Research Article

Received: 24 July 2023 Revised: 20 March 2024 Accepted article published: 9 April 2024 Published online in Wiley Online Library: 22 April 2024

(wileyonlinelibrary.com) DOI 10.1002/jsfa.13521

Rapid analysis of wheat gluten composition using a triple ELISA

Clemens Schuster,[a,b] Julien Huen,[c] Thomas Weiss[d] and Katharina Anne Scherf[b,e*]

Abstract

BACKGROUND: Gluten composition is an important quality parameter of wheat flour. Reversed-phase high-performance liquid chromatography (RP-HPLC) is a state-of-the-art method for its analysis. As this is a very labour-intensive and time-consuming procedure, alternative faster methods are desirable. Enzyme-linked immunosorbent assay (ELISA) is a high-throughput method often used for the analysis of gluten traces in gluten-free products. In this proof-of-principle study, we introduce an experimental triple ELISA for the relative quantitation of gliadins, high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) of one wheat flour extract.

RESULTS: The results of 80 common wheat flour samples obtained from the triple ELISA and RP-HPLC were correlated. The results for gliadins ($r = 0.69$) and HMW-GS ($r = 0.81$) showed a medium and high correlation, respectively. Only a very weak correlation of ELISA and RP-HPLC results was observed for LMW-GS ($r = 0.49$). Results for glutenins ($r = 0.69$) and gluten ($r = 0.72$) had a medium correlation. The gliadin/glutenin ratio ($r = 0.47$) and LMW-GS/HMW-GS ratio ($r = 0.40$) showed a weak or no correlation. The gliadin, LMW-GS and gluten contents were lower and the HMW-GS content was higher in the ELISA measurement compared to RP-HPLC.

CONCLUSION: The quantitation of gliadins and HMW-GS by the experimental triple ELISA showed comparable results to RP-HPLC, whereas no strong correlation between the results from the two methods was found for LMW-GS. Overall, the experimental triple ELISA is suitable for relative gluten quantitation, especially for the analysis of large sample sets. Further work will focus on improving the experimental procedure of the ELISA.

Keywords: gliadins; glutenins; high-molecular-weight glutenin subunits; low-molecular-weight glutenin subunits; reversed-phase high-performance liquid chromatography

INTRODUCTION

The analysis of the gluten composition of wheat (*Triticum aestivum*) flour is important because gluten is one of the major determinants for end-use quality. Wheat gluten consists of gliadins (α-, γ-, ω1,2- and ω5-gliadins), which are important for dough viscosity, and glutenins (high-molecular-weight (HMW-GS) and low-molecular-weight (LMW-GS) glutenin subunits), which are important for dough elasticity. The ratio of gliadins to glutenins is considered to be very important for the baking quality of wheat flour.[1,2] One state-of-the-art method to analyse the composition of wheat proteins is to sequentially extract albumins/globulins (ALGL), gliadins and glutenins according to their solubility following modified Osborne fractionation.[3,4] Quantitation is performed by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection,[4] using a separate run for each of the three protein fractions. The RP-HPLC method gives precise information about the qualitative and quantitative gluten composition[5-7] (Fig. 1), but it is labour-intensive and time-consuming taking 3370 min (about 56.2 h) for 40 samples. Therefore, it is desirable to develop complementary rapid high-throughput methods in order to gain information on wheat protein composition and predict end-use quality. Rapid spectroscopic and rheological approaches have been reported to predict the protein content and other quality parameters of wheat flour. For example,

* *Correspondence to: KA Scherf, Leibniz Institute for Food Systems Biology at the Technical University of Munich, Lise-Meitner-Straße 34, 85354 Freising, Germany, E-mail: katharina.scherf@tum.de*

a *TUM School of Life Sciences, Technical University of Munich, Freising, Germany*

b *Leibniz Institute for Food Systems Biology at the Technical University of Munich, Freising, Germany*

c *ttz Bremerhaven, Bremerhaven, Germany*

d *R-Biopharm AG, Darmstadt, Germany*

e *TUM School of Life Sciences, Professorship of Food Biopolymer Systems, Technical University of Munich, Freising, Germany*

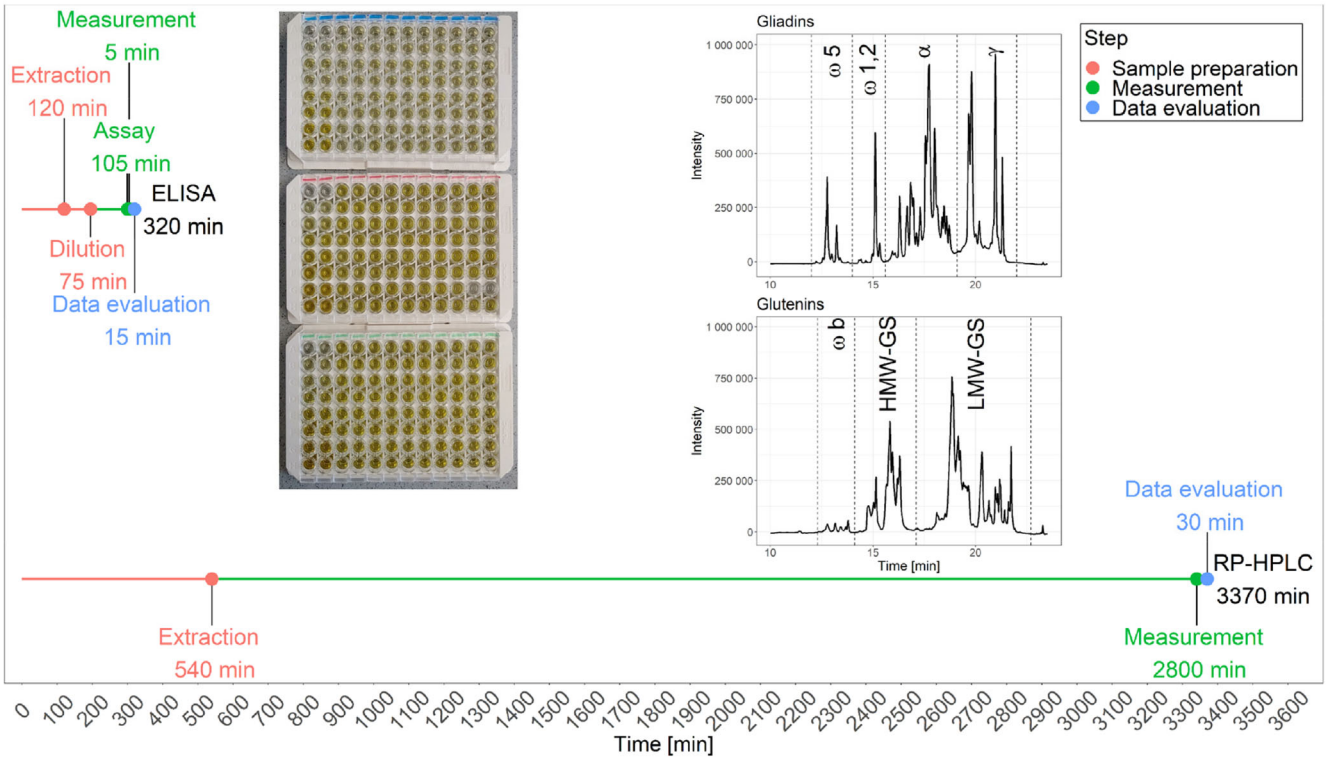

Figure 1. Time required for ELISA and RP-HPLC procedures to quantitate the gluten composition of 40 samples.

Marti *et al.*[8] showed correlations between gluten aggregation parameters and gluten proteins and conventional rheological parameters (e.g. dough stability, extensibility, dough strength).[8,9] Schuster *et al.*[10] recently published a comprehensive study about the link between baking quality and wheat protein composition. In this context, a partial least squares regression model was calculated to quantitate gluten proteins using the parameters evaluated from gluten aggregation curves. Several studies used NIR spectroscopy to quantitate the gluten, gliadin or glutenin contents.[11-13]

Many approaches focus on speeding up the quantitation process by minimizing analysis time. Another possibility is the development of methods to analyse a large number of samples in parallel. Enzyme-linked immunosorbent assays (ELISAs) are commonly used as high-throughput methods. Based on the antigen–antibody reaction, the ELISA is a highly specific and sensitive method and is therefore well established to quantitate allergens in food.[14] Several assays are available to quantitate gluten traces in supposedly gluten-free products.[15,16] For gluten-free products, the Codex Alimentarius defines a threshold of 20 mg kg^{-1} of gluten. Existing ELISA methods, for example, based on the R5 or G12 monoclonal antibodies, are designed to quantitate gliadin epitopes that are immunogenic for celiac disease patients.[17,18] Recently, several studies also used ELISA to estimate the immunoreactivity of different wheat flours.[19-21] As the antibodies mainly react with gliadins, the gluten content is calculated by duplication of the gliadin content analysed in each sample. The RIDASCREEN® Total Gluten assay uses distinct monoclonal antibodies to detect gliadins, HMW-GS and LMW-GS and therefore quantitates the total gluten content more comprehensively compared to the RIDASCREEN® Gliadin assay that has been established as a standard method by different international standardization organizations for many years.[22-25]

In the work reported here, we developed an experimental triple ELISA based on the RIDASCREEN® Total Gluten assay[22] for research purposes to separately quantitate gliadins, HMW-GS and LMW-GS in parallel. Gluten proteins are considered highly important for determining the baking quality of wheat flour. The crude protein content[26] or the gluten content, for example, wet gluten content,[27] are commonly used as indirect parameters for quality assessment. In recent studies, the gluten composition has been increasingly considered to be important when discussing baking quality.[28] The stepwise extraction of the three Osborne fractions followed by RP-HPLC is a comparatively labour-intensive and time-consuming method. To speed up the quality assessment of wheat flours, complementary fast and high-throughput methods are desirable. In this proof-of-principle study, we evaluated a new ELISA procedure to show the feasibility of quantitating the gliadin, HMW-GS and LMW-GS contents of wheat flour in parallel after a single-step extraction.

MATERIALS AND METHODS

Wheat samples

For this study, 80 German winter wheat (*Triticum aestivum*) flour samples were analysed. Sixteen samples were commercially available blended wheat flours of type 550 (ash content of 0.51% to 0.63% based on dry matter, according to the German flour classification system) without additives from nine different mills. The wheat grains for the commercially available flours were harvested in 2018 and 2020. The remaining 64 samples were single-cultivar grains of 23 different cultivars from different growing locations in

Journal of The Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Germany and harvest years. Grains of the single-cultivar flours were obtained from the harvest years 2018, 2019 and 2020. Single-cultivar grain samples were milled on a Quadrumat senior mill (Brabender, Duisburg, Germany) to yield white flour of type 550. A bran duster (Brabender) was used to increase flour yield.[10] More detailed information on the samples has already been reported.[10]

RP-HPLC for the quantitation of gliadins, HMW-GS and LMW-GS

Wheat protein composition was quantitated according to Wieser *et al.*[4] and recently described by Schuster *et al.*[10] The flour samples were subsequently extracted twice with 1 mL of Na_2HPO_4/KH_2PO_4 buffer (0.67 mol L^{-1}, pH = 7.6) containing 0.4 mol L^{-1} NaCl (10 min at 22 °C), three times with 0.5 mL of 60% (v/v) aqueous ethanol (10 min at 22 °C) and twice with 1 mL of 0.1 mol L^{-1} Tris/HCl buffer (pH = 7.6)/1-propanol (50/50 v/v) containing 2 mol L^{-1} urea and 10 mg mL^{-1} dithiothreitol (30 min at 60 °C) to extract ALGL, gliadins and glutenins, respectively.

A Jasco XLC HPLC system (Jasco Deutschland GmbH, Pfungstadt, Germany) was used for the analysis of all fractions. Separation of protein fractions was performed on a Dionex Acclaim 300 C_{18} (3 µm, 2.1 × 150 mm) column at 60 °C using water with 0.1% trifluoroacetic acid (TFA) and acetonitrile (ACN) with 0.1% TFA as eluents. The flow rate was 0.2 mL min^{-1}. Linear gradients were used as summarized in Table 1. A solution of the reference gliadin (2.5 mg mL^{-1}) from the Prolamin Working Group (PWG)[29] was used for calibration by injecting 5, 10, 15 and 20 µL.

Triple ELISA for quantitation of gliadins, HMW-GS and LMW-GS

Sample extraction

For extraction, 250 mg of flour was suspended in 2.5 mL of Cocktail (patented; R-Biopharm, Darmstadt, Germany) and 7.5 mL of 80% (v/v) aqueous ethanol. The samples were incubated for 40 min at 50 °C and thoroughly shaken in between after 20 min of incubation. Then the samples were shaken upside down for 1 h at 22 °C and centrifuged at 4600 × *g* at 4 °C for 10 min. Before starting the ELISA, the clear supernatant was diluted in three subsequent steps by 1:50. This results in a total dilution of the sample extract of 1:125 000. The dilution buffer contained Total Gluten Buffer (R-Biopharm), 80% (v/v) aqueous ethanol and Cocktail (96/3/1 v/v/v).[22]

Assay design

The experimental triple ELISA performed in this study is a sandwich ELISA developed based on the RIDASCREEN® Total Gluten ELISA by R-Biopharm. For the detection of gliadins, HMW-GS and LMW-GS from a single extract, monoclonal antibodies were immobilized on three separate 96-well plates. The R5 antibody is used to detect the gliadins. HMW-GS are detected by the HMW antibody and LMW-GS by the LMW 1 and 2 antibodies, as capture and horseradish peroxidase-conjugated antibody, respectively. The assay is used to determine intact gluten proteins. Standard solutions containing a total gluten extract from four wheat varieties are used for calibration.[22]

Assay procedure

The triple ELISA consists of three assays performed in parallel (Fig. 2). Three different 96-well plates and conjugates with specific antibodies for each analyte were necessary for the simultaneous quantitation of gliadins, HMW-GS and LMW-GS. The following steps were conducted for all three 96-well plates with a time-shift of 5 to 10 min. The diluted sample extracts and gluten standards (100 µL) were pipetted into the cavities of the 96-well plates in technical duplicates and incubated for 30 min. To ensure a quick transfer onto the three different plates, the use of uncoated preplates is highly recommended. After washing (three times with 250 µL of washing buffer), the 96-well plates were incubated with 100 µL of the respective conjugate for 30 min. Then the plates were washed again three times with 250 µL of washing buffer and incubated in the dark for 10 min with 100 µL of substrate. A combined substrate/chromogen solution containing 3,3′,5,5′-tetramethylbenzidin and hydrogen peroxide was used for the triple ELISA. The enzyme reaction was stopped by adding 100 µL of stop solution. Absorption at 450 nm was measured using a GloMax Discover microplate reader (Promega, Madison, USA). For calibration, the absorption was correlated to the gluten concentration of eight standard solutions using a four-parameter

Table 1. Linear gradients for RP-HPLC analysis of albumins/globulins, gliadins and glutenins

Analyte	Time (min)	Mobile phase A (water + 0.1% TFA) (%)	Mobile phase B (ACN + 0.1% TFA) (%)
Albumins/globulins	0.0	100	0
	0.4	100	0
	0.5	80	20
	5.0	40	60
	5.1	10	90
	9.0	10	90
	9.1	100	0
	25.0	100	0
Gliadins and glutenins	0.0	100	0
	0.4	100	0
	0.5	76	24
	15.0	44	56
	15.1	10	90
	19.1	10	90
	19.2	100	0
	35.0	100	0

Journal of The Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

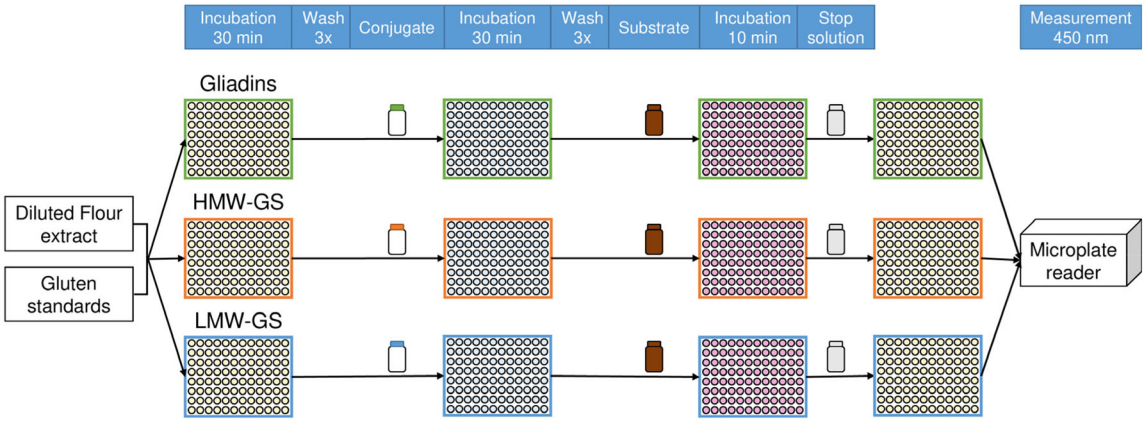

Figure 2. Experimental procedure of the triple ELISA.

function. The standards contained 0, 3.125, 6.25, 12.5, 25, 50, 100 and 200 mg g^{-1} of gluten, including the sample dilution factor. All three ELISAs use the same standard material containing gliadins as well as HMW-GS and LMW-GS. Since the antibodies in the three ELISAs primarily react with gliadins, LMW-GS or HMW-GS, respectively, in the standards and the samples, each ELISA quantifies one fraction. However, the focus of this experimental ELISA was not the absolute quantitation of gliadins, LMW-GS and HMW-GS, but the relative quantitation of the three fractions. The glutenin content was calculated as the sum of HMW-GS and LMW-GS. The gluten content is the sum of gliadin, HMW-GS and LMW-GS contents.

Data analysis

All analyses were performed in triplicate. The evaluation of ELISA results was performed in the RIDASOFT Win.NET software (R-Biopharm) using a distinct evaluation method for each analyte. Within the methods, the dilution factors were considered to calculate the content of gliadins, HMW-GS and LMW-GS from the mean extinction of the technical duplicates. To compensate for interplate differences during the assay procedure, three independent extracts of each sample were analysed on different plates for each analyte. The linear Pearson correlation coefficients were calculated in R studio using the Hmisc package. Correlations were classified as very weak ($0.41 \le r < 0.54$), weak ($0.54 \le r < 0.67$), medium ($0.67 \le r < 0.78$) and strong ($r \ge 0.78$). Significant correlations were determined on a significance level of $P < 0.05$. Mean values are given as the arithmetic mean.

RESULTS AND DISCUSSION

Gluten protein content and composition

Within the sample set comprising 80 winter wheat flours, the content of gliadins ranged from 33.8 to 74.9 mg g^{-1} (median = 53.2 mg g^{-1}) analysed with RP-HPLC and from 20.1 to 88.8 mg g^{-1} (median = 50.0 mg g^{-1}) with ELISA (Table 2). The HMW-GS and LMW-GS contents of the RP-HPLC quantitation ranged from 4.3 to 12.1 mg g^{-1} (median = 8.0 mg g^{-1}) and from 13.5 to 24.3 mg g^{-1} (median = 18.8 mg g^{-1}), respectively. Using the ELISA, the contents of HMW-GS and LMW-GS ranged from 6.0 to 17.0 mg g^{-1} (median = 10.0 mg g^{-1}) and from 3.2 to 23.6 mg g^{-1} (median = 10.7 mg g^{-1}), respectively. Glutenins were calculated as the sum of HMW-GS and LMW-GS and ranged from 18.9 to 36.1 mg g^{-1} (median = 26.7 mg g^{-1}) and from 11.6 to 36.4 mg g^{-1} (median = 20.8 mg g^{-1}) quantitated by RP-HPLC and ELISA, respectively.

The gluten content determined by RP-HPLC was between 58.3 and 111.2 mg g^{-1} (median = 82.0 mg g^{-1}) and between 35.8 and 111.0 mg g^{-1} (median = 69.2 mg g^{-1}) by ELISA. The gliadin/glutenin ratio was between 1.3 and 2.9 (median = 2.0) and the LMW-GS/HMW-GS ratio was between 1.8 and 3.5 (median = 2.4) for RP-HPLC (Table 2). For the ELISA, the gliadin/glutenin ratio ranged from 0.8 to 7.5 (median = 2.3) and the LMW-GS/HMW-GS ratio ranged from 0.4 to 2.3 (median = 1.1). Therefore, the protein composition of the analysed wheat flour samples was within a typical range for white flours (T550) of German winter wheat, judging by the RP-HPLC results.[7]

Correlation of the two gluten quantitation methods

To evaluate if ELISA is suitable to quantitate the gluten composition in wheat flour, the results of the triple ELISA and those of RP-HPLC as a reference method were correlated (Fig. 3). All evaluated correlations were highly significant based on a significance level of 5%. The Pearson correlation coefficients were $r = 0.69$ ($P = 1.2 \times 10^{-12}$) and $r = 0.81$ ($P = 2.2 \times 10^{-16}$) for gliadins and HMW-GS, respectively, and the results showed a medium and high correlation, respectively. The LMW-GS content analysed by ELISA showed only a very weak correlation to the results obtained by RP-HPLC ($r = 0.49$, $P = 4.7 \times 10^{-6}$). The glutenin and gluten content were also compared to those quantitated with RP-HPLC. The results of both methods had a medium correlation ($r = 0.67$, $P = 1.4 \times 10^{-11}$ and $r = 0.72$, $P = 8.1 \times 10^{-14}$). The gliadin and LMW-GS contents tended to be lower as determined by ELISA compared to RP-HPLC (Fig. 4(D),(E)).

The difference of ELISA and RP-HPLC contents was calculated for each analyte. For gliadins the difference ranged from −24.3 to 39.6 mg g^{-1} (mean = −4.3 mg g^{-1}). On average, the gliadin content measured by ELISA was lower by 9%. The relative difference of ELISA and RP-HPLC decreased with higher gliadin content (Fig. 4(A)), indicating that the results of ELISA and RP-HPLC matched better. This effect might be random, because the number of samples with a high gliadin content above 65 mg g^{-1} was quite low. The difference of HMW-GS contents (ELISA *versus* RP-HPLC) ranged from −0.9 to 4.9 mg g^{-1} (mean = 2.1 mg g^{-1}). The mean result was 28% higher in ELISA compared to RP-HPLC. The decreasing trend of the relative difference between ELISA and RP-HPLC of the HMW-GS showed that the absolute difference was constant for the analysed range (Fig. 4(B)). Only two samples

Table 2. Summary of protein contents determined by RP-HPLC and ELISA ($n = 80$)

	RP-HPLC		ELISA	
Analyte	Range	Median	Range	Median
Gliadins (mg g^{-1})	33.8–74.9	53.2	20.1–88.8	50.0
HMW-GS (mg g^{-1})	4.3–12.1	8.0	6.0–17.0	10.0
LMW-GS (mg g^{-1})	13.5–24.3	18.8	3.2–23.6	10.7
Glutenins (mg g^{-1})	18.9–36.1	26.7	11.6–36.4	20.8
Gluten (mg g^{-1})	58.3–111.2	82.0	35.8–111.0	69.2
Gliadins/glutenins	1.3–2.9	2.0	0.8–7.5	2.3
LMW-GS/HMW-GS	1.8–3.5	2.4	0.4–2.3	1.1

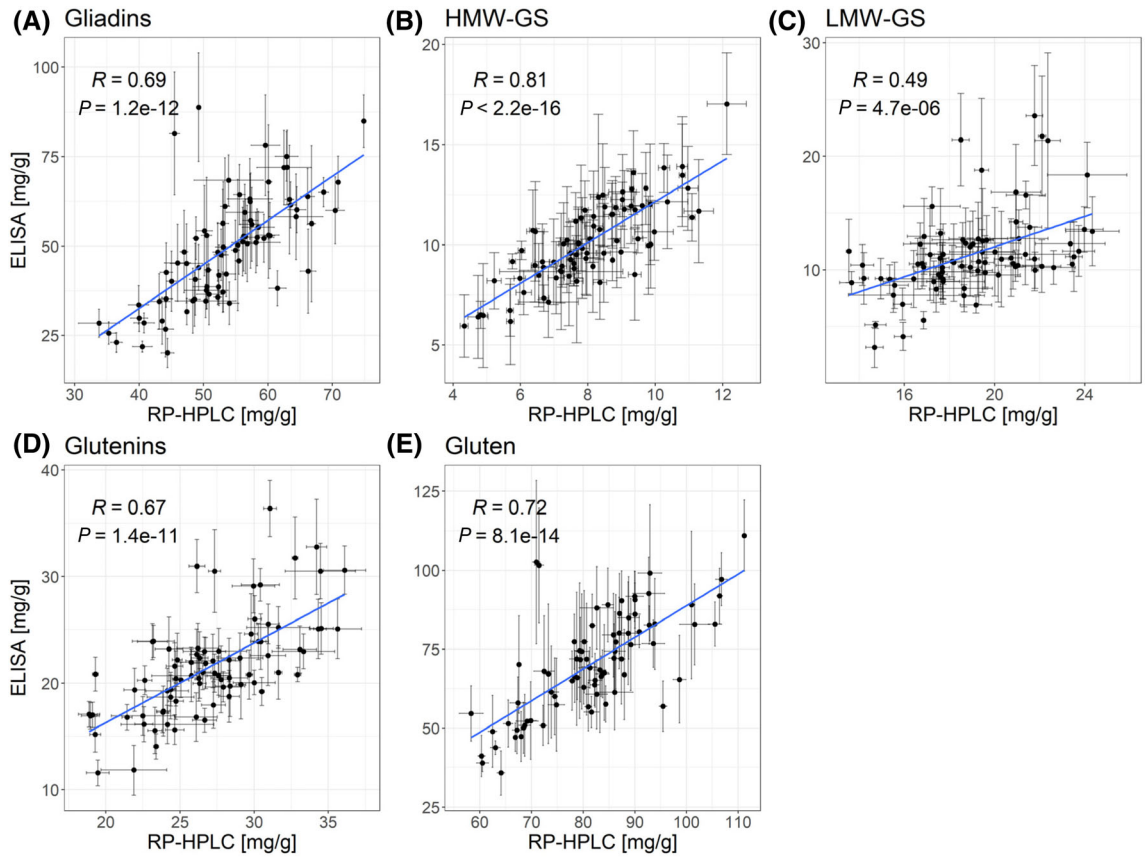

Figure 3. Linear correlation of the content of gliadins (A), HMW-GS (B), LMW-GS (C), glutenins (D) and gluten (E) analysed by ELISA and RP-HPLC.

had a lower HMW-GS content by ELISA compared to RP-HPLC. For LMW-GS, the difference ranged from −13.0 to 3.0 mg g^{-1} (mean = −7.6 mg g^{-1}). On average, the ELISA result was 40% lower compared to RP-HPLC for LMW-GS. The relative difference remained almost constant for the entire analysed range of LMW-GS (Fig. 4(C)). The difference of ELISA and RP-HPLC for glutenins ranged from −12.1 to 5.3 mg g^{-1} (mean = −5.5 mg g^{-1}). The glutenin content was on average 20% lower by ELISA (Fig. 4(C)). The lower result for LMW-GS dominated the glutenin content, because LMW-GS account for approximately 60% of glutenins.[6,30] The relative difference of the glutenins increased with increasing content, indicating that the absolute difference tends to increase.

The difference of the gluten content ranged from −38.5 to 31.6 mg g^{-1} (mean = −11.3 mg g^{-1}). The gluten content was also lower by ELISA compared to RP-HPLC with an average of 14%. For gluten, the same as for gliadins and HMW-GS was observed and the relative difference of ELISA and RP-HPLC decreased with increasing gluten content.

Furthermore, the gliadin/glutenin and LMW-GS/HMW-GS ratios were calculated for ELISA and RP-HPLC (Fig. 5). Pearson correlation coefficients for the gliadin/glutenin and LMW-GS/HMW-GS ratios were $r = 0.47$ ($P = 1.1 \times 10^{-5}$) and $r = 0.40$ ($P = 2.5 \times 10^{-4}$), respectively, indicating only a very weak or no correlation between the results of both methods. Of all samples, 66% had a higher gliadin/glutenin ratio determined by ELISA compared to RP-HPLC. The lower result for the glutenin content (−20%) exceeded that of gliadins (−9%) and therefore a larger ratio of gliadins/glutenins resulted compared to RP-

Journal of The Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

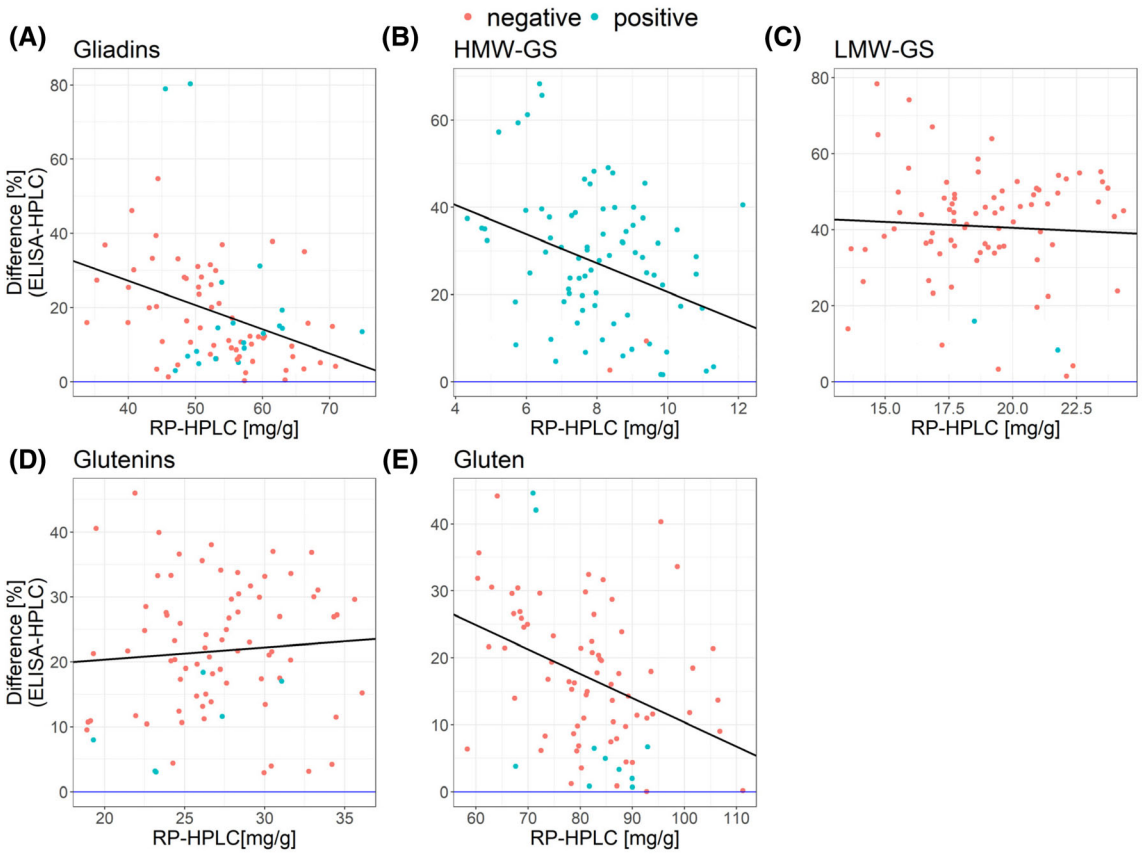

Figure 4. Absolute difference (in %) of gliadins (A), HMW-GS (B), LMW-GS (C), glutenins (D) and gluten (E) quantitated by ELISA minus RP-HPLC compared to the respective content quantitated by RP-HPLC. Negative values are indicated in red, positive values in blue.

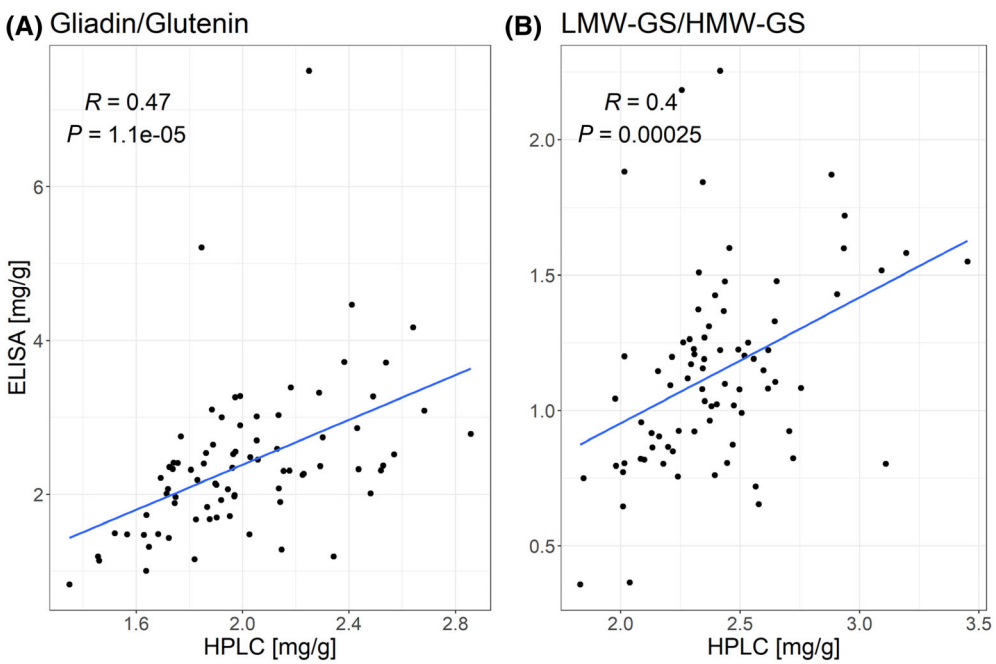

Figure 5. Linear correlation of gliadin/glutenin and LMW-GS/HMW-GS ratios quantitated by ELISA and RP-HPLC.

HPLC. The gliadin/glutenin ratio determined by ELISA was on average 18% higher than that determined by RP-HPLC. As expected, considering the ELISA results for LMW-GS and HMW-GS, the LMW-GS/HMW-GS ratio was on average −52% lower for all analysed samples using ELISA compared to RP-HPLC.

The correlations of the content analysed via ELISA and RP-HPLC show that the results for gliadins and HMW-GS were acceptable. As the higher result for HMW-GS was relatively constant for all samples, determination of the recovery rate for each assay should be adequate to eliminate this error. The current assay procedure for the quantitation of LMW-GS in wheat flour samples led to different results. The gluten content as the sum of gliadins, HMW-GS and LMW-GS also showed a strong correlation between both methods, even though the LMW-GS content was lower in ELISA.

Advantages and limitations of triple ELISA compared to RP-HPLC

With different specific antibodies for the quantitation of gliadins, HMW-GS and LMW-GS, these three fractions can be analysed from one sample extract. Specific antibodies are important to ensure the analytical specificity of the ELISA. The R5 antibody has a low cross-reactivity against HMW-GS. The HMW antibody has a low cross-reactivity against gliadins. Neither the R5 antibody nor the HMW antibody show cross-reactivity against LMW-GS. The LMW antibodies have considerable cross-reactivity against gliadins, but none for HMW-GS. The cross-reactivity of the LMW antibodies against gliadins could be a reason for the low correlation of the HPLC and ELISA results for the LMW-GS. In contrast, the specificity of the RP-HPLC method depends on the solubility of the different wheat proteins in the respective solvent and on the retention time, because UV detection at 210 nm only detects the peptide bond, but not any specific protein. This is why liquid chromatography–tandem mass spectrometry analyses revealed the presence of many different gluten and non-gluten proteins in different gluten protein types, even after preparative HPLC purification.[31]

As the quantitation of gluten traces is the typical scope of application, commercially available ELISA kits are optimized for concentration ranges around 20 mg kg^{-1}.[22,32] The gluten concentration in regular wheat flour exceeds this concentration range by far, requiring a very high dilution of the sample extracts to avoid saturation. The dilution was performed in three subsequent steps by diluting 100 µL of the sample extract or the previous dilution with 4.9 mL of dilution buffer (1:50), successively, resulting in a final dilution of 1:125 000. This constitutes one limitation of the current procedure, because these pipetting steps require time and reduce precision. Variation of the dilution factor for each sample may limit reliability of the ELISA results when comparing different samples. To maintain comparability, the same dilution factor was chosen for all samples. Samples were diluted in a serial dilution containing three individual steps. Even if errors in each dilution step may be small, these could add up. Therefore, the same diluted sample extract was used for analysis on all three 96-well plates in one run. If significant errors had occurred during dilution, this should have been evident in all three fractions, allowing such samples to be eliminated as outliers from the triplicates.

For RP-HPLC, ALGL, gliadins and glutenins are extracted in three subsequent steps. Each extraction step is performed at least twice, to ensure complete extraction of the respective protein fraction. Even if there is no interest in analysing ALGL, this fraction needs to be removed when following the protocol described above.[4] One advantage is that sample dilution is usually not necessary for RP-HPLC analysis, because lower injection volumes can be used if the detector reaches saturation.

To improve the determination of the gluten composition, alternative methods need to save time or reduce workload compared to the RP-HPLC method. At this experimental stage, the triple ELISA is still quite time-consuming and labour-intensive, mostly due to several dilution and pipetting steps and incubation times. To ensure enough time for washing and applying all reagents to the 96-well plates, a time shift of 5 to 10 min between the three assays was necessary. RP-HPLC does not include critical incubation times, but a distinct RP-HPLC run is necessary to analyse all fractions. After starting the HPLC system, it is sufficient to check the system regularly. Nevertheless, well-trained personnel are necessary to operate the HPLC system.

The ELISA determines proteins containing specific epitopes that bind to the immobilized antibodies. When measuring the extinction at 450 nm, the gluten fractions are quantitated without any further information on the gluten protein types in each fraction. In comparison, the chromatograms of each RP-HPLC run contain detailed peak profiles indicating the composition of α-, γ-, ω1,2- and ω5-gliadins as well as HMW-GS and LMW-GS. The gluten content, the gliadin/glutenin ratio and the LMW-GS/HMW-GS ratio are most commonly used to evaluate gluten quality, so that the details provided by RP-HPLC are often not needed.[6,33] Therefore, the information on gliadins, HMW-GS and LMW-GS provided by the triple ELISA is sufficient. Current research shows that gluten quality, that is, gluten composition, is important for the functional properties of wheat flour rather than the gluten content.[10,28] Commonly used quick methods for wheat flour quality assessment (e.g. crude protein content, wet gluten content, etc.) do not provide the desired information about gluten quality.

The coefficient of variation (CV) for a threefold determination of the ELISA was up to 20%. Compared to earlier reports, a CV of 10–15% can be expected for ELISA.[34,35] The larger CV observed here probably derives from the high sample dilution (in total 1:125 000). Optimization of the experimental procedure for the ELISA will likely reduce the CV. For RP-HPLC, a CV of 10% is acceptable.[36] Further experimental validation including recovery and trueness of both methods is recommended.

For an economical application of the ELISA, especially the dilution procedure needs further optimization to reduce the susceptibility to errors and the amount of waste. Using the presented sample extraction procedure, each sample is diluted with approximately 15 mL of dilution buffer, but only 100 µL of the final diluted extract is needed per cavity. The comparatively large dilution volumes were chosen to minimize pipetting errors. To analyse 40 samples in triplicate, three 96-well plates are necessary, which are not reusable. To avoid cross-contamination of cavities, fresh pipette tips should be used for each pipetting step, resulting in a large amount of solvent and plastic waste. Therefore, the assay procedure is quite expensive, regarding the required consumables. The extraction procedure for RP-HPLC analysis was already optimized using only 2 mL of each solvent. If samples are filtered before injection, the HPLC column should last for at least 1000 sample injections. Optimized solvent usage for extraction and reusability of the analytical column make the RP-HPLC a cheaper analysis compared to the ELISA, not considering the cost for purchasing the HPLC instrument.

The time required for both methods was estimated based on our practical experience after optimizing the workflow (Fig. 1). The extraction times of both methods had the largest difference, because they depend on how many samples are extracted simultaneously. We estimated extraction times for extracting 40 samples simultaneously. As the ELISA does not quantitate ALGL, the measurement time of the RP-HPLC for this fraction was excluded. The estimated time scales show that ELISA is approximately

Journal of The Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

10 times faster compared to RP-HPLC. If only a small sample set (10 samples or less) needs to be analysed, the time required for the ELISA does not significantly decrease as most of the analysis time is defined by the assay procedure itself. The major workload for the analysis of flour samples using RP-HPLC is due to the stepwise extraction. Once the HPLC sequence is started, it is only necessary to monitor the system at regular time intervals, whereas the ELISA is labour-intensive throughout the whole process. Furthermore, the measurement time could be reduced by switching from an HPLC to an ultraperformance liquid chromatography system.[37]

If the gluten composition of a large sample set (more than 10 samples) needs to be analysed, the triple ELISA is suitable as a fast method. Quantitation results of gliadins, HMW-GS and gluten were comparable in ELISA and RP-HPLC, as strong and moderate correlations were found for the two methods. Quantitation results of LMW-GS showed only a very weak correlation for both methods. There are several potential reasons for this deviation. First, the different extractions probably lead to different extraction efficiencies. Second, the largest difference is the detection system, based on retention times followed by UV measurement for RP-HPLC compared to the detection of specific amino acid sequences by antibodies in the ELISA. Thus, the result in the ELISA correlates to the frequency of these sequences. As mentioned above, some cross-reactivities were observed for the used antibodies, which do not pose a problem for quantitation of the total gluten protein content, but are essential for the detection of separate gluten fractions. However, the HPLC method also has limitations in specificity, as discussed above. Finally, different calibrations have an influence as well (PWG-gliadin for RP-HPLC and gluten extract for ELISA). Because PWG-gliadin predominantly contains gliadins (ω5-, ω1,2-, α- and γ-gliadins) and only traces of HMW-GS and LMW-GS,[29] it cannot be used for calibration of the ELISA to quantitate HMW-GS and LMW-GS. Due to the selectivity of the monoclonal antibodies, gliadins cannot be detected with the HMW-GS and LMW-GS ELISA. In order to use the same calibrator for all three ELISAs, a total gluten extract was used for calibration. In order to minimize variety-specific protein composition, a mixture of four different varieties was used. The calibration of the experimental ELISA is not yet meant for absolute quantitation and could be improved, especially for LMW-GS. For calibration of the RP-HPLC method, the UV absorption of the peptide bond at 210 nm is used. As shown by Wieser *et al.*,[4] calibration curves using gliadins, HMW-GS and LMW-GS were almost identical. Therefore, calibration of glutenin proteins is also possible using the PWG-gliadin.

It is not surprising that the largest differences between the two methods were observed for the LMW-GS. All three protein fractions of gliadins, HMW-GS and LMW-GS consist of different gluten protein types, which themselves consist of many individual proteins. Compared to the gliadins and HMW-GS, the LMW-GS are a very heterogeneous protein group.[30,38] It is therefore plausible that the above-mentioned differences between the two methods (extraction, detection, specificity and calibration) had the highest impact on quantitation of LMW-GS.

CONCLUSION

Gluten content and composition are important parameters regarding the quality assessment of wheat flour. An experimental high-throughput triple ELISA method was developed based on specific antibodies for gliadins, HMW-GS and LMW-GS. We showed that it is feasible to determine the gluten composition after a single-step extraction of wheat flour. The results for gliadins and HMW-GS were highly correlated to the respective amounts quantitated via RP-HPLC as a reference method. The LMW-GS were only very weakly correlated to the results of the RP-HPLC method. The ELISA procedure to determine the gluten composition of 40 wheat samples is 10 times faster compared to the commonly used modified Osborne fractionation followed by RP-HPLC. Further work will focus on improving the experimental procedure of the ELISA to make it more easily applicable in routine analyses.

ACKNOWLEDGEMENTS

We thank Alexandra Axthelm (Leibniz-LSB@TUM) for excellent technical assistance as well as Sophie Ballmann and Niklas Weber (R-Biopharm) for the development and validation of the R&D ELISA Kits used for this research project.

FUNDING INFORMATION

This IGF Project of the FEI was supported via AiF within the programme for promoting the Industrial Collective Research (IGF) of the German Ministry of Economics and Climate Action (BMWK), based on a resolution of the German Parliament. Project AiF 20283N. Open Access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

CS, JH and KAS have no conflict of interest to declare. TW is an employee of R-Biopharm AG.

REFERENCES

1 Amend T and Belitz HD, Microstructural studies of gluten and a hypothesis on dough formation. *Food Struct* **10**:277–288 (1991).
2 Singh H and MacRitchie F, Application of polymer science to properties of gluten. *J Cereal Sci* **33**:231–243 (2001).
3 Osborne TB and Harris IF, The chemistry of the protein-bodies of the wheat kernel. Part I. The protein soluble in alcohol and its glutaminic acid content. *Am J Physiol Legacy* **13**:35–44 (1905).
4 Wieser H, Antes S and Seilmeier W, Quantitative determination of gluten protein types in wheat flour by reversed-phase high-performance liquid chromatography. *Cereal Chem* **75**:644–650 (1998).
5 Pronin D, Börner A, Weber H and Scherf KA, Wheat (*Triticum aestivum* L.) breeding from 1891 to 2010 contributed to increasing yield and glutenin contents but decreasing protein and gliadin contents. *J Agric Food Chem* **68**:13247–13256 (2020).
6 Thanhaeuser SM, Wieser H and Koehler P, Correlation of quality parameters with the baking performance of wheat flours. *Cereal Chem* **91**: 333–341 (2014).
7 Geisslitz S, Wieser H, Scherf KA and Koehler P, Gluten protein composition and aggregation properties as predictors for bread volume of common wheat, spelt, durum wheat, emmer and einkorn. *J Cereal Sci* **83**:204–212 (2018).
8 Marti A, Augst E, Cox S and Koehler P, Correlations between gluten aggregation properties defined by the GlutoPeak test and content of quality-related protein fractions of winter wheat flour. *J Cereal Sci* **66**:89–95 (2015).
9 Marti A, Ulrici A, Foca G, Quaglia L and Pagani MA, Characterization of common wheat flours (*Triticum aestivum* L.) through multivariate

analysis of conventional rheological parameters and gluten peak test indices. *LWT Food Sci Technol* **64**:95–103 (2015).
10 Schuster C, Huen J and Scherf KA, Comprehensive study on gluten composition and baking quality of winter wheat. *Cereal Chem* **100**: 142–155 (2023).
11 Wesley IJ, Larroque O, Osborne BG, Azudin N, Allen H and Skerritt JH, Measurement of gliadin and glutenin content of flour by NIR spectroscopy. *J Cereal Sci* **34**:125–133 (2001).
12 Fox G and Manley M, Applications of single kernel conventional and hyperspectral imaging near infrared spectroscopy in cereals. *J Sci Food Agric* **94**:174–179 (2014).
13 Schuster C, Huen J and Scherf KA, Prediction of wheat gluten composition via near-infrared spectroscopy. *Curr Res Food Sci* **6**:100471 (2023).
14 Taylor SL, Nordlee JA, Niemann LM and Lambrecht DM, Allergen immunoassays: considerations for use of naturally incurred standards. *Anal Bioanal Chem* **395**:83–92 (2009).
15 Scherf KA and Poms RE, Recent developments in analytical methods for tracing gluten. *J Cereal Sci* **67**:112–122 (2016).
16 Xhaferaj M, Alves TO, Ferreira MSL and Scherf KA, Recent progress in analytical method development to ensure the safety of gluten-free foods for celiac disease patients. *J Cereal Sci* **96**:103114 (2020).
17 Morón B, Cebolla Á, Manyani H, Álvarez-Maqueda M, Megías M, Thomas MC *et al*., Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. *Am J Clin Nutr* **87**:405–414 (2008).
18 Valdés I, García E, Llorente M and Méndez E, Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol. *Eur J Gastroenterol Hepatol* **15**:465–747 (2003).
19 Hajas L, Scherf KA, Török K, Bugyi Z, Schall E, Poms RE *et al*., Variation in protein composition among wheat (*Triticum aestivum* L.) cultivars to identify cultivars suitable as reference material for wheat gluten analysis. *Food Chem* **267**:387–394 (2018).
20 Escarnot E, Gofflot S, Sinnaeve G, Dubois B, Bertin P and Mingeot D, Reactivity of gluten proteins from spelt and bread wheat accessions towards A1 and G12 antibodies in the framework of celiac disease. *Food Chem* **268**:522–532 (2018).
21 Pronin D, Börner A and Scherf KA, Old and modern wheat (*Triticum aestivum* L.) cultivars and their potential to elicit celiac disease. *Food Chem* **339**:127952 (2021).
22 Lacorn M, Weiss T, Wehling P, Arlinghaus M and Scherf K, Quantification of wheat, rye, and barley gluten in oat and oat products by ELISA RIDASCREEN® total gluten: collaborative study, first action 2018.15. *J AOAC Int* **102**:1535–1543 (2019).
23 ICC Standard No. 182. International Association for Cereal Science and Technology, RIDASCREEN® Gliadin – Enzyme immunoassay for the quantitative analysis of gliadins and corresponding prolamines in unprocessed and processed foods (2016).
24 AACCI Approved Method 38-50.01, Gluten in Corn Flour and Corn-Based products by Sandwich ELISA.
25 Lacorn M, Dubois T, Weiss T, Zimmermann L, Schinabeck T-M, Loos-Theisen S *et al*., Determination of gliadin as a measure of gluten in food by R5 Sandwich ELISA RIDASCREEN® gliadin matrix extension: collaborative study 2012.01. *J AOAC Int* **105**:442–455 (2022).
26 ICC Standard No. 167. International Association for Cereal Science and Technology, Determination of crude protein in grain and grain products for food and feed by the Dumas combustion principle (2000).
27 ICC Standard No. 137/1. International Association of Cereal Science and Technology, Mechanical Determination of the wet gluten content of wheat flour (Glutomatic) (1994).
28 Gabriel D, Pfitzner C, Haase NU, Hüsken A, Prüfer H, Greef J-M *et al*., New strategies for a reliable assessment of baking quality of wheat – rethinking the current indicator protein content. *J Cereal Sci* **77**:126–134 (2017).
29 van Eckert R, Berghofer E, Ciclitira PJ, Chirdo F, Denery-Papini S, Ellis HJ *et al*., Towards a new gliadin reference material – isolation and characterisation. *J Cereal Sci* **43**:331–341 (2006).
30 Wieser H, Koehler P and Scherf KA, Chemistry of wheat gluten proteins: quantitative composition. *Cereal Chem* **100**:36–55 (2023).
31 Lexhaller B, Colgrave ML and Scherf KA, Characterization and relative quantitation of wheat, rye and barley gluten protein types by LC-MS/MS. *Front Plant Sci* **10**:1530 (2019).
32 Koehler P, Schwalb T, Immer U, Lacorn M, Wehling P and Don C, AACCI approved methods technical committee report: collaborative study on the immunochemical determination of intact gluten using an R5 sandwich ELISA. *Cereal Foods World* **58**:36–40 (2013).
33 Laidig F, Piepho H-P, Hüsken A, Begemann J, Rentel D, Drobek T *et al*., Predicting loaf volume for winter wheat by linear regression models based on protein concentration and sedimentation value using samples from VCU trials and mills. *J Cereal Sci* **84**:132–141 (2018).
34 Schall E, Bugyi Z, Hajas L, Török K and Tömösközi S, Applicability of ELISA methods for high gluten-containing samples. *Acta Alimentaria* **48**:365–374 (2019).
35 Scherf KA, Gluten analysis of wheat starches with seven commercial ELISA test kits: up to six different values. *Food Anal Methods* **10**: 234–246 (2017).
36 Pronin D, Geisslitz S, Börner A and Scherf KA, Fingerprinting of wheat protein profiles for improved distinction between wheat cultivars and species. *Cereal Chem* **97**:999–1009 (2020).
37 Yu Z, Han C, Yan X, Li X, Jiang G and Yan Y, Rapid characterization of wheat low molecular weight glutenin subunits by ultraperformance liquid chromatography (UPLC). *J Agric Food Chem* **61**:4026–4034 (2013).
38 Dupont FM, Vensel WH, Tanaka CK, Hurkman WJ and Altenbach SB, Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. *Proteome Sci* **9**:10 (2011).

