

# Impact of the preparation procedure on gliadin, glutenin and gluten contents of wheat starches determined by RP-HPLC and ELISA

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Received: 16 December 2015 / Revised: 9 March 2016 / Accepted: 30 March 2016 / Published online: 15 April 2016  
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**Abstract** Celiac disease patients rely on safe gluten-free products, and the use of purified wheat starch (WSt) as part of the diet is usually well tolerated. However, uncertainties about residual gluten amounts in WSt remain, because ELISAs target the alcohol-soluble prolamins (gliadin) fraction of gluten, but hardly detect the alcohol-insoluble glutelins (glutenin) fraction. Therefore, gliadin, glutenin and gluten contents of WSt prepared from doughs on a laboratory scale were monitored by RP-HPLC. WSt washed with water from optimally or overmixed doughs had the lowest gluten contents. Gliadins were removed more extensively than glutenins during consecutive washing steps, so that gluten contents analyzed by ELISA were lower than those by RP-HPLC in 17 out of 24 WSt samples. The inability to detect glutelins by ELISA may thus lead to an underestimation of gluten contents in WSt. These findings highlight the need for improved analytical methods capable of detecting both prolamins and glutelins in processed food samples.

**Keywords** Celiac disease · Enzyme-linked immunosorbent assay (ELISA) · Gliadin · Gluten analysis · Reversed-phase high-performance liquid chromatography (RP-HPLC) · Wheat starch

## Introduction

Approximately 0.9 % of the world population is affected by celiac disease (CD), which is a chronic immune-mediated

inflammatory disease of the upper small intestine in genetically susceptible persons triggered by the ingestion of gluten proteins from wheat, rye, barley and possibly oats [1, 2]. A strict lifelong gluten-free diet (GFD) is the essential treatment for CD patients to prevent small intestinal villous atrophy and subsequent malabsorption, malnutrition and severe complications [3]. The tolerable level of gluten for most CD patients is reported to be between 10 and 50 mg daily, although particularly sensitive or recovering patients may tolerate even less than 10 mg/day [4]. This average tolerable gluten level is reflected by the threshold of 20 mg/kg for gluten-free products laid down in Codex Standard 118-1979 [5]. Gluten-free foods may be made either from naturally gluten-free ingredients or from gluten-containing ingredients that have been specially processed to remove gluten below 20 mg/kg [6]. Wheat starch (WSt), obtained after separation of wheat flour into starch, gluten and by-products using the dough, dough–batter or batter process [7], may be additionally purified by repetitive washing and centrifugation steps to render it gluten-free or low in gluten (<100 mg/kg). Its composition of ≈75 % large lenticular-shaped A granules and ≈25 % small round B granules (each by weight) makes it unique compared to other starch sources and contributes to favorable textural properties of baked goods [8, 9]. The use of purified WSt in gluten-free foods is generally well-accepted in northern Europe and the UK [10]. However, doubts about its safety for CD patients remain, especially in southern Europe, the USA and Canada [11–13], because early studies reported possible adverse effects of a WSt-containing GFD [14, 15]. Later studies reported that the dietary response to a WSt-based GFD was as good as that to a naturally GFD [16]. The daily ingestion of wheat-based starch hydrolysates, glucose syrups and maltodextrins containing only trace amounts of gluten had no harmful effect on the small intestinal mucosa

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[17]. WSt in the GFD also had no effect on persistent duodenal intraepithelial lymphocytosis [18].

The gluten content of foods is most commonly measured by enzyme-linked immunosorbent assays (ELISAs) using antibodies that have been raised against the alcohol-soluble prolamins (gliadin in the case of wheat) fraction of gluten [19]. Although also CD-active [20], the alcohol-insoluble glutelin (glutenin in the case of wheat) fraction is not targeted and the prolamins content multiplied by a factor of 2 to obtain the gluten content [5]. However, this factor resulted in an underestimation of gluten contents in industrial WSt, because gliadin contents were exceptionally low [21]. Due to their partial solubility in aqueous systems, gliadins may be depleted more extensively during starch processing than glutenins. Despite this knowledge, the gliadin and glutenin contents of WSt depending on the preparation procedure and successive washing steps have so far not been investigated in great detail.

Therefore, the aim of this study was to analyze gliadin and glutenin contents of WSt prepared from doughs on a laboratory scale. Dough mixing times and wash solutions during gluten–starch separation were varied. Gliadin and glutenin contents of the starches were measured by RP-HPLC at the beginning and after each of three successive washing steps, and the results were compared to those for gliadins by competitive ELISA. The main factors influencing crude protein, gliadin, glutenin and gluten contents were evaluated. A special focus was laid on gliadin-to-glutenin (gli/glu) ratios to assess whether the analysis of gliadins by ELISA may lead to an underestimation of gluten contents.

## Materials and methods

### Materials

Acetonitrile (HPLC grade) was from J. T. Baker (Deventer, The Netherlands), dithiothreitol was from Serva (Heidelberg, Germany), and disodium hydrogen phosphate, ethanol, potassium dihydrogen phosphate, 2-propanol, sodium chloride, tris(hydroxymethyl)aminomethane (Tris) and urea were purchased in 'pro analysis' quality from Merck (Darmstadt, Germany). PWG-gliadin [22] was kindly provided by Prof. Dr. Peter Koehler, Chairman of the Working Group on Prolamins Analysis and Toxicity. Water for chromatographic separations was purified using a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany). Grains of the German winter wheat cultivar Akeur (harvest 2013) were donated by I.G. Pflanzenzucht (Munich, Germany) and milled into flour using a Quadrumat Junior mill (Brabender, Duisburg, Germany). After sieving (mesh size 200  $\mu\text{m}$ ), the flour was allowed to rest for 2 weeks prior to

the determination of moisture and ash contents according to ICC Standards 110/1 and 104/1, respectively [23, 24]. The flour had 13.3 % moisture and 0.50 % ash.

### Preparation of wheat doughs

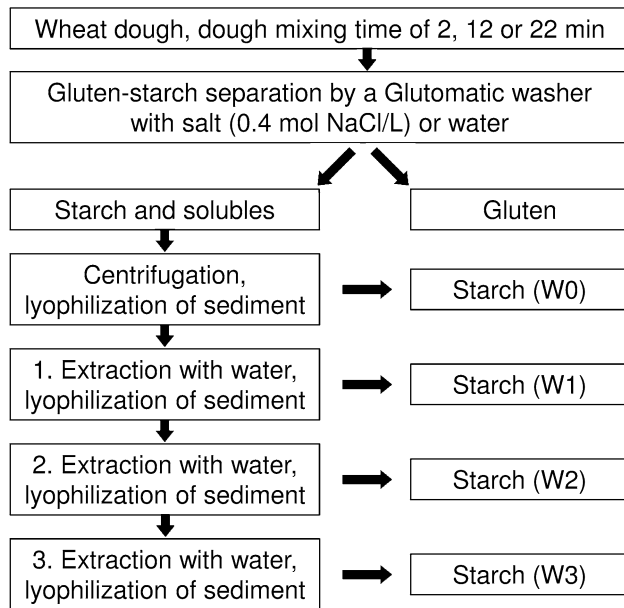
Water absorbance and dough development time (22 °C, 550 Brabender Units) were determined in a Microfarinograph (Brabender) on the basis of 10 g of wheat flour [25]. Using the optimal water absorbance (61 %), three different doughs were prepared on the basis of 300 g of wheat flour in a Farinograph (Brabender) equipped with a Z blade mixer. One dough was mixed for only 2 min (undermixed), one was mixed to the optimum consistency for 12 min, and one was mixed for 22 min (overmixed). After removal from the mixer, the doughs were allowed to rest for 5 min at 22 °C and  $\geq 60$  % relative humidity and split into 19 g portions.

### Preparation of wheat starches

Two 19 g portions (a and b) of each wheat dough (mixing times 2, 12 and 22 min) were washed once with 530 ml distilled water for 10 min by a Glutomatic 2200 gluten washer (Pertin, Hägersten, Sweden) [26]. In a similar experiment, two 19 g portions of each wheat dough were washed with 530 ml salt solution (0.4 mol/l NaCl) by the gluten washer. The effluent of each portion containing starch and solubles was collected, centrifuged (3550 $\times$ g, 25 min, 22 °C) and the sediment lyophilized (Gamma 1–20, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). Of the homogenized sediment, 1.5 g (W0) was set aside and the rest was subsequently extracted with a fivefold excess of water by vortexing for 30 s and vigorous magnetic stirring for 30 min at 22 °C. This suspension was centrifuged (3550 $\times$ g, 25 min, 22 °C) and the sediment lyophilized (W1). This procedure was repeated twice to yield the W2 and W3 starch sediments (Fig. 1). The starches are designated in the following way, kneading time (2, 12 or 22 min)/glutomatic wash solution (water or salt)/washing step (W0, W1, W2 or W3), for example, 2 min/water/W0.

### Determination of salt, crude protein and gliadin contents

The sodium contents of starches that had been washed from doughs with 0.4 mol/l NaCl solution were determined using a sodium-selective electrode (ISE) [27]. Fifty milligrams of starch (2, 12 or 22 min/salt/W0–W3) was suspended in 3 ml purified water and vortexed for 5 min followed by the addition of 3 ml total ionic strength adjustment buffer (TISAB, 1 mol/l Tris), vortexing and centrifugation (3550 $\times$ g, 1 min, 22 °C). The sodium concentrations



**Fig. 1** Overview of the preparation procedure of wheat starches. Dough mixing times (2, 12 or 22 min) and glutomatic wash solutions (water or 0.4 mol/l NaCl solution) were varied. After the initial gluten–starch separation, the wheat starches (W0) underwent three successive washing steps with water (W1–W3)

were measured in the clear supernatant by ISE and calibrated by external eight-point standard calibration in the range of 5–5000 mg sodium/l. Sodium-free dry masses of lyophilized starches were calculated. The nitrogen contents of WSt were measured by the Dumas combustion method using a TruSpec nitrogen analyzer (Leco, Moenchengladbach, Germany). The nitrogen content was multiplied with a factor of 5.7 to calculate the crude protein (CP) content (ICC Standard 167) [28]. Gliadin contents were quantitated by ELISA using the RIDASCREEN® Gliadin competitive kit (R-Biopharm, Darmstadt, Germany). WSt was extracted with 60 % ethanol strictly according to the manufacturers' instructions for starch samples. The subsequent ELISA procedure was carried out exactly as described in the kit manual. Additional dilutions of sample extracts were made as appropriate. WSt (a and b) were each extracted in duplicate, and each extract was applied into two cavities of the 96-well plate ( $n = 8$ ).

#### Extraction of albumins/globulins, gliadins and glutenins from wheat starches

WSt (100 mg) was extracted sequentially with salt solution ( $2 \times 0.20$  ml; 0.4 mol/l NaCl with 0.067 mol/l  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.6) for 10 min at 22 °C (albumins/globulins), with 60 % (v/v) ethanol ( $3 \times 0.15$  ml) for 10 min at 22 °C (gliadins) and with glutenin extraction solution ( $3 \times 0.15$  ml; 50 % (v/v) 1-propanol/0.1 mol/l Tris–HCl,

pH 7.5, containing 0.06 mol/l (w/v) dithiothreitol and 2 mol/l (w/v) urea) for 30 min at 60 °C under nitrogen (glutenins) according to Wieser et al. [29] with some minor modifications. Each extraction step started with vortex mixing for 2 min at 22 °C followed by magnetic stirring. The suspensions were centrifuged ( $3550 \times g$ , 25 min, 22 °C) and the respective supernatants were combined and made up to 0.5 ml with the extraction solvent. All supernatants were filtered (Whatman™ Spartan 13/0.45 RC, GE Healthcare, Freiburg, Germany) and analyzed by RP-HPLC. WSt (a and b) were each extracted in duplicate ( $n = 4$ ). For the purpose of comparing the extracts from starches with the original wheat flour, 100 mg of wheat flour (cv. Akteur, 2013) was also sequentially extracted as described above, with the only difference that single extraction volumes were  $2 \times 1$  ml for albumins/globulins and glutenins and  $3 \times 0.5$  ml for gliadins, and the final volume was made up to 2 ml.

#### Reversed-phase high-performance liquid chromatography

All fractions were analyzed by RP-HPLC with the following conditions [25]: instrument, Jasco XLC with Chrom-Pass software for instrument control and data analysis (Jasco, Gross-Umstadt, Germany); column, Acclaim™ 300 C18 (particle size 3  $\mu\text{m}$ , pore size 30 nm,  $2.1 \times 150$  mm, Thermo Fisher Scientific, Braunschweig, Germany); temperature, 60 °C; injection volume, 30  $\mu\text{l}$  for albumins/globulins and glutenins, 10  $\mu\text{l}$  for gliadins; elution solvents, trifluoroacetic acid (TFA) (0.1 %, v/v) in water (A) and TFA (0.1 %, v/v) in acetonitrile (B); linear gradient, 0 min 20 % B, 20 min 60 % B for albumins/globulins; 0 min 24 % B, 30 min 56 % B for gliadins and glutenins; flow rate, 0.2 ml/min; detection, UV absorbance at 210 nm; calibration reference PWG-gliadin [22] in the range of 1.2–34.9  $\mu\text{g}$ . After each run, the column was cleaned with 90 % B for 5 min and equilibrated with the starting solvent for 10 min. All quantitative values are given as g/100 g based on flour or WSt. Percentages of gluten protein types and non-gluten proteins are given based on CP contents. Recovery rates, limits of detection (LOD) and limits of quantitation (LOQ) of the RP-HPLC were assessed by spiking one sample of gluten-free wheat starch (gfWSt, Merck, Darmstadt, confirmed to contain <10 mg/kg of gluten by RIDASCREEN® Gliadin competitive and <5 mg/kg of gluten by RIDASCREEN® Gliadin) with different amounts of wheat flour (cv. Akteur, 2013) to yield 0.25, 0.20, 0.10, 0.05 and 0.03 g gliadin per 100 g of gfWSt, corresponding to 0.13, 0.10, 0.05, 0.025 and 0.01 g glutenin and 0.38, 0.30, 0.15, 0.08 and 0.04 g gluten per 100 g of gfWSt. The wheat flour and gfWSt mixture with the highest gluten content were subsequently diluted down with gfWSt to yield the four lower

gluten amounts [30]. All mixtures were mixed for 24 h using an overhead shaker (Turbula, Willy A. Bachofen Maschinenfabrik, Muttenz, Switzerland) and five replicates of the gfWSt and the five spiked samples were extracted and analyzed in duplicate by RP-HPLC as described above. Recoveries were calculated from the peak areas of the RP-HPLC chromatograms using PWG-gliadin as calibration reference and comparison to the calculated amounts of the samples. LODs were calculated from the signal of the gfWSt + 3 times the standard deviation of the gfWSt sample ( $n = 10$ ), and the LOQs were the signal of the gfWSt + 10 times the standard deviation of the gfWSt sample, for the gliadin and glutenin extracts, respectively [31, 32].

### Data analysis

Three-way analysis of variance (ANOVA) with kneading time, wash solution and washing step as factors and Tukey's test ( $p < 0.05$ ) was used to identify significant differences between these three factors and to identify the main effects that influence CP, gliadin, glutenin and gluten contents. Significant differences between washing steps within one set of starch samples were identified by one-way ANOVA with washing step as factor and Tukey's test ( $p < 0.05$ ). Two-way ANOVA with washing step and analytical method (ELISA vs. HPLC) as factors and Tukey's test ( $p < 0.05$ ) revealed significant differences between both analytical methods. CP, gliadin, glutenin and gluten contents were correlated using Pearson product-moment correlations. Correlation coefficients  $>0.78$ ,  $0.67$ – $0.78$ ,  $0.54$ – $0.66$  and  $<0.54$  were considered as strong, medium, weak and no correlations, respectively [25]. All statistical analyses were carried out with SigmaPlot 12.0 (Systat Software, San Jose, CA, USA).

## Results

### Qualitative profiles of gliadins and glutenins extracted from wheat starches, recovery and limits of detection and quantitation

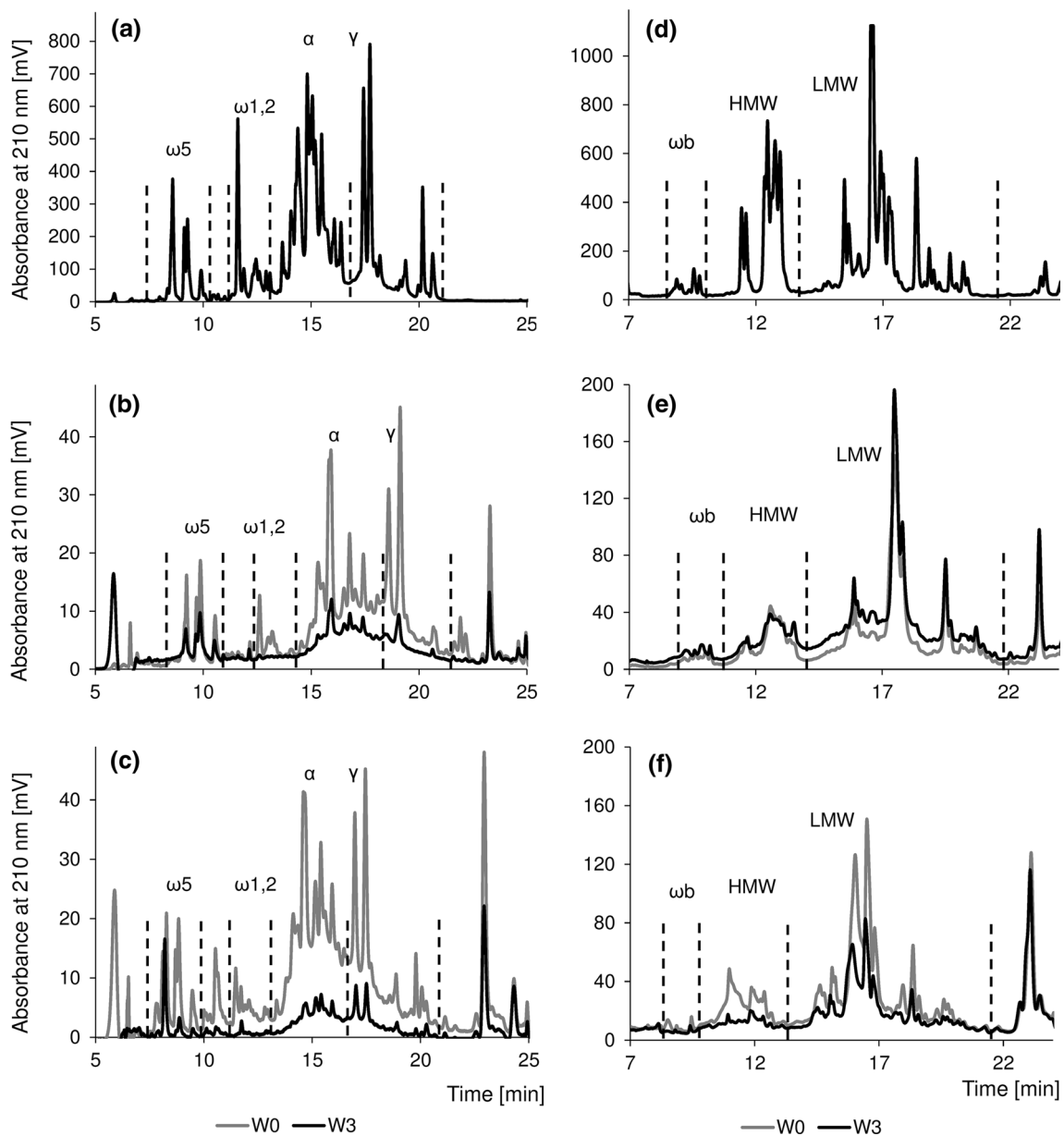
The wheat flour (cultivar Akteur, 2013) used for dough preparation and spiking of gfWSt contained  $13.13 \pm 0.01$  g/100 g CP composed of 1.30 g/100 g albumins/globulins (9.9 %), 7.59 g/100 g gliadins (57.8 %, composed of 5.4 %  $\omega 5$ -, 5.0 %  $\omega 1,2$ -, 30.0 %  $\alpha$ - and 17.4 %  $\gamma$ -gliadins), 3.72 g/100 g glutenins [28.3 %, composed of 1.2 %  $\omega b$ -gliadins, 9.4 % high molecular weight glutenin subunits (HMW-GS) and 17.7 % low molecular weight glutenin subunits (LMW-GS)] and 0.52 g/100 g residual proteins (4.0 %; all percentages referred to CP

content). The resulting gli/glu ratio of 2.0 lay within the range of 1.5–3.1 reported for common wheat [21], and the RP-HPLC chromatograms showed wheat-specific profiles for the gliadin and glutenin fractions [33] (Fig. 2a, d) comparable also to the reference PWG-gliadin [22].

Any residual water- and salt-soluble proteins were first removed from WSt by two subsequent extraction steps with saline. The gliadin fractions extracted from WSt 12 min/salt and 12 min/water directly after laboratory-scale gluten–starch separation (W0) also showed typical elution profiles. In contrast to this, only a few discernible peaks (e.g.,  $\gamma$ -gliadins) were visible after the third washing step (W3) (Fig. 2b, c). The glutenin fractions of the same WSt revealed characteristic profiles comparable to those of wheat flour, although some peaks within one group of proteins (HMW-GS) were less clearly separated, supposedly due to the lower concentration. The difference between washing steps W0 and W3 was less obvious for the glutenins than for the gliadins, because the peak profiles were almost the same in W0 and W3 for WSt 12 min/salt and only slightly reduced in W3 compared to W0 for WSt 12 min/water (Fig. 2e, f). Corresponding observations were made for the gliadin and glutenin fractions extracted from steps W0 and W3 of WSt 2 min/salt, 2 min/water, 22 min/salt and 22 min/water (chromatograms are not shown). Recovery rates were 93–98 % for gliadins and 91–99 % for glutenins calculated for the samples spiked at 0.38, 0.30 and 0.15 g gluten. The LODs and LOQs were 0.012 and 0.04 g/100 g for gliadins and 0.018 and 0.06 g/100 g for glutenins, respectively.

### Quantitation of gliadin, glutenin and gluten contents by RP-HPLC in wheat starches separated with salt solution after dough mixing times of 2, 12 and 22 min

The gliadin contents of WSt obtained from undermixed dough after gluten–starch separation with 0.4 mol/l NaCl solution (2 min/salt) ranged from  $0.23 \pm 0.07$  g/100 g (W0) to  $0.15 \pm 0.03$  g/100 g (W3). Although the gliadin content tended to be lower in W3, only the difference to W1 was significant, but not to W0 and W2. The glutenin contents were between  $0.42 \pm 0.07$  g/100 g (W0) and  $0.38 \pm 0.01$  g/100 g (W3) and showed no significant differences between the successive washing steps (Fig. 3a). The resulting gli/glu ratios of the 2 min/salt WSt were 0.5, 0.5, 0.6 and 0.4 after the W0, W1, W2 and W3 washing steps, respectively. The gluten content, which is the sum of gliadin and glutenin contents, decreased significantly from W0 ( $0.65 \pm 0.07$  g/100 g) and W1 ( $0.67 \pm 0.03$  g/100 g) to W3 ( $0.54 \pm 0.02$  g/100 g). The average CP content ( $1.03 \pm 0.05$  g/100 g) stayed constant despite more extensive washing, so that the amount of non-gluten proteins in those WSt was as high as



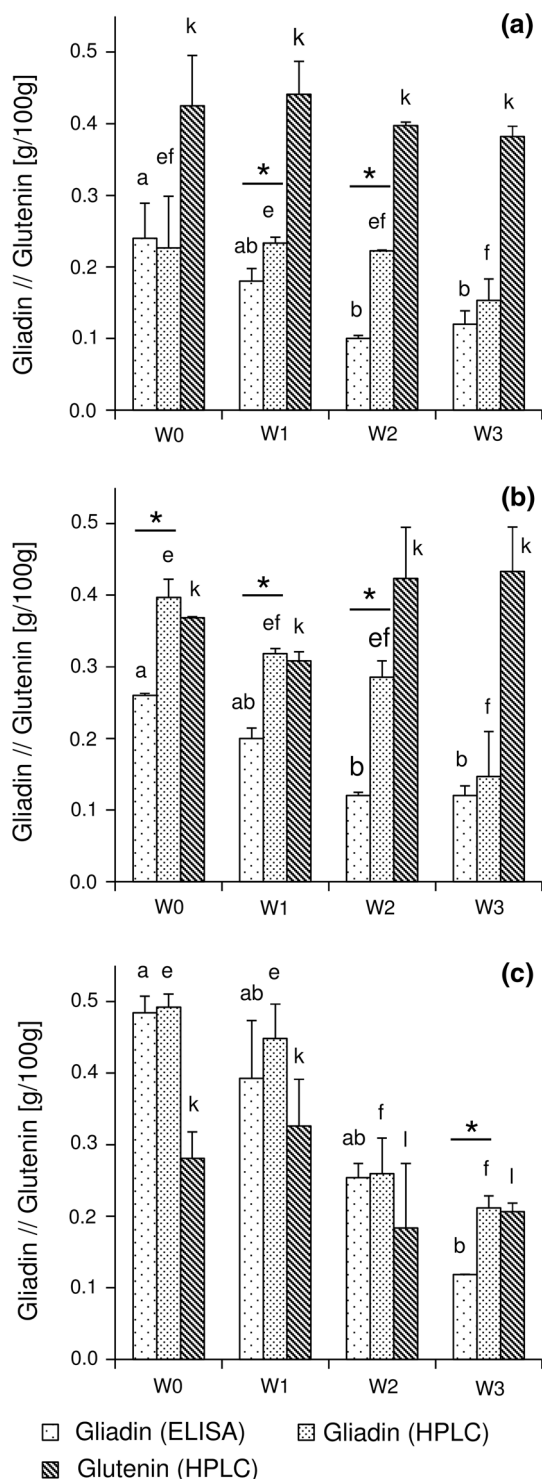
**Fig. 2** Qualitative RP-HPLC profiles of gliadin and glutenin extracts from wheat starches (WSt) and the original wheat flour. **a** Gliadin extract from wheat flour cultivar Akteur, **b** gliadin extracts from WSt 12 min/salt/W0 (gray) and W3 (black), **c** gliadin extracts from WSt

12 min/water/W0 (gray) and W3 (black), **d** glutenin extract from wheat flour cultivar Akteur, **e** glutenin extracts from WSt 12 min/salt/W0 (gray) and W3 (black), **f** glutenin extracts from WSt 12 min/water/W0 (gray) and W3 (black)

37.4 % in W0 and 50.0 % in W3 (Fig. 4a). These non-gluten proteins were considered to be the sum of residual albumins and globulins (quantitated by RP-HPLC) as well as non-extractable internal starch proteins [9]. Compared to the original wheat flour, the percentages of gliadin types in WSt 2 min/salt/W0 were all lower with 3.2 %  $\omega$ 5-, 1.5 %  $\omega$ 1,2-, 9.8 %  $\alpha$ - and 7.3 %  $\gamma$ -gliadins, whereas among glutenin types HMW-GS (7.6 %) was only slightly reduced, and LMW-GS (30.2 %) was higher.

WSt prepared from dough mixed to the optimum consistency (12 min/salt) had gliadin contents of  $0.40 \pm 0.03$  g/100 g (W0) down to  $0.15 \pm 0.06$  g/100 g (W3), showing a significant decrease from W0 to W3. In contrast, glutenin contents were  $0.37 \pm 0.01$  g/100 g (W0) and  $0.43 \pm 0.06$  g/100 g (W3) with no significant differences between washing steps (Fig. 3b). The gli/glu ratios decreased from 1.1 to 1.0, 0.7 and 0.3 with each washing step W0, W1, W2 and W3, respectively. A significant reduction in the gluten content was observed between





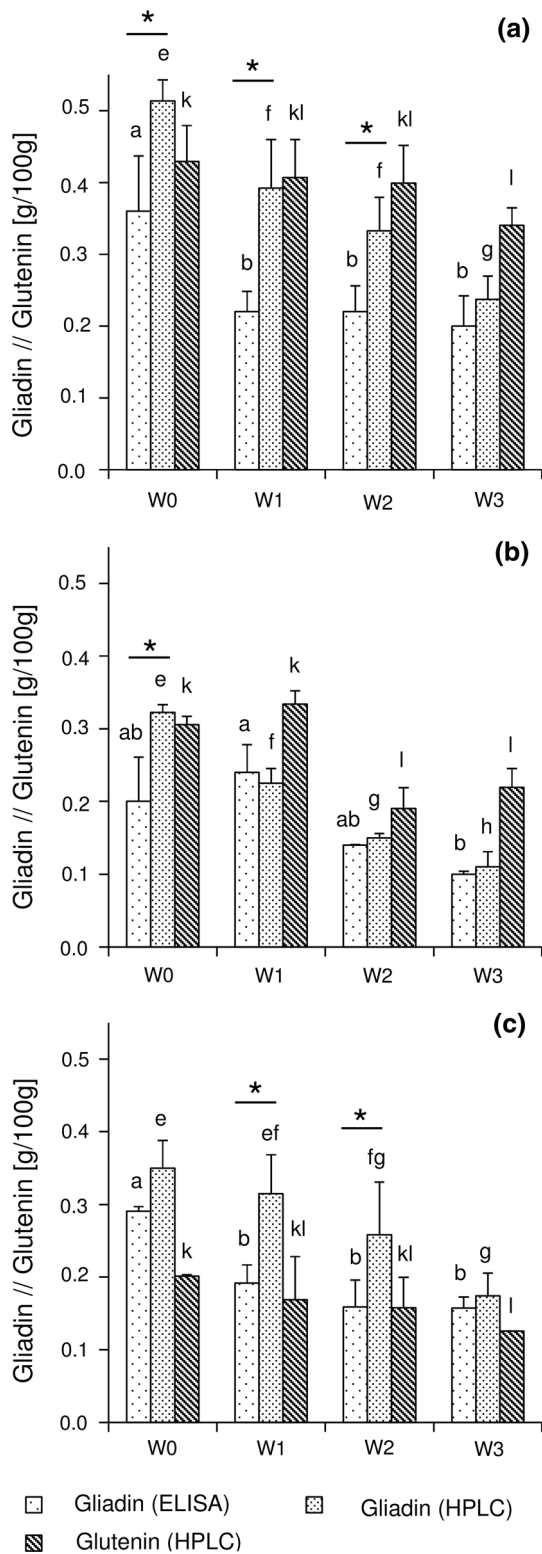
**Fig. 3** Gliadin contents of wheat starches (WSt) analyzed by competitive ELISA and HPLC as well as glutenin contents of WSt analyzed by HPLC. **a** WSt 2 min/salt/W0–W3. **b** WSt 12 min/salt/W0–W3. **c** WSt 22 min/salt/W0–W3. Lower-case letters (a–b, e–f and k–l) indicate significant differences between washing steps within gliadin contents (ELISA), gliadin contents (HPLC) and glutenin contents (HPLC), respectively. Asterisks indicate significant differences between gliadin contents depending on the analytical method (ELISA vs. HPLC) within one washing step

W0 ( $0.77 \pm 0.01$  g/100 g) and W3 ( $0.58 \pm 0.06$  g/100 g) (Fig. 4b). The percentages of gluten protein types in WSt 12 min/salt/W0 were 5.2 %  $\omega$ 5-, 2.9 %  $\omega$ 1,2-, 20.9 %  $\alpha$ - and 14.6 %  $\gamma$ -gliadins, 8.7 % HMW-GS and 29.9 % LMW-GS. These decreased to 3.3 %  $\omega$ 5-, 1.0 %  $\omega$ 1,2-, 7.3 %  $\alpha$ - and 4.2 %  $\gamma$ -gliadins in 12 min/salt/W3, but HMW-GS (8.9 %) and LMW-GS (33.7 %) remained constant, while the percentage of non-gluten proteins increased from 14.0 to 37.8 % (average CP content of  $0.92 \pm 0.03$  g/100 g).

When WSt was washed out from overmixed dough (22 min/salt), the gliadin contents decreased significantly from  $0.49 \pm 0.02$  g/100 g in W0 to  $0.21 \pm 0.02$  g/100 g in W3. Here, the glutenin and CP contents also showed significant decreases from  $0.28 \pm 0.04$  g/100 g in W0 to  $0.21 \pm 0.01$  g/100 g in W3 for glutenins (Fig. 3c) and from  $1.00 \pm 0.04$  g/100 g in W0 to  $0.63 \pm 0.06$  g/100 g for CP (Fig. 4c). WSt 22 min/salt/W0 contained 5.1 %  $\omega$ 5-, 4.3 %  $\omega$ 1,2-, 24.5 %  $\alpha$ - and 15.2 %  $\gamma$ -gliadins, 7.3 % HMW-GS and 18.7 % LMW-GS, which is comparable to the original flour, except for a significant reduction in  $\alpha$ -gliadins and HMW-GS. After the third washing step (W3), this composition had changed to 3.4 %  $\omega$ 5-, 2.5 %  $\omega$ 1,2-, 16.4 %  $\alpha$ - and 11.3 %  $\gamma$ -gliadins, 5.6 % HMW-GS and 25.3 LMW-GS. The similarity of gluten compositions between flour and WSt 22 min/salt/W0 was reflected in a comparable gli/glu ratio of 1.8, but this ratio decreased to 1.4, 1.4 and 1.0 depending on the washing steps W1, W2 and W3, respectively. The gluten contents were similar in W0 and W1 ( $0.77 \pm 0.06$  g/100 g), but were significantly reduced in W2 ( $0.44 \pm 0.07$  g/100 g), with no further reduction in W3 ( $0.42 \pm 0.01$  g/100 g).

#### Quantitation of gliadin, glutenin and gluten contents by RP-HPLC in wheat starches separated with water after dough mixing times of 2, 12 and 22 min

When undermixed doughs were subjected to gluten–starch separation with water (2 min/water), the gliadin content in the W0 WSt was  $0.51 \pm 0.03$  g/100 g and this amount fell significantly to  $0.24 \pm 0.03$  g/100 g in the W3 WSt. The glutenin contents were  $0.43 \pm 0.05$  g/100 g in W0 and  $0.34 \pm 0.02$  g/100 g in W3 (Fig. 5a). This also resulted in a significant reduction in glutenins from W0 to W3, but the extent of the decrease (0.09 g/100 g) was smaller than for gliadins (0.27 g/100 g). This trend was confirmed by the gli/glu ratios of the 2 min/water WSt which decreased from 1.2 to 1.0, 0.8 and 0.7 across the W0, W1, W2 and W3 washing steps, respectively. In contrast to the 2 min/salt WSt, where the CP content was constant, the CP content of the 2 min/water WSt also decreased significantly from  $1.25 \pm 0.01$  g/100 g (W0) to  $0.78 \pm 0.07$  g/100 g (W3). Just the same, the initial gluten content in WSt of  $0.94 \pm 0.04$  g/100 g (W0) went down significantly



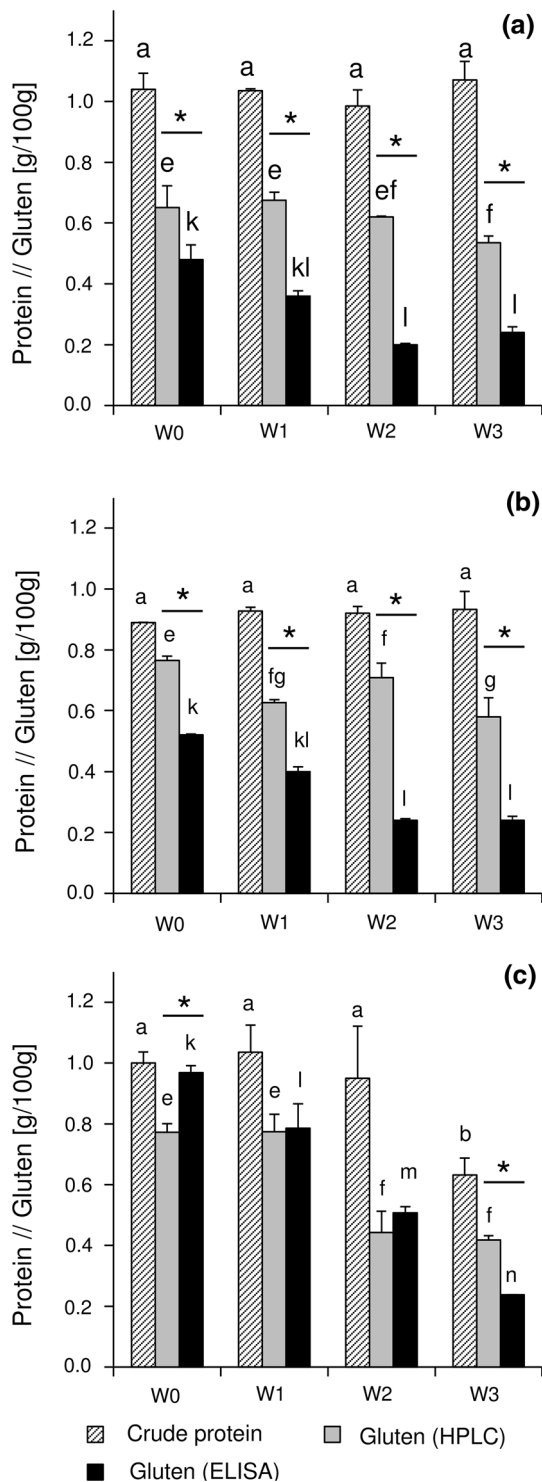
**Fig. 4** Gliadin contents of wheat starches (WSt) analyzed by competitive ELISA and HPLC as well as glutenin contents of WSt analyzed by HPLC. **a** WSt 2 min/water/W0–W3. **b** WSt 12 min/water/W0–W3. **c** WSt 22 min/water/W0–W3. Lower-case letters (a–b, e–h and k–l) indicate significant differences between washing steps within gliadin contents (ELISA), gliadin contents (HPLC) and glutenin contents (HPLC), respectively. Asterisks indicate significant differences between gliadin contents depending on the analytical method (ELISA vs. HPLC) within one washing step

proteins remained constant within 2 min/water/W0–W3 at an average percentage of 24.8 %.

WSt washed with water from dough mixed to the optimum consistency (12 min/water) contained CP amounts of  $0.89 \pm 0.01$  g/100 g (W0) and  $0.59 \pm 0.13$  g/100 g (W3), gliadin amounts of  $0.32 \pm 0.01$  g/100 g (W0) and  $0.11 \pm 0.02$  g/100 g (W3) and glutenin amounts of  $0.31 \pm 0.01$  g/100 g (W0) and  $0.22 \pm 0.03$  g/100 g (W3) (Fig. 5b), resulting in gluten amounts of  $0.63 \pm 0.01$  g/100 g (W0) and  $0.33 \pm 0.02$  g/100 g (W3) (Fig. 6b). The differences between W0 and W3 were all significant for CP, gliadin, glutenin and gluten contents. Although repetitive washing removed both gliadins and glutenins, the gli/glu ratios were again diminished from 1.1 to 0.7, 0.8 and 0.5 in 12 min/water W0, W1, W2 and W3, respectively, indicating that gliadins were eliminated more extensively than glutenins. The percentage of non-gluten proteins in WSt 12 min/water was comparatively high with 29.2 % (W0) and 44.5 % (W3). Within gluten, the contents of protein types were 5.1 %  $\omega$ -5-, 3.2 %  $\omega$ 1,2-, 15.8 %  $\alpha$ - and 11.2 %  $\gamma$ -gliadins, 8.1 % HMW-GS and 24.0 % LMW-GS in WSt 12 min/water/W0.

Aqueous gluten–starch separation of overmixed dough (22 min/water) yielded WSt with CP contents of  $0.80 \pm 0.03$  g/100 g (W0) and  $0.64 \pm 0.10$  g/100 g (W3), gliadin contents of  $0.35 \pm 0.04$  g/100 g (W0) and  $0.17 \pm 0.03$  g/100 g (W3), glutenin contents of  $0.20 \pm 0.01$  g/100 g (W0) and  $0.13 \pm 0.01$  g/100 g (W3) (Fig. 5c) and total gluten contents of  $0.55 \pm 0.02$  g/100 g (W0) and  $0.30 \pm 0.02$  g/100 g (W3) (Fig. 6c). There were no significant differences between washing steps for CP contents, but gliadins, glutenins and gluten decreased significantly from WSt 22 min/water W0 to W3. The gli/glu ratios were 1.7, 1.9, 1.6 and 1.4 in the 22 min/water WSt for the washing steps W0, W1, W2 and W3, respectively. The gluten proteins extracted from WSt 22 min/water/W0 consisted of 5.4 %  $\omega$ -5-, 4.4 %  $\omega$ 1,2-, 21.1 %  $\alpha$ - and 12.8 %  $\gamma$ -gliadins, 4.4 % HMW-GS and 19.2 % LMW-GS. Non-gluten proteins made up 31.1 % of the protein fraction in WSt 22 min/water/W0, and this percentage increased to 53.4 % in W3. Compared to the original flour, all WSt contained less than one tenth of CP, ranging from 0.59 to 1.25 g/100 g overall.

to  $0.58 \pm 0.03$  g/100 g (Fig. 6a). The gluten composition in the 2 min/water/W0 WSt was 4.9 %  $\omega$ -5-, 3.8 %  $\omega$ 1,2-, 17.4 %  $\alpha$ - and 13.1 %  $\gamma$ -gliadins, 7.4 % HMW-GS and 25.2 % LMW-GS. The overall content of non-gluten



**Fig. 5** Crude protein and gluten contents of wheat starches (WSt) analyzed by HPLC (gluten as sum of gliadins and glutenins) and by competitive ELISA (gluten as gliadins multiplied by 2). **a** WSt 2 min/salt/W0–W3. **b** WSt 12 min/salt/W0–W3. **c** WSt 22 min/salt/W0–W3. Lower-case letters (a–b, e–g and k–n) indicate significant differences between washing steps within crude protein contents, gluten contents (HPLC) and gluten contents (ELISA), respectively. Asterisks indicate significant differences between gluten contents depending on the analytical method (ELISA vs. HPLC) within one washing step

### Main factors influencing crude protein, gliadin, glutenin and gluten contents of wheat starches

The identification of the main factors influencing CP, gliadin, glutenin and gluten contents of WSt was done by three-way ANOVA over all data (24 WSt samples in total) with dough mixing time (2, 12 or 22 min), wash solution (salt or water) and washing step (W0, W1, W2 or W3) as factors. In general, the dough mixing time affected CP, gliadin, glutenin and gluten contents of the WSt and the comparisons of 2 versus 22, 2 versus 12 and 12 versus 22 min were significant for all four parameters with only one exception (12 vs. 22 min for CP contents). After a dough mixing time of 2 min, the CP was higher than after 12 min or 22 min. Within gliadins, the contents were highest after a mixing time of 22 min and lowest after 12 min, with 2 min in between. For glutenins and total gluten, longer mixing times led to lower contents.

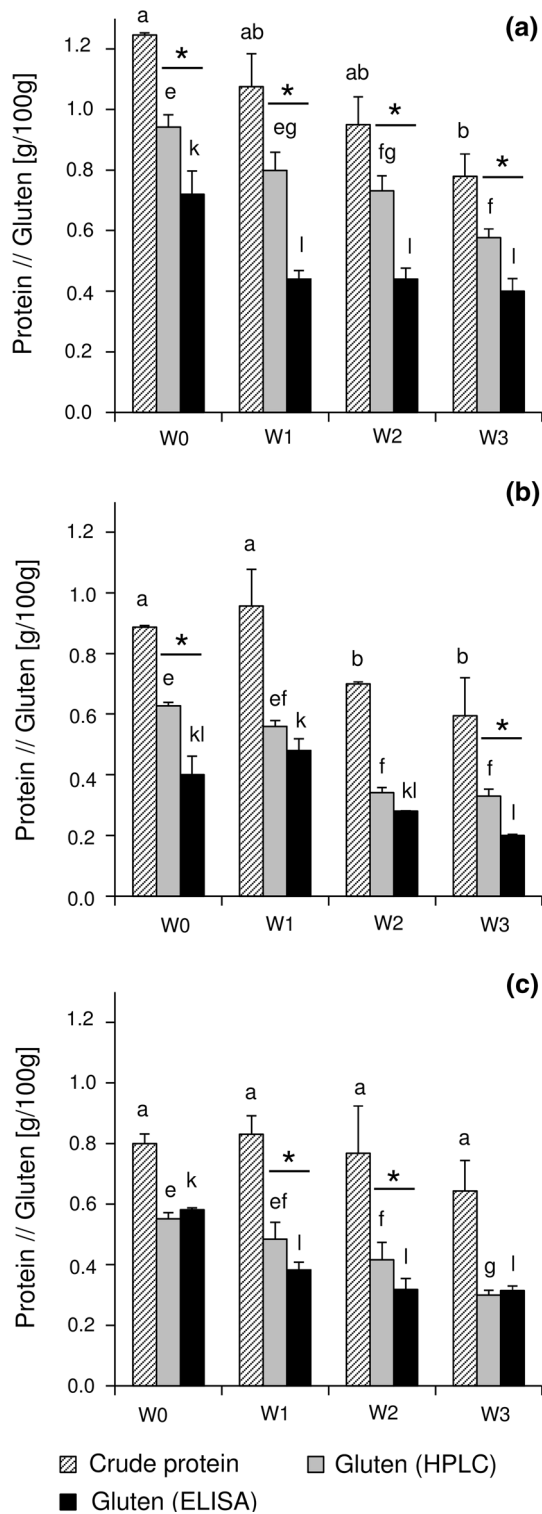
Overall, the wash solution was responsible for significant differences between CP, glutenin and gluten, but not for gliadin contents of WSt. CP, glutenin and gluten contents of WSt were all higher when salt solution was used for gluten–starch separation instead of water.

Within the successive washing steps of WSt, significant reductions were observed from W0 to W3, W1 to W2 and W1 to W3 for all four parameters. The values for gliadin and gluten contents were significantly decreased when comparing W0 versus W1, but not for CP and glutenin contents. With the exception of CP contents, gliadin, glutenin and gluten contents were significantly reduced from W0 to W2. The last washing step (W2 vs. W3) caused a significant decrease in CP, gliadin and gluten, but not glutenin contents.

Additionally, the simple main effects influencing CP, gliadin, glutenin and gluten contents of WSt directly after gluten–starch separation (W0) were evaluated. Different dough mixing times had no significant influence on CP contents of WSt when salt solution was used for gluten–starch separation. When water was used, the CP content was significantly higher with a dough mixing time of 2 min compared to 12 and 22 min. When comparing wash solutions after a dough mixing time of 2 min, washing with water resulted in significantly higher CP contents in WSt than washing with salt solution. No significant differences in CP contents due to wash solution were observed for the other mixing times 12 and 22 min.

The gliadin contents of WSt (W0) were significantly influenced by both dough mixing time and wash solution. With salt solution, the gliadin contents increased with each prolongation of mixing time. The opposite was true for water, because the gliadin contents decreased from 2 to 12 min, but there was no further difference between 12 and 22 min. Within the same dough mixing time of 2 min, the





**Fig. 6** Crude protein and gluten contents of wheat starches (WSt) analyzed by HPLC (gluten as sum of gliadins and glutenins) and by competitive ELISA (gluten as gliadins multiplied by 2). **a** WSt 2 min/water/W0–W3. **b** WSt 12 min/water/W0–W3. **c** WSt 22 min/water/W0–W3. Lower-case letters (a–b, e–g and k–l) indicate significant differences between washing steps within crude protein contents, gluten contents (HPLC) and gluten contents (ELISA), respectively. Asterisks indicate significant differences between gluten contents depending on the analytical method (ELISA vs. HPLC) within one washing step

significant reduction in glutenin contents. The same observation was made for WSt washed with water. The gluten contents of WSt were the same when doughs were mixed for 2 min and washed with salt solution or water. However, at the 12 and 22 min mixing times, the glutenin contents were higher when salt solution was used compared to water.

The sum of gliadin and glutenin contents corresponds to the gluten content of WSt. When salt solution was used, the gluten content of WSt (W0) was lower with a dough mixing time of 2 min compared to 12 and 22 min. There was no difference between 12 and 22 min, because gliadin contents increased from 12 to 22 min, while glutenin contents decreased. With water, the gluten content of WSt decreased significantly from 2 to 12 min and further to 22 min, because both gliadins and glutenins also decreased. Within the same dough mixing time of 2 min, the gluten contents followed the same trends as the gliadin contents. Washing with salt solution resulted in lower gluten contents in WSt compared to water, whereas the opposite was true within 12 and 22 min.

All in all, mixing the dough to its optimum consistency or even longer (overmixed) is favorable to achieve low residual gluten contents in WSt. Water is recommended as wash solution for gluten–starch separation, and subsequent washing steps with water are suitable to reduce gluten contents even further.

**Comparison of gliadin and gluten contents analyzed by RP-HPLC and competitive ELISA**

In addition to the RP-HPLC analyses, the gliadin contents of the WSt were measured by competitive ELISA, which is recommended for starch samples by the test kit manufacturer. The ELISA results for gliadin contents (Fig. 4) were multiplied by a factor of 2 to obtain the ELISA results for gluten contents (Fig. 6), as recommended by legislation [5]. In contrast to this, HPLC gluten contents are expressed as sum of gliadin and glutenin contents analyzed by HPLC. Taking the data from all 24 WSt samples into account, gliadin contents quantitated by ELISA showed a medium correlation ( $r = 0.763$ ) to gliadin contents, no correlation ( $r = 0.150$ ) to glutenin contents and only a

salt-washed WSt had lower gliadin contents compared to the water-washed WSt, but the opposite was found within 12 and 22 min.

WSt (W0) separated with salt solution contained the highest amounts of glutenins when the dough mixing time was 2 min and each prolongation of mixing time caused a

weak ( $r = 0.599$ ) correlation to gluten contents obtained by HPLC. Different effects were seen within each of the six sets of WSt samples. Strong correlations between gliadin contents by ELISA and gliadin ( $r > 0.822$ ) and gluten ( $r > 0.789$ ) contents by HPLC were found for the 12 min/salt and 22 min/salt WSt. For the 2 min/water and 22 min/water WSt, ELISA gliadin contents and HPLC gliadin ( $r = 0.766$  and  $r = 0.728$ , respectively) and gluten ( $r = 0.745$  and  $r = 0.775$ , respectively) contents showed medium correlations. Within the 12 min/water WSt, gliadin contents analyzed by ELISA and HPLC were not correlated ( $r = 0.536$ ), but ELISA gliadin contents had a medium correlation to HPLC gluten contents ( $r = 0.672$ ). No correlations between ELISA and HPLC results were observed within the 2 min/salt WSt.

A comparison of ELISA and HPLC results for gliadin contents in all 24 WSt samples revealed no significant differences in 11 out of 24 WSt. In the other 13 WSt, the gliadin contents quantitated by ELISA were significantly lower than those analyzed by HPLC (Figs. 3, 5). For gluten contents, no significant differences between ELISA and HPLC results were found for only 6 out of 24 WSt. Except for one WSt sample, the gluten contents measured by ELISA were significantly lower than by HPLC (17 out of 24 WSt) (Figs. 4, 6). The six WSt samples with no significant differences depending on the quantitation method (22 min/salt/W1–W2, 12 min/water/W1–W2, 22 min/water/W0 and W3) and the one exception with a higher gluten content by ELISA than by HPLC (22 min/salt/W0) all showed a good agreement between ELISA and HPLC results for gliadin contents (no significant differences) and gli/glu ratios of 0.7 up to 1.8. For the other 17 WSt samples, two effects were observed. On the one hand, the ELISA gliadin contents were mostly lower than the HPLC gliadin contents. On the other hand, the ELISA was not suitable for the detection of glutenins, so that large discrepancies in gluten contents were seen between ELISA and HPLC in WSt samples with particularly low gli/glu ratios of 0.6 down to 0.3, for example, in the WSt samples 2 min/salt/W0–W3, 12 min/salt W2–W3 and 12 min/water/W3.

## Discussion

The gluten contents of WSt directly after laboratory-scale gluten–starch separation in a Glutomatic washer ranged from 5500 to 9400 mg/kg (HPLC) or 4000 to 9700 mg/kg (ELISA) in the six different W0 samples. These values were in the same order of magnitude as those reported by Walter et al. [26], who used the same washing procedure. Commercial industrially purified WSt typically had gluten contents ranging from 5 to 360 mg/kg (ELISA) [9]. This indicates that the laboratory-scale preparation, originally

intended for the determination of the wet gluten content of wheat flours (ICC Standard 137/1) [34], is less effective in separating gluten and starch. Another reason may be that the entire starch residue was taken after centrifugation for further analyses, including the top streams carrying the gluten fractions and squeegee starch. Usually, these top streams are removed from the dense bottom stream containing prime starch (A-starch) [7]. One positive aspect of these comparatively high gluten contents in the WSt was that gliadin and glutenin extracts could be analyzed by HPLC to monitor their compositions and contents subject to washing procedures. The qualitative profiles of gliadins and glutenins extracted from WSt revealed that all gluten protein types were present in the WSt, which is in accordance with earlier findings based on two-dimensional gel electrophoresis and mass spectrometry [9].

Depending on dough mixing time and wash solution (water vs. salt solution), the contents of gluten protein fractions and types in WSt were affected in different ways. Repetitive washing steps (W1–W3) led to decreases of gliadins, glutenins and gluten, but gliadins were depleted more extensively than glutenins, because the gli/glu ratios decreased in all six sets of WSt, independent of the initial gluten composition in the W0 WSt. Gli/glu ratios  $< 1$  were already described for starch samples [21] and semiquantitative data of four WSt also indicated that more glutenin than gliadin appeared to be on the granule surface [9]. In the present study, up to 37 and 53 % non-gluten proteins were found in the W0 and W3 WSt, respectively. Although these were not investigated further, these were comprised of up to 21 % albumins/globulins (enzymes, enzyme inhibitors) and presumably internal proteins (starch synthases) [9]. Within gluten protein types, the contents of  $\omega 5$ -gliadins in the W0 WSt were reduced by 0 % (22 min/water) up to 41 % (2 min/salt) compared to wheat flour, those of  $\omega 1,2$ -gliadins by 12 % (22 min/water) up to 70 % (2 min/salt), those of  $\alpha$ -gliadins by 18 % (22 min/salt) up to 67 % (2 min/salt), those of  $\gamma$ -gliadins by 13 % (22 min/salt) up to 58 % (2 min/salt) and those of HMW-GS by 14 % (12 min/water) up to 53 % (22 min/water). On the contrary, the contents of LMW-GS increased by 36 % (12 min/water) up to 71 % (2 min/salt). Successive washing steps (W1–W3) tended to further decrease  $\omega 5$ -,  $\omega 1,2$ -,  $\alpha$ - and  $\gamma$ -gliadin contents, whereas the contents of HMW- and LMW-GS remained mostly unaffected.

WSt washed from undermixed doughs (2 min) had the highest gluten contents, which can be explained by the fact that the gluten network in the dough was not yet fully developed. The coarse, random network of large gluten strands found at the beginning of dough development has not yet aggregated and polymerized, so that gluten–starch separation would be less clear-cut. With continued mixing, the starch granules become less firmly attached to

gluten, and most of the starch is readily removable from optimally mixed doughs [35]. This effect was observed for the 12 min WSt washed from dough with its optimum consistency, where gluten contents were lower compared to the 2 min WSt. Overmixing typically results in a disruption of the gluten film. The wheat flour employed in this study was rather tolerant to overmixing, because the farinogram still indicated 530 Brabender Units at a dough mixing time of 22 min. Therefore, the gluten film appeared to be still intact, resulting in the lowest gluten contents in WSt washed from overmixed doughs. In summary, washing starches from optimally mixed or overmixed doughs with water yielded the lowest residual gluten contents in WSt.

ELISAs with antibodies targeting the gliadin fraction are most commonly used for gluten quantitation. Because antibody reactivities toward glutenins are low, the gliadin content is duplicated to obtain the gluten content, assuming a gli/glu ratio of 1 [5]. If otherwise untreated wheat flour is present, the duplication of quantitative values for gliadin tends to result in an overestimation of gluten, because actual gli/glu ratios (1.5–3.1) were higher than 1 [21]. However, gli/glu ratios well below 1 (down to 0.3) were found in this investigation due to gliadin depletion during washing of WSt. In 17 out of 24 WSt samples, gluten contents analyzed by ELISA were significantly lower than those by HPLC, resulting in an underestimation of gluten contents by up to 68 % (2 min/salt/W2). If gli/glu ratios are below 1, duplication of the ELISA gliadin content will result in too low values for gluten contents, because glutenins are not taken into account.

For further investigations into the issue of changes in gluten composition during starch processing, it would be interesting to monitor gliadin and glutenin contents of WSt from different production stages during industrial purification. This was not the focus of this study, because the LODs and LOQs of the RP-HPLC–UV method were clearly too high, but further efforts are currently underway to improve the sensitivity of detection, for example, by using fluorescence or by looking at characteristic gluten marker peptides with a comprehensive LC–MS/MS method. Then the results obtained on a laboratory scale could be compared to those from industrially purified WSt, even though the effect of gliadin depletion seen here is expected to occur in both processes, because monomeric gliadins are presumably easier to solubilize in aqueous solutions than polymeric glutenins [3]. A novel alternative for gluten detection based on voltammetry may be further developed to enable the sensitive analysis of gluten traces [36].

The consumption of WSt as part of the GFD seems to be safe for the vast majority of CD patients [16], but analytical methods should be able to reliably detect both gliadins and glutenins, not only in WSt, but also in other processed products from which the prolamins fraction may have been

partially removed. The treatment of WSt with a prolyl endopeptidase from *Aspergillus niger* (AN-PEP) is a promising option to completely degrade gluten and remove its CD-toxicity [26], and this could be incorporated into the industrial purification process.

## Conclusion

Dough mixing time, wash solution for gluten–starch separation and repetitive washing steps significantly influenced the compositions and contents of residual gluten in WSt samples. The lowest residual gluten contents in WSt were achieved after gluten–starch separation with water using optimally or overmixed doughs. Subsequent washing steps caused a further reduction in gluten contents, with gliadins being removed more extensively than glutenins. The GFD with a maximal intake of 20 mg gluten per day is a vital necessity for CD patients [37], and HMW-GS were shown to stimulate T-cell expression and exacerbate CD in vivo [38]. The assessment of the safety of gluten-free products is based on analytical methods to reliably quantitate gluten traces, but glutelins cannot be analyzed accurately with the currently available ELISA methods. As highlighted in the present study, the failure to detect glutelins may lead to an underestimation of gluten contents. Therefore, improved immunological and non-immunological methods for gluten detection are urgently needed, and further research will focus on developing independent reference methods.

**Acknowledgments** The author would like to thank Ms. Angelika Grassl, Ms. Anneliese Koehler and Ms. Alexandra Axthelm for excellent technical assistance.

## Compliance with ethical standards

**Conflict of interest** None.

**Compliance with ethics requirements** This article does not contain any studies with human or animal subjects.

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