\omega = 1$ rad/s and n' to the power law parameter [23].

Following the frequency sweep, a creep-recovery test was performed by applying a constant shear stress of 200 Pa for 600 s, followed by a 1200 s recovery phase. Compliance of the samples during the creep phase was fitted using the Burgers Model (Eq. 2),

$$J(t) = J_0 + J_1\left(1 - e^{\frac{t}{\lambda}}\right) + \frac{t}{\eta_0} \quad (2)$$

where J_0 corresponds to the instantaneous compliance, J_1 to the viscoelastic compliance, λ to the retardation time and η_0 to the zero shear viscosity [24].

2.8. Baking

To assess the functionality of VG as a dough enhancer, commercial German wheat flour (type 550) was supplemented with 5.5 % (w/flour dry weight) VG. Before the baking trials, dough development time and water absorption of each formulation were assessed based on ICC 115/1 using a DoughLAB (Perten Instruments GmbH, Hamburg, Germany). Using the respective values, kneading of wheat dough enriched with VG was conducted in a 300 g scale Farinograph (F2, Brabender GmbH & Co. KG, Duisburg, Germany) with addition of 1.5 % (w/flour weight) dry yeast. After kneading, the dough rested for 10 min before dividing, molding, and placing it into baking tins. The 175 g pieces were proofed at 30 °C and 80 % relative humidity, followed by baking for 22 min at 220 °C in a deck oven (Matador Store 12.8, Werner & Pfleiderer GmbH, Sohland, Germany) with 0.5 L steam injection. After cooling at room temperature for 2 h, the breads were characterized in terms of specific volume using a volumeter (TexVol BVM-L370, Perten Instruments AB, Stockholm, Sweden) and crumb hardness (TVT-300 XP, Perten Instruments AB, Stockholm, Sweden equipped with a 5 kg load cell). Crumb hardness was determined in accordance with Brandner et al. [25] by compressing two bread slices of 12.5 mm thickness by 40 % after exceeding a trigger force of 0.05 N using a compression plunger (diameter = 25 mm) at a compression speed of 1 mm/s. The compression was conducted in two cycles with a 5 s rest between the first and the second compression cycle. The force recorded over time was used to determine the crumb hardness as the peak force exerted during the first compression cycle.

2.9. Statistics

Statistical tests, principal component analysis (PCA) and partial least squares regression (PLSR) were performed with OriginPro, Version 2023 (OriginLab Corporation, Northampton, MA, USA). To identify the influence of drying temperature or concentration and type of salt, significant differences were determined by analysis of variance (ANOVA) with Tukey's test ($p \leq 0.05$) or Kruskal-Wallis test as a non-parametric test followed by Dunn's Test as a post-hoc test on a significance level of $\alpha = 0.05$. To identify interactions between process and after-treatment parameters, a linear model was applied to analyse the data (Posit Software, PBC, Boston, MA, USA), incorporating both main and interaction effects between the considered parameters. By including first order interaction terms, potential interactions that indicate how combinations of specific parameters lead to synergistic effects were identified.

3. Results & discussion

3.1. Effect of process and after-treatment parameters on gluten protein composition

The crude protein content determined by Dumas ranged from 77.9 % to 90.6 % (Table S1) and was lowest for VG (50 °C, 3 % NaCl, 40 °C) and highest for VG (50 °C, 0.1 M NaCl, FD). Osborne fractionation was performed to determine the gluten protein composition of all samples. The content of gliadins, consisting of the gluten protein types ω5-, ωb-, ω1,2-, α- and γ-gliadins, varied between 384.4 mg/g and 491.7 mg/g. The content of glutenins, including low-molecular-weight glutenin subunits (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS), ranged from 263.0 mg/g to 357.2 mg/g (Fig. 2, Table S2).

Samples washed with water at 50 °C contained more glutenins, but less gliadins than samples washed at 20 °C and vice versa. This was particularly evident in samples without salt and in samples that were dried at 40 °C. This trend was less pronounced in samples which were treated with salt and then freeze-dried. In order to make these trends clearer and achieve better comparability between the samples, the ratio of gliadins and glutenins (glia/glut) was considered, which ranged from 1.12 to 1.78 (Fig. 2B, Table S2). All samples treated at 50 °C showed a lower or similar glia/glut ratio than the corresponding sample treated at 20 °C, except those treated with 0.5 % $CaCl_2$ (w/wet gluten weight). In relative terms, a lower glia/glut ratio is indicative of the presence of more protein aggregates. In particular, the addition of NaCl to the 20 °C washing water and the after-treatment of gluten with NaCl resulted in a high glia/glut ratio. Samples treated with both NaCl in the washing water and in the after-treatment step showed the highest glia/glut ratio regardless of temperature. In addition, VG (20 °C, 3 % NaCl, 0.1 M NaCl, FD) showed a significant difference compared to the separate treatments of the washing water in VG (20 °C, 0.1 M NaCl, FD) or in the after-treatment in VG (20 °C, 3 % NaCl, FD). This indicates an enhancing effect of the combination of NaCl treatment in the washing process and after-treatment.

Contradictory results were obtained in the literature regarding the influence of salts on the composition of gluten. Fu et al. found more gliadins but less insoluble proteins in NaCl-washed gluten, while Chen et al. extracted less gliadins and more glutenins in gluten from NaCl-

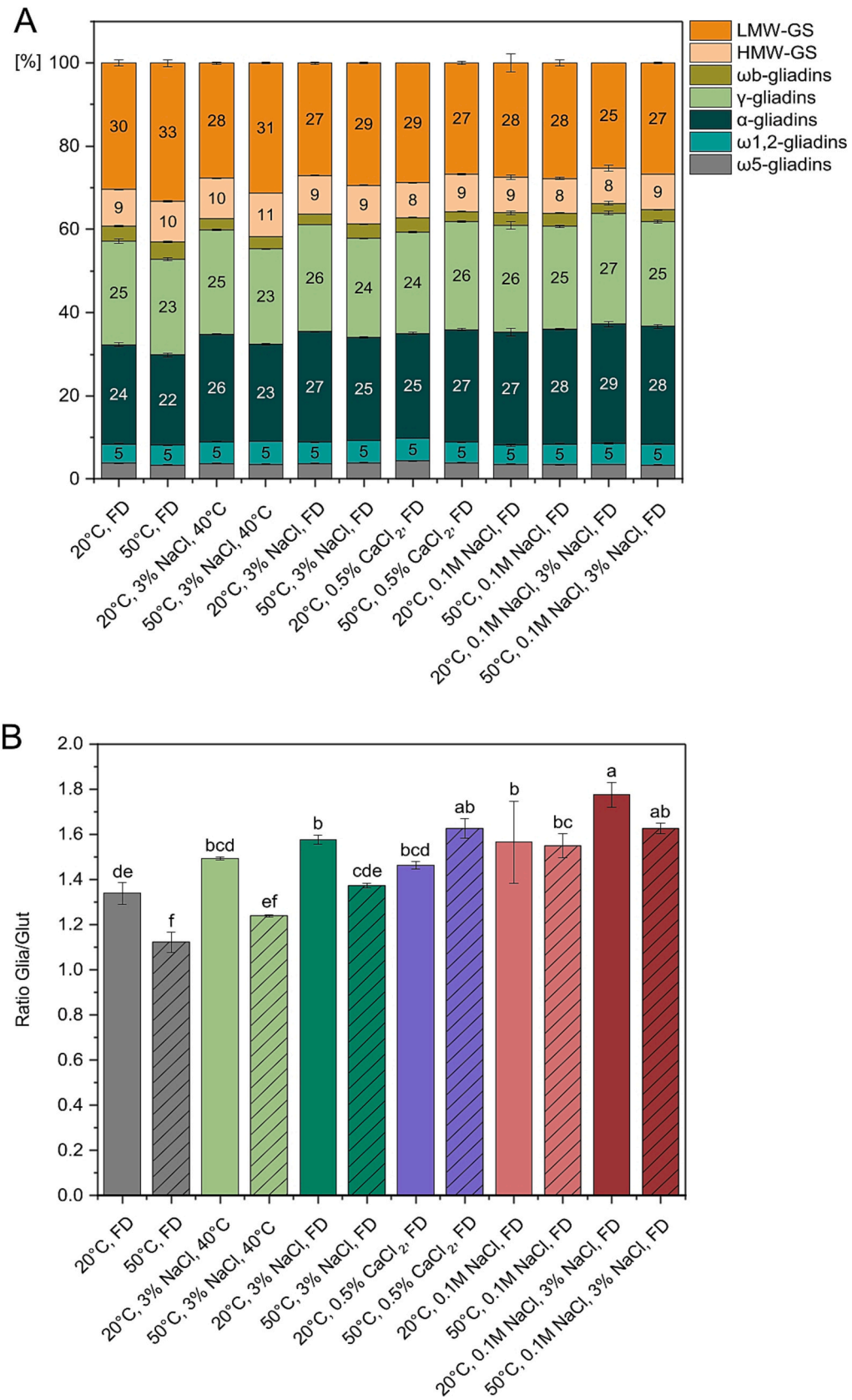

Fig. 2. Content of low-molecular-weight glutenin subunits (LMW-GS), high-molecular-weight glutenin subunits (HMW-GS), gluten protein types ω5-, ωb-, ω1,2-, α- and γ-gliadins (A) and gliadin-to-glutenin (glia/glut) ratio (B), in salt-treated vital gluten samples and control samples without salt dried at 40 °C or freeze-dried (FD). All values are given as means ± standard deviation ($n = 3$). Values below 4 % are not shown. Significant differences between vital gluten samples are indicated by small superscript letters of the mean values (ANOVA, Tukey test, $p < 0.05$).

treated dough [8,10]. These opposing findings may be attributed to variations in sample preparation or to the protein composition of the raw material flour, which contributes to gluten properties [26]. Furthermore, the findings of previous publications can hardly be compared with the results of our study, as the existing literature did not assess the influence of salts in after-treatment processes of pre-isolated gluten.

3.2. Effect of process and after-treatment parameters on SDSS and GMP

The molecular size distribution of gluten proteins was determined by SDSS/GMP fractionation. The SDSS content was lowest for VG (50 °C, 0.5 % $CaCl_2$, FD) (363.3 mg/g) and highest for VG (50 °C, FD) (597.9 mg/g). The GMP content, including LMW-GMP and HMW-GMP, ranged from 45.5 mg/g in VG (50 °C, 0.5 % $CaCl_2$, FD) to 264.5 mg/g in VG (50 °C, 0.1 M NaCl, FD) (Fig. 3, Table S3). Overall, the SDSS content tended to be higher for samples treated at 20 °C whereas the GMP content was low in these samples, especially for HMW-GMP. This trend did not apply to the samples treated with 0.5 % $CaCl_2$ (w/wet gluten weight).

These effects became clearer when comparing the ratio of SDSS and GMP (Fig. 3C, Table S3). For all samples treated at 50 °C, the SDSS/GMP ratio was lower, except for those with 0.5 % $CaCl_2$ (w/wet gluten weight). The lower the SDSS/GMP ratio, the higher the relative amount of large gluten protein aggregates in the VG. The SDSS/GMP ratio for samples with the combination of NaCl in both the process parameters and after-treatment process did not differ significantly from the samples without any salt added. However, this combined treatment differed significantly from the separate treatment with NaCl either in the washing process or in the after-treatment process when washed at 20 °C. This might be due to a coupled effect of NaCl in both processing steps leading to a higher amount of GMP and thus a lower SDSS/GMP ratio.

Looking at the absolute values of the SDSS and GMP content as well as the sum of both, it is noticeable that the extractability was significantly lower for salt-treated and freeze-dried samples (Fig. 3B, Table S3). However, the extractability of SDSS and GMP was better for VG (20 °C, 0.5 % $CaCl_2$, FD) than for VG (50 °C, 0.5 % $CaCl_2$, FD). Rombouts et al. found that the SDSS extractability of hydrated VG heated at 50 °C for 120 min did not decrease. Furthermore, the loss of extractability cannot be explained by the formation of new disulphide bonds. These bonds are reversible under reducing conditions and disulphide cross-linked proteins would therefore be soluble in the GMP-fraction [27]. The loss of extractability is therefore not primarily influenced by the temperature, but mainly by the addition of salt.

Salts may lead to the formation of non-reducible, non-SS cross-links by direct interaction between the proteins charged amino acid side chains or the polypeptide backbone [12,28–31]. Assessing the individual influences of single ions is difficult, as the detectable effects of anions and cations are not additive but synergistic, meaning that each salt has a specific effect on proteins [29]. The resulting effect on the properties of the protein is determined by the ion type and salt concentration [9,28,32].

3.3. Effect of process and after-treatment parameters on free and bound thiol groups

The Ellman assay was used to determine the content of free and bound thiol groups in the VG. The content of free thiols (SH) was between 1.04 μmol/g protein to 1.91 μmol/g protein and the content of bound thiols (SS) ranged from 17.13 μmol/g protein to 27.02 μmol/g protein (Fig. 4, Table S4). The SH content was influenced by the salt treatment and was significantly lower for the samples washed at 50 °C than in the corresponding sample washed at 20 °C. At a washing water temperature of 20 °C, most of the salt-treated samples differed significantly from the samples without salt. A significant difference was seen in all corresponding salt-treated samples except VG (20 °C, 3 % NaCl, 40 °C) and VG (50 °C, 3 % NaCl, 40 °C). This indicates a major effect of the drying temperature in the reduction of the SH content even if the sample was already exposed to higher washing water temperature. VG (20 °C, 0.5 % $CaCl_2$, FD) and VG (50 °C, 0.5 % $CaCl_2$, FD) showed a different behaviour than the NaCl-treated samples. VG (50 °C, 0.5 % $CaCl_2$, FD) exhibited a higher SH content than VG (20 °C, 0.5 % $CaCl_2$, FD) and both samples differed significantly from the non-salt-treated samples washed at the respective temperature.

High washing water temperatures and after-treatment with salt led to a significant decrease in SS content. VG (20 °C, 0.5 % $CaCl_2$, FD) and VG (50 °C, 0.5 % $CaCl_2$, FD) were both significantly lower compared to the corresponding control sample. No treatment led to the formation of new SS bonds. Chen et al. and Tuhumury et al. also found no increase in SS content in gluten of NaCl-treated dough [10,26].

For better comparability, the ratio of SS and SH was calculated. The SS/SH ratio was between 11.62 and 25.54 (Fig. 4C, Table S4). The SS/SH ratio was significantly higher for most samples washed at 50 °C compared to the respective sample washed at 20 °C. A high SS/SH ratio indicates a high relative content of bound thiols in the sample and thus a more strongly cross-linked protein network. This hypothesis is confirmed by the higher GMP content and lower SDSS content in samples washed at 50 °C. This results in a lower SDSS/GMP ratio in samples washed at 50 °C (Fig. 3, Table S3).

No statistical difference was found between VG (50 °C, 3 % NaCl, 40 °C) and VG (50 °C, 3 % NaCl, FD). This could be due to the fact that thermal treatment affects the gluten protein composition, regardless of the timing of heat exposure during the production process.

3.4. Effect of process and after-treatment parameters on the water retention capacity

The WRC of gluten depends on the composition [22] and protein conformation, determining the hydrophobicity and hydrophilicity. Both temperature and salt treatment influenced the WRC (Fig. 5, Table S5). The most substantial impact originated from the after-process treatment with 3 % NaCl (w/wet gluten weight), reducing the WRC by 14.6 % (where 14.6 % represents the relative reduction compared to 20 °C, FD). In comparison, exposure to NaCl during the washing process resulted in a lower WRC reduction. The combined effect of NaCl treatment during and after the washing process did not intensify the reduction of the WRC compared to the only after-process treatment. The reduction of the WRC in the presence of NaCl was previously described by Beck et al. [33], who attributed this effect to the competition between water and ions for the protein side chains. The presence of salts can thereby induce a shielding of electrostatic interactions of proteins, leading to an increase in hydrophobic interactions and increased surface hydrophobicity [34,35]. Consequently, the WRC of gluten proteins may be reduced.

Increasing the washing water temperature to 50 °C decreased the WRC by 8.3 % in non-salt-treated samples. This effect is well-known and it is related to the formation of aggregates limiting the water absorption and a reduced swelling capacity of the glutenins in particular. The occurrence of aggregates as the result of heat treatment during the VG production process was indicated by a decrease in both the SDSS content and the content of free thiols (see 3.2 and 3.3).

Interestingly, the combined application of a higher washing water temperature and salt treatments did not intensify the reduction in WRC. In contrast, the difference between the 20 °C and 50 °C samples became minor. In the case of the after-treatment with 0.5 % $CaCl_2$ (w/wet gluten weight), even a higher WRC at 50 °C washing water temperature was observed. No differences were found between FD and hot air-dried samples, indicating that a hydrothermal treatment has a stronger functional impact than hot air-treatment. This can be attributed to the reduced molecular mobility in dry samples, leading to an occurrence of structural changes at higher temperatures [36]. In general, both chloride salts and hydrothermal treatment resulted in a reduced WRC, indicating the impact of the process parameters on the protein conformation.

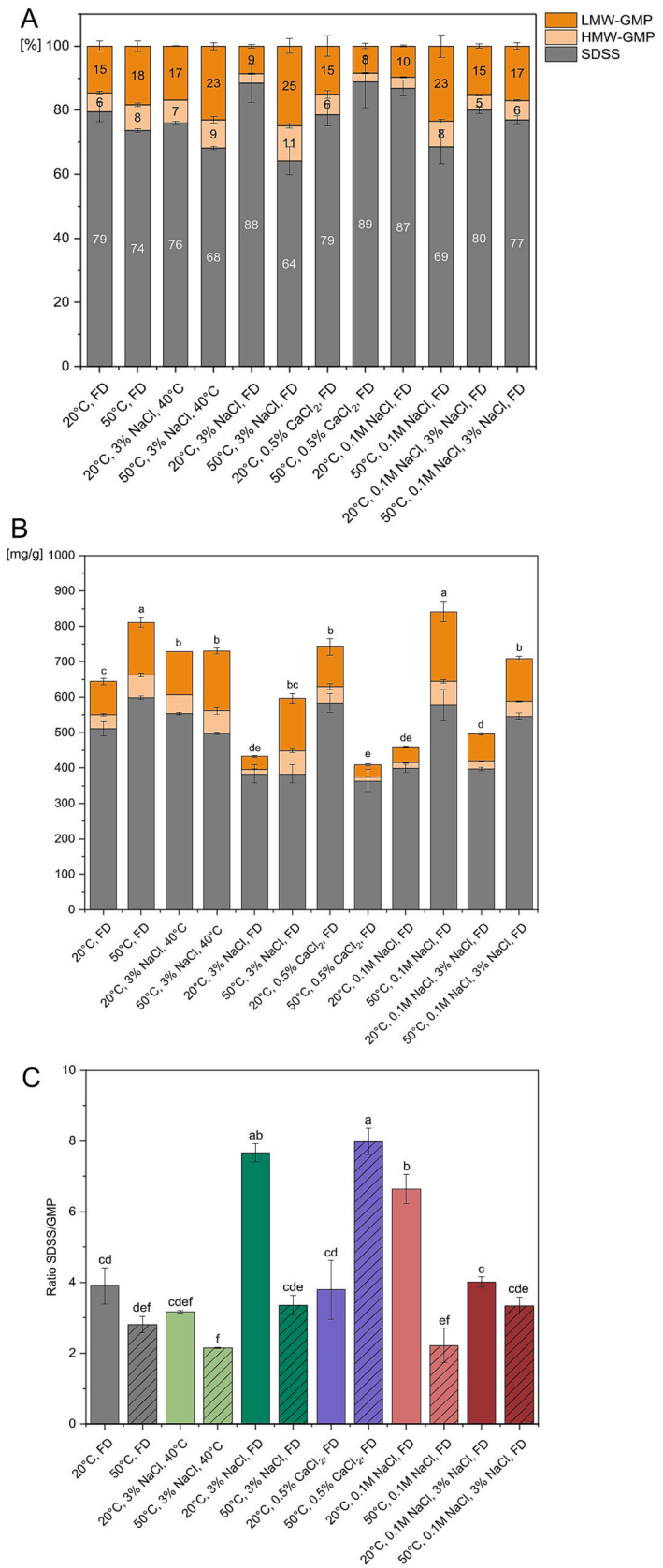

Fig. 3. Relative (A) and absolute (B) content of low-molecular weight glutenin macropolymer (LMW-GMP), high-molecular weight glutenin macropolymer (HMW-GMP) and ratio of SDS-soluble proteins (SDSS) and glutenin macropolymer (SDSS/GMP) (C) in salt-treated vital gluten samples and control samples without salt dried at 40 °C or freeze-dried (FD). All values are given as means ± standard deviation (n = 3). Values below 4 % are not shown. Significant differences between vital gluten samples are indicated by small superscript letters of the mean values (ANOVA, Tukey test, $p < 0.05$). Significant differences in (B) refer to the total amount of protein.

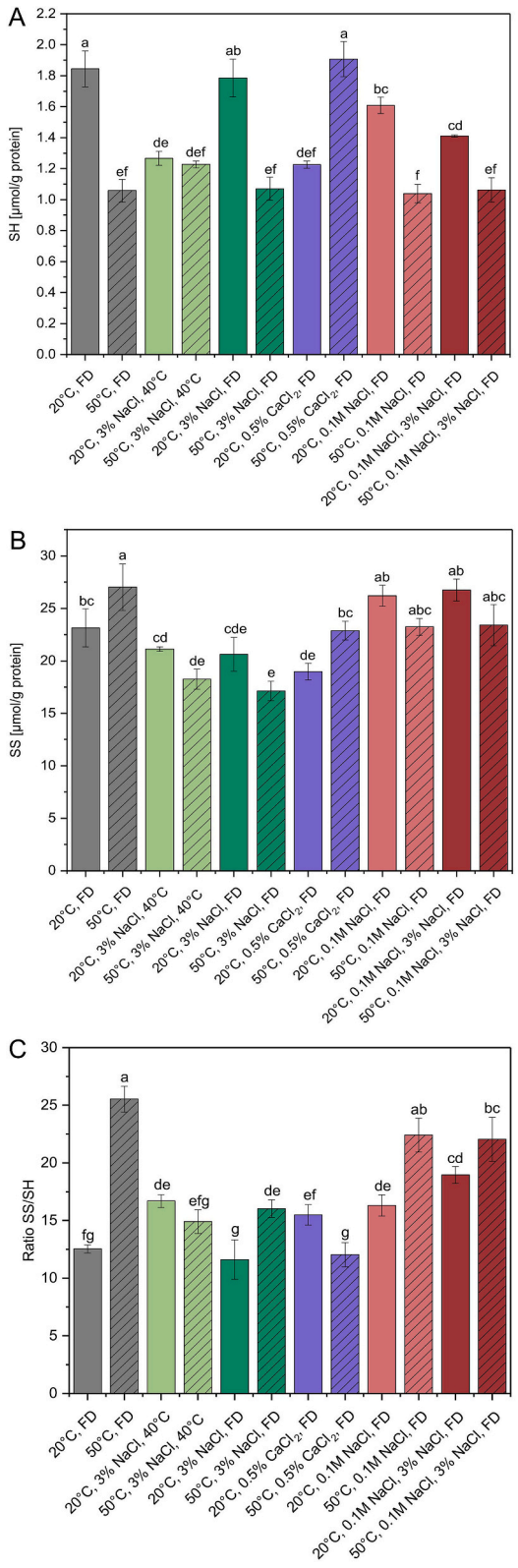

Fig. 4. Content of free (SH) (A) and bound (SS) (B) thiol groups and ratio of bound and free thiols (SS/SH) (C) in salt-treated vital gluten samples and control samples without salt dried at 40 °C or freeze-dried (FD). All values are given as means ± standard deviation (n = 3). Values below 4 % are not shown. Significant differences between vital gluten samples are indicated by small superscript letters of the mean values (ANOVA, Tukey test, $p < 0.05$).

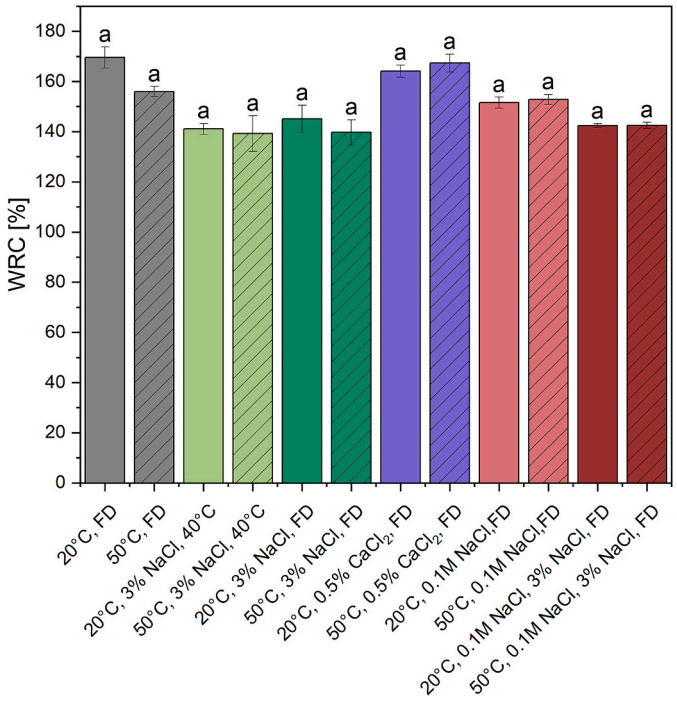

Fig. 5. Mean values of the water retention capacity of vital gluten samples in dependence of the applied process and after-treatment parameters. All values are given as means ± standard deviation (n = 3). Significant differences between vital gluten samples are indicated by small superscript letters of the mean values (Kruskal-Wallis test, Dunn's test, $p < 0.05$).

3.5. *Effect of process and after-treatment parameters on the rheological behaviour*

The viscoelastic behaviour of the gluten samples was characterized using small amplitude oscillatory and large deformation tests. Thermal stress exerted during the VG production resulted in a pronounced increase of the storage (G') and loss modulus (G"). A marked increase in the power law model parameter G'_0 further indicates this (Fig. 6). The power law parameter n' did not appear to be affected by thermal treatment alone. The large deformation behaviour was altered similarly as the zero shear viscosity η_0 increased, indicating a higher resistance to flow. Additionally, the pure elastic part J_0 decreased, indicating an increase in the rigidity of the sample and a lower degree of crosslinking. We reasoned that the increase in the moduli and rigidity originates from increasing aggregation of the proteins. This is consistent with the results obtained for the SSDS/GMP ratio and SS/SH ratio, both indicating an enhanced level of aggregation after thermal treatment.

In contrast, the presence of salts resulted in a decrease of G'_0, indicating a less solid-like behaviour. Interestingly, G" mainly remained unaffected by NaCl treatment during or after the washing process, while G' decreased markedly in the presence of NaCl and $CaCl_2$. However, n' increased drastically, indicating an increasing frequency dependency of G' due to a more fluid character of the samples. The same is observed by the reduction of η_0, indicating a highly deformable system with a low relative recovery [37]. The higher flowability can be explained by the lower water absorption potential of the salt-treated samples, resulting in a higher amount of unbound water in the system and, thus, reducing the zero shear viscosity, as deduced by Sun [37]. However, the protein network itself appears to be reinforced as applying NaCl or $CaCl_2$ during or after washing results in a more elastic, crosslinked gluten network. This effect originates from the well-known fact that NaCl strengthens protein-protein interactions as increasing salt concentrations lead to a shielding of the intrinsic positive charge of the protein. The resulting reduction of repulsive interactions leads to a strengthened network with

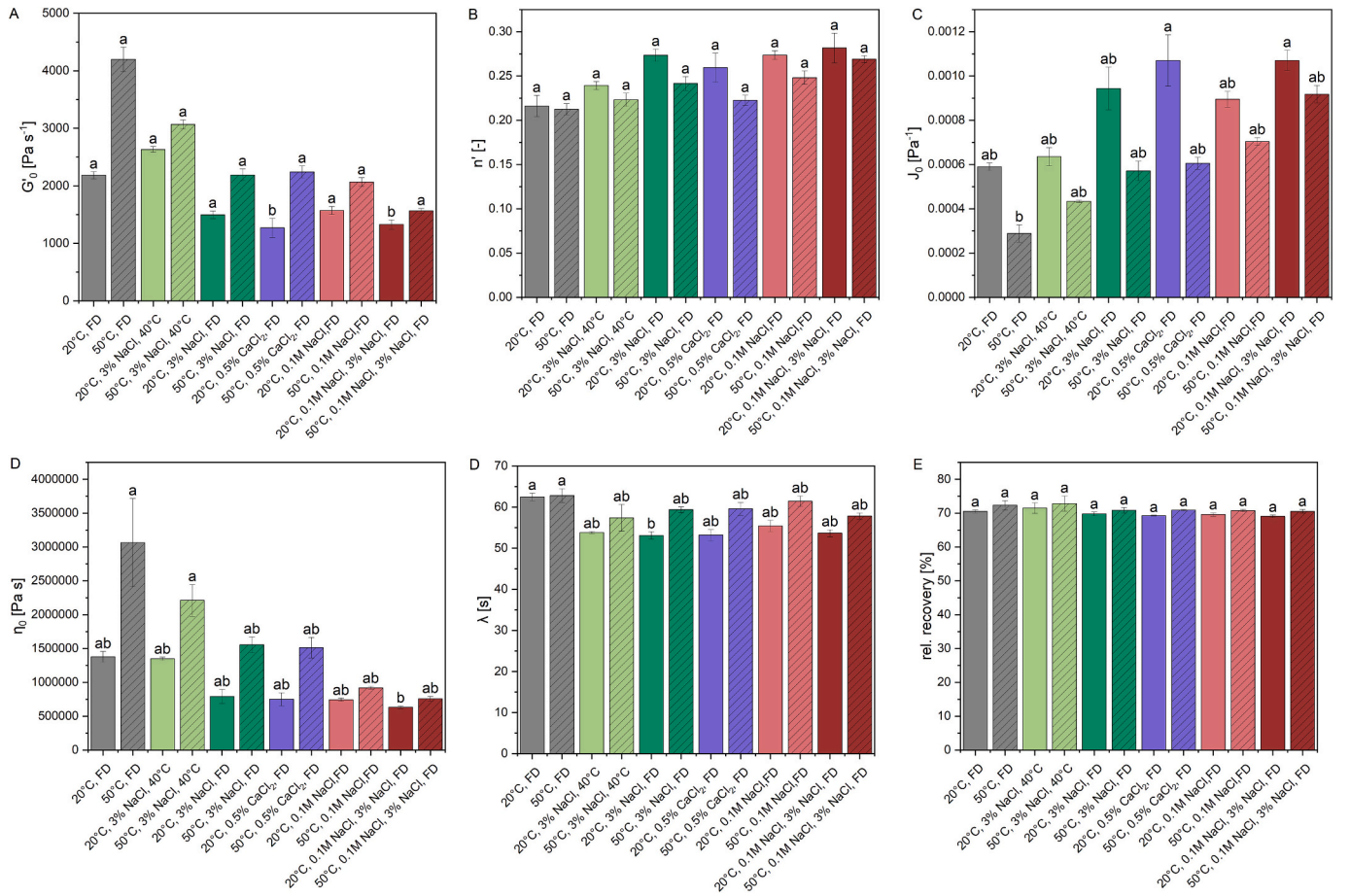

Fig. 6. Comparison of the viscoelastic properties and large deformation behaviour between the vital gluten samples obtained by applying various process and after-treatment parameters during the vital gluten production. (A) Intercept of the power law model for G'_0, (B) power law constant n', (C) instantaneous compliance J_0, (D) the zero shear viscosity η_0, (E) the retardation time λ resulting from the fitting of the creep phase of the creep-recovery test, and (F) the relative recovery as the ratio between the maximum compliance at the end of the recovery phase and the maximum compliance at the end of the creep phase. All values are given as means ± standard deviation (n = 3). Significant differences between vital gluten samples are indicated by small superscript letters of the mean values (Kruskal-Wallis test, Dunn's test, $p < 0.05$).

an enforced elastic character [33]. The stronger crosslinking further leads to a reduction of the retardation times, indicating a faster stress reduction in this more rigid network [38]. The strongest re-enforcing effect can be observed for $CaCl_2$, followed by the combined during and after washing-treatment with NaCl, which probably results in the highest NaCl concentration in the material. The combined application of the higher washing water temperature and salt treatments resulted in a mixed effect because the negative effect of the hydrothermal treatment was balanced out in the presence of salts. However, the application of thermal stress during and after the washing process could not be reverted by the presence of NaCl as indicated by the lower J_0 of the (50 °C, 3 % NaCl, 40 °C) VG compared to the (50 °C, 3 % NaCl, FD) VG.

3.6. Effect of process and after-treatment parameters on the bread quality

As breadmaking represents a primary application of VG, the effect of process and after-treatment parameters on the bread quality was assessed. Thus, the extracted VG were added at 5.5 % (w/flour dry weight) while bread making and bread quality was evaluated in terms of crumb hardness and specific volume. An increase in crumb hardness, accompanied by a decreased specific volume, became evident for VG undergoing hydrothermal treatment during washing (Fig. 7, Table S7). In contrast, applying salts during or after the washing process, significantly decreased crumb hardness and increased the specific volume, especially in the case of $CaCl_2$. The positive effect of a 0.5 % $CaCl_2$ (w/wet gluten weight) addition after the washing process was as pronounced as applying a 0.1 M NaCl-solution during the washing process combined with an addition of 3 % NaCl (w/wet gluten weight) after the washing process. The combined application of NaCl during and after the gluten washing process was the most effective way to positively influence the quality of VG as a bread improver.

The application of chloride salts during or after the washing process was further shown to reduce the adverse effects induced by hydrothermal stress during the washing process, leading to a crumb hardness comparable to the 20 °C, FD-VG. As the course of the crumb hardness seemed highly related to the elasticity of the VG derived from the large deformation testing, a linear fit was applied. The fit revealed a linear relation (Pearson's $r = 0.82$, $p < 0.05$, RSME = 0.14) between J_0 and crumb hardness, indicating a high predictability of the final bread quality from large deformation rheometry.

3.7. Identification of similarities and differences between vital gluten samples depending on the treatment

Principal component analysis (PCA) was used to identify similarities between samples treated with different process and after-treatment parameters based on their rheological behaviour and gluten protein composition (Fig. 8).

Three principal components (PC) accounted for 81.4 % of the variation of the data. PC1 accounted for 45.8 % of the variation, PC2 for

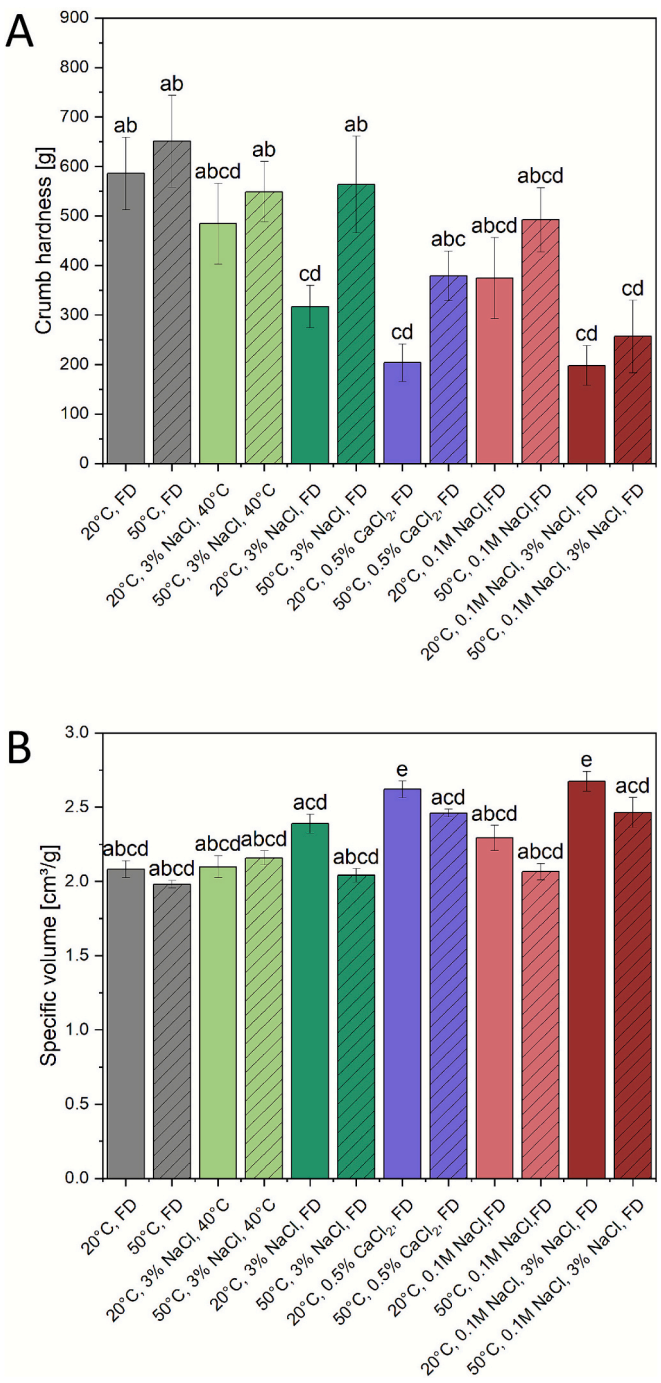

Fig. 7. Mean values of the crumb hardness (A) and specific volume (B) resulting from the baking test with the vital gluten samples. The results are presented in dependence of the process and after-treatment parameters applied during the vital gluten production. All values are given as means ± standard deviation (n = 3). Significant differences between vital gluten samples are indicated by small superscript letters of the mean values (Kruskal-Wallis test, Dunn's test, $p < 0.05$).

19.8 % and PC3 for 15.8 %. Fig. 8 shows the score plot and loading plot of PC1 and PC2, which accounted for 65.7 % of the variability. In the score plot, the formation of a cluster is visible. Three of the NaCl- and heat-treated samples clustered in the bottom-right. According to the loading plot, these samples were similar in terms of moisture. Along PC1, the samples of this cluster as well as VG (50 °C, 0.1 M NaCl, 3 % NaCl, FD) were closer to the 20 °C control sample than to the 50 °C control sample or at least shifted in the direction of the 20 °C control sample. VG (50 °C, 0.1 M NaCl, FD) was particularly close to the 20 °C control sample. This supports the hypothesis that emerged from the analysis of the analytical-chemical and rheological data: heat effects that lead to a change in protein composition and rheological properties and functionality as a dough enhancer can be partially compensated by treatment with salt. VG (20 °C, 0.1 M NaCl, FD) and VG (20 °C, 3 % NaCl, FD) were in similar positions along the PC1 axis but were more split up along the PC2 axis. This shows that the timing of salt addition influenced the properties of the VG to a different extent.

The score plot of PC1 and PC3 showed no particular clustering of the samples. This proportion of variability can be explained by differences in SH content, SS/SH ratio, SDSS/GMP ratio and SDSS and GMP content of the VG, as these parameters influenced PC3 the most. Most corresponding samples, which were close in the score plot of PC1 and PC2, were more widely spread along PC3. VG (20 °C, 0.5 % $CaCl_2$, FD) and VG (50 °C, 0.5 % $CaCl_2$, FD) for instance were further apart along PC3 than PC2. This suggests that the temperature of the washing water is associated with parameters like SH content, SDSS/GMP ratio, glia/glut ratio, gliadin content, specific volume and J_0/J_1.

In both score plots, the samples treated with $CaCl_2$ were not close to the respective NaCl-treated sample. The PCA thus visualizes the analytical results that even though the concentration of the two salts is not the same, $CaCl_2$ causes different effects in the after-treatment than NaCl. However, due to the small sample size of the sample set, no assumption can be made about a systematic influence of monovalent or kosmotropic and divalent or chaotropic salts. Nevertheless, the observations made suggest that the salts interact in different ways with the charged side chains of the protein, its amino acid backbone and its hydration layer [29,30,39].

3.8. Prediction of rheological properties and functionality of gluten protein as a dough enhancer based on gluten composition

At last, the predictability of the bread quality was assessed based on the analytical and functional data. To identify the parameters describing the VG functionality, a PCA was conducted with the results of the WRC test, the parameters extracted from the rheological tests, and the baking tests. Three principal components (PC) described 94.3 % of the variance, where PC1 captured 74.5 %, PC2 13.5 %, and PC3 6.4 % of the data variation (Fig. S1). The first PC strongly positively correlated with crumb hardness, the power law parameter G'_0, the zero shear viscosity, and the relative recovery. On the other hand, negative correlations appeared with J_0 and J_1, the power law exponent n', and the specific volume with this component. PC2 was positively correlated with the WRC. Consequently, all results in PC1 explained a major share of the rheological properties and functionality as a dough enhancer of the VG. Subsequently, the parameters correlating with PC1 were predicted by data on protein composition using partial least squares regression (PLSR) with the number of components using cross-validation. PLSR was able to predict 61 % of the variance of the VG functionality (rheological properties and functionality as a dough enhancer) using the compositional data. According to the variable importance in projection (VIP) scores, the glutenin content, glia/glut ratio, HMW of GMP, and SDSS/GMP ratio are considered to be important for the projection of the model. An approach to reduce the complexity of the model by only using these parameters was successfully conducted and resulted in a PLSR model predicting 84 % of the variance. Additional attempts to further reduce the complexity of the model indicated a negligible importance of the glia/glut ratio, as the explained variance remained the same after the exclusion of this parameter. The linear regression equation modelling

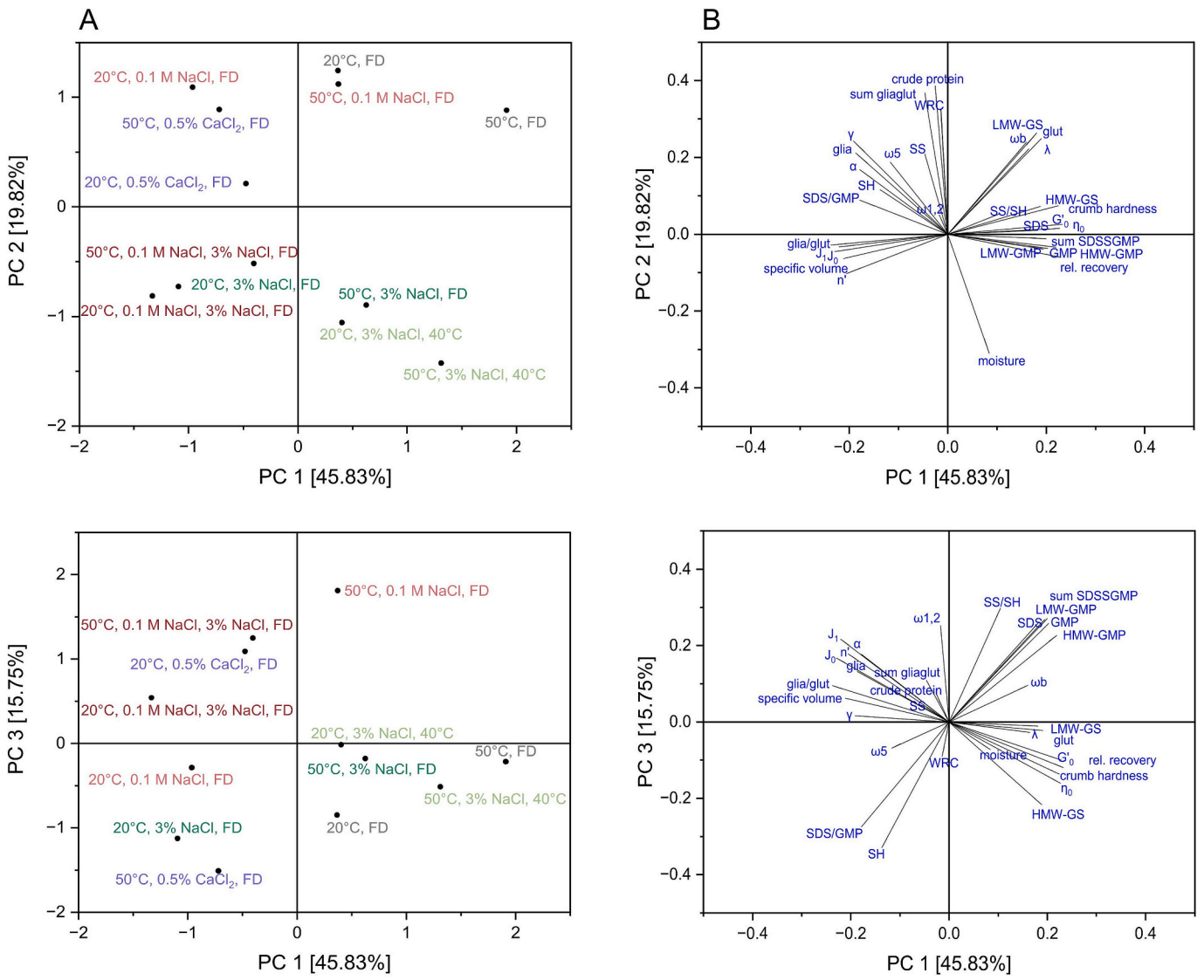

Fig. 8. Score plot (A) and loading plot (B) of the principal component analysis (PCA) with analytical and rheological parameters of salt-treated vital gluten samples, control samples without salt dried at 40 °C or freeze-dried (FD) and baking experiments. Content of free (SH) and bound (SS) thiol groups, ratio of bound and free thiols (SS/SH), content of gliadins (glia) and gluten protein types ω5-, ωb-, ω1,2-, α- and γ-gliadins, content of glutenins (glut), low-molecular-weight glutenin subunits (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS), sum of gliadins and glutenins (sum gliaglut), gliadin-to-glutenin (glia/glut) ratio, content of SDS-soluble proteins (SDSS), content of glutenin macropolymer (GMP), low-molecular-weight glutenin macropolymer (LMW-GMP) and high-molecular-weight glutenin macropolymer (HMW-GMP), sum of SDS-soluble proteins and glutenin macropolymer (sum SDSSGMP), ratio of SDS-soluble proteins and glutenin macropolymer (SDSS/GMP), the water retention capacity (WRC), the relative recovery (rel. recovery), the intercept of the power law model for G' (G'_0), the power law constant (n'), the instantaneous compliance (J_0), the viscoelastic compliance (J_1), the zero shear viscosity η_0 and the retardation time (λ) resulting from the fitting of the creep phase of the creep-recovery test.

VG functionality (resembled by the first PC as abovementioned) was the following:

$$PC1 = 0.126 \times Glut + 0.049 \times HMW - GMP + 0.157 \times SDSS/GMP - 6.089 \quad (3)$$

Thus, it was possible to predict gluten functionality (rheological properties and functionality as a dough enhancer) based on the glutenin content (Glut), the HMW fraction of GMP, and the SDSS/GMP ratio.

3.9. Identification of synergistic effects

The previous section suggested the presence of synergistic effects between the washing water temperature and the presence of chloride salts. To further investigate the plausibility of interactions between process parameters, a linear model was applied to identify potential two-way interactions. As shown in section 3.8, the VG rheological behaviour and functionality as a dough enhancer could be predicted based on the gluten composition. Additionally, VG functionality was successfully described by PC1, explaining the majority of the variance of the rheological properties and functionality as a dough enhancer of the VG.

Therefore, a linear model was fitted to PC1 using five main effects: the washing water temperature and four treatment processes, namely replacing distilled water with 0.1 M NaCl solution during the extraction, an after-treatment with 3 % NaCl (w/wet gluten weight), an after-treatment with 0.5 % $CaCl_2$ (w/wet gluten weight), and a combination of 0.1 M NaCl solution during the extraction and a NaCl after-treatment process. The model treated the washing and after-treatment processes as categorical variables and included the main effects as well as the interaction terms. The linear model ($p < 0.01$) fit PC1 with an adjusted R^2 of 0.98 and a root mean square error of 0.42. The model is

Table 1
Results of the linear model with interaction terms. The indicated significance code symbols refer to the following values: '***' 0.001, '**' 0.05, '*' 0.1.

Coefficients	Estimate	Standard Error	t value	Pr(>|t|)	Significance
(Intercept)	4.5876	0.1469	31.237	< 2e-16	***
Washing water temperature	2.6078	0.2037	12.807	< 2e-16	***
0.1 M NaCl	−4.5510	0.2077	21.911	< 2e-16	***
3 % NaCl	−3.7930	0.2077	18.262	< 2e-16	***
0.5 % $CaCl_2$	−4.4299	0.2077	21.328	< 2e-16	***
0.1 M NaCl, 3 % NaCl	−7.0206	0.2077	33.801	< 2e-16	***
Washing water temperature x 0.1 M NaCl	−0.5798	0.2880	−2.013	0.04952	**
Washing water temperature x 3 % NaCl	0.5687	0.2880	1.974	0.05386	*
Washing water temperature x 0.5 % $CaCl_2$	0.6996	0.2880	2.429	0.01878	**
Washing water temperature x 0.1 M NaCl, 3 % NaCl	−1.0914	0.2880	−3.789	0.00041	***

summarized in Table 1.

The results suggest significant impacts of the five main effects and their interaction terms. It is evident that the impact of an increase in washing water temperature, and the presence of chloride salts have opposing influences on PC1. Considering the interaction between the washing water temperature and the treatment with chloride salts, interactions are present for each combination. However, slope of the interactive term (washing water temperature x 3 % NaCl) is not significantly different from zero on a 5 % significance level. Contrarily, the interaction terms (washing water temperature x 0.1 M NaCl), (washing water temperature x 0.5 % $CaCl_2$), and (washing water temperature x 0.1 M NaCl, 3 % NaCl) are statistically significant. The greatest effect was found for the interaction term (washing water temperature x 0.1 M NaCl, 3 % NaCl), indicating that the combined effect of the addition of chloride salts during the washing process and the after-treatment is more impactful.

Thus, the model supports the previously stated hypothesis that there are synergistic effects between thermal treatment and chloride salt addition. The results suggest that the observed adverse effects of thermal stress during the washing process can be partially mitigated by adding chloride salts during the washing process or as an after-treatment.

4. Conclusion

The modification of process and after-treatment parameters with heat treatment and chloride salts caused a synergistic effect on gluten protein composition and rheological properties and functionality as a dough enhancer. Gluten protein composition was mainly affected by temperature during gluten washing or drying. Moreover, heat treatment had an adverse effect on the rheological properties and functionality of gluten protein as a dough enhancer. At the same time, specific bread volume and crumb hardness were positively influenced by salt-treated VG. The addition of 0.5 % $CaCl_2$ (w/wet gluten weight) to wet gluten had a clear effect on VG protein composition, resulting in the highest bread volume and a major reduction in crumb hardness. One of the key findings of our study was that effects of heat treatment are partially reversible by the incorporation of chloride salts during the gluten extraction process.

Partial least squares regression revealed that the rheological behaviour of the VG samples and the baking results can be predicted by the variables glutenin content, HMW-GMP and SDSS/GMP ratio. Consequently, the variability of rheological properties and functionality of gluten protein as a dough enhancer can be explained by the protein composition to a large extent. This synergistic effect was further supported by a linear model with first-order interaction terms. Limitations of the study include that it focused on certain pre-defined variations, used only one wheat flour for VG production and was performed on lab-scale, which could only partly model industrially relevant production settings. Further research could include salts with different anions and/or different concentrations as well as more industrially relevant drying parameters. Future work would also benefit from including additional complementary analyses such as microstructural investigations, e.g., using confocal laser scanning microscopy.

Understanding how different parameters in the washing process and after-treatment affect gluten protein composition and the rheological properties and functionality of gluten protein as a dough enhancer, enables a more targeted production design of VG and opens up new opportunities for end product development.

Abbreviations

ANOVA analysis of variance
GMP glutenin macropolymer
HMW-GS high-molecular-weight glutenin subunits
HMW-GMP high-molecular-weight glutenin macropolymer
LMW-GS low-molecular-weight glutenin subunits
LMW-GMP low-molecular-weight glutenin macropolymer
PCA principal component analysis
PC principal component
PWG Prolamin Working Group
SDSS SDS-soluble protein
SH free thiol groups
SS bound thiol groups
VG vital gluten

CRediT authorship contribution statement

Nina Hoeller: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thekla Alpers:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Kerstin Holtz:** Writing – review & editing, Methodology, Investigation. **Iain Whitehead:** Writing – review & editing, Formal analysis, Conceptualization. **Thomas Becker:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Informed consent

Not applicable.

Compliance with ethical standards

Not applicable.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.149678.

Data availability

Data will be made available on request.

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