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Absolute quantification of gluten protein groups and their relation to wheat baking quality

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ABSTRACT

Wheat gluten proteins are key determinants of baking quality. While untargeted proteomics enables relative quantification, absolute quantities of specific gluten protein groups are not available so far. We developed a targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) stable isotope dilution assay to quantify eleven individual gluten protein groups based on isotope-labeled internal standards corresponding to selected marker peptides. The comparison of targeted and untargeted measurements revealed differences in protein composition, likely arising from different MS/MS acquisition strategies and protein assignment. We correlated the absolute protein content with baking quality traits in a multiple advanced generation intercross wheat population comprising 394 inbred lines. None of the individual groups correlated strongly with any baking quality trait. Six groups (α-gliadin 2, γ-gliadin 1, low-molecular-weight glutenin subunit (LMW-GS) 3, and high-molecular-weight glutenin subunits (HMW-GS) 2–4) showed weak to moderate associations (r = 0.32–0.64), mainly with grain protein content, sedimentation value, and wet gluten content. LMW-GS 3 represents the rare i-type containing eight cysteine residues. Loaf volume was only weakly to moderately correlated, primarily with HMW-GS 1 (r = 0.41) and HMW-GS 3 (r = 0.40), supporting the superior effect of Dy10. By contrast, HMW-GS 5 (Dx2, Dx5) showed little effect, consistent with a stronger influence of y-type glutenin subunits. Summing up the protein content across groups increased correlation strengths, yet baking quality remains a complex trait shaped by multiple proteins and non-protein factors.

1. Introduction

Common wheat (*Triticum aestivum*) is one of the most important staple crops for human nutrition worldwide. During milling, the nutrient-rich endosperm is separated from the bran and germ to obtain white flour. When flour is mixed with water, a viscoelastic gluten protein network is formed which provides the structural basis for leavened bread and other baked goods (Shewry et al., 2002). The flour proteins are therefore key determinants of wheat baking quality. Wheat flour proteins are classified into distinct fractions based on their solubility, known as the Osborne fractions. These include albumins (water-soluble), globulins (salt-soluble), gliadins (soluble in aqueous alcohols), and glutenins (insoluble), with gliadins and glutenins together forming wheat gluten (Osborne, 1908). Gliadins can be further subdivided into α-, γ-, ω1,2-, and ω5-gliadins, whereas glutenins are classified into low-molecular-weight (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS) (Wieser et al., 1998).

With respect to dough and baking properties, the different gluten proteins fulfill distinct functions. Gliadins mainly contribute to dough viscosity and extensibility, whereas glutenins determine dough strength and elasticity (Wieser, 2007). Consequently, the ratio of gliadins to glutenins is a major factor influencing dough characteristics. HMW-GS are closely associated with baking quality (Orth & Bushuk, 1972). This, together with the elucidation of essential functional properties of HMW-GS in dough, laid the foundation for classifying HMW-GS loci and alleles according to their impact on flour quality. For example, the combination Dx5 + Dy10 is associated with superior baking performance, while Dx2 + Dy12 is linked to poorer quality (Payne, 1987).

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Moreover, x-type HMW-GS have been shown to exert a stronger effect on dough properties than y-types (Anderson & Bekes, 2011). Numerous studies have examined the influence of glutenins on baking quality, though often based on limited datasets or specific regional samples. A large-scale study by Guzmán et al., which analyzed 2550 bread wheat lines, confirmed that HMW-GS alleles *Glu-A1a*, *Glu-A1b*, *Glu-B1al*, *Glu-B1i*, *Glu-B1f*, *Glu-D1d* (corresponds to subunit Dx5 + Dy10) and LMW-GS alleles such as *Glu-A3*, *Glu-A3d*, *Glu-A3f*, *Glu-B3c*, and *Glu-B3d* are associated with higher loaf volume, whereas HMW-GS alleles *Glu-A1c*, *Glu-B1a*, *Glu-B1d*, *Glu-D1a* and LMW-GS alleles *Glu-A3e* and *Glu-B3j* are correlated with inferior baking quality (Guzmán et al., 2022).

The assessment of wheat flour baking quality is most reliably achieved through standardized baking tests, such as those described in ICC Standard No. 131 (ICC Standard No 131, 1980). Among the various parameters obtained from these tests, loaf volume is widely regarded as the most informative single indicator of baking performance. It is therefore frequently used as a reference trait in studies investigating correlations with other physicochemical, rheological, or compositional characteristics, as well as in evaluations of predictive markers for baking quality. However, despite their high reliability, standardized baking tests are time-consuming, labor-intensive, and require substantial sample quantities, which greatly limits their suitability for routine analytical workflows or high-throughput screening.

To circumvent these limitations, several indirect quality parameters are commonly employed to estimate the baking potential of wheat flour. These include crude protein content, wet gluten content, gluten index, sedimentation value (Zeleny test), falling number, kernel hardness, and water absorption capacity. Crude protein content remains the most widely used proxy for baking quality (Finney & Barmore, 1948; ICC Standard No 164, 2000). Wet gluten and gluten index provide information on the quantity and strength of gluten, while the sedimentation value reflects the swelling capacity and quality of gluten proteins (ICC Standard No 116/1, 1994; ICC Standard No 137/1, 1994; ICC Standard No 155, 1980). The falling number serves as an indicator of α-amylase activity, whereas kernel hardness influences milling behavior and dough properties, and water absorption specifies the optimal flour-to-water ratio required to create standardized dough systems (ICC Standard No 107/1, 1995; ICC Standard No 115/1, 1992). Together, these indirect parameters offer practical, rapid alternatives for assessing wheat quality, although they cannot fully substitute the comprehensive insights gained from standardized baking trials.

Due to its high selectivity, sensitivity, and versatility, liquid chromatography with tandem mass spectrometry (LC-MS/MS) represents a promising approach for the detection and quantification of wheat proteins (Scherf & Poms, 2016). Dupont et al. (2011) were the first to assign wheat flour proteins to specific gene sequences and to quantify them relatively, identifying 157 proteins in the cultivar Butte 86, including gliadins and glutenins (Dupont et al., 2011). In 2016, Bromilow et al. reported 26 gliadins and 37 glutenins in the cultivar Hereward (Bromilow et al., 2016). In a subsequent 2023 study, 19 glutenins and 23 gliadins were identified across ten cultivars, focusing on genetically stable proteins consistently detected across genotypes and environments (Afzal et al., 2023).

The identification of the proteome of an organism with untargeted proteomics is essential to understand its biology. Complementary targeted approaches that determine the absolute content of proteins can describe the proteome and its dynamics more comprehensively (Hoofnagle et al., 2011). Targeted wheat proteomics analyses by Colgrave et al. (Colgrave et al., 2015) and Martinez-Esteso et al. (Martinez-Esteso et al., 2016) focused on the detection of wheat peptides as potential contaminants in non-wheat samples, selecting peptide markers that were absent in other cereal species. Another targeted approach aimed at quantifying total wheat gluten content using chymotryptic peptide markers (Schalk et al., 2018). However, to date no method has been reported that applies a stable isotope dilution assay (SIDA) to the analysis of individual gluten protein groups.

The primary aim of this study was to develop a targeted SIDA method for specific gluten protein groups and to apply it to the eight way advanced generation intercross (MAGIC) wheat population BMWpop (Stadlmeier et al., 2018) in order to quantify protein content and assess correlations of specific proteins with baking quality traits.

2. Materials and methods

2.1. Chemicals and standards

All reagents, salts and solvents were obtained from Acros Organics (Geel, Belgium), Alfa Aesar (Ward Hill, MA, USA), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Thermo Fisher Scientific (Waltham, MA, USA) and VWR Chemicals (Radnor, PA, USA). Trypsin (from bovine pancreas, TPCK-treated, enzyme activity according to manufacturer: ≥10,000 U/mg protein) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Unlabeled (marker peptides P1–P11) and stable isotope labeled (internal standards IS1–IS11) peptides (Table 1) were synthesized by GenScript (Piscataway, NJ, USA). The heavy peptides contained fully [^{13}C]- and [^{15}N]-labeled amino acids. The purity was specified by the manufacturer. For stock solutions (1 mg/mL in water or dimethyl sulfoxide), the peptides were solubilized according to the manufacturer's guidelines and stored at −80 °C prior to use.

2.2. Plant material

For targeted LC-MS/MS SIDA method development, flours of nine wheat cultivars and breeding lines were used: Ambition, FIRL3565, Bussard, Event, Format, Julius, BAYP4535, Potenzial and RGT Reform. Besides RGT Reform, they represent the parental wheat lines of the MAGIC population BMWpop. This population was developed at Bavarian State Research Center for Agriculture and comprises 394 recombinant inbred lines that represent a large part of allelic diversity of the German wheat gene pool (Stadlmeier et al., 2018). RGT Reform is one of the most commonly grown wheat varieties in Germany and serves as a comparative standard. Using the final method, 403 wheat lines comprising the BMWpop, its parental lines, and the cultivar RGT Reform were investigated.

All samples were retrieved from a field trial conducted by Strube D&S GmbH in Söllingen, Germany, in 2018. The experiment was laid out as an alpha lattice with two complete blocks and 86 incomplete blocks with a plot size of 6 m^2. BMWpop lines were grown in two replications, whereas the parental line Julius and the standard RGT Reform were grown in 14 and 18 replications. All other parents were replicated four times. Grains were cleaned using a 2.2 mm sieve and milled using a Bühler MLU-202 laboratory mill (Bühler AG, Uzwil, Switzerland), resulting in flours of type 550 according to the German flour classification system (ash content of 0.51 % to 0.63 % based on dry matter).

2.3. Grain protein content and baking quality parameters

Quality traits were determined following standard methods of the International Association for Cereal Science and Technology (ICC) at the Bavarian State Research Center for Agriculture: grain protein content by near-infrared reflectance spectroscopy (NIRS; ICC No. 159), kernel hardness by NIRS (ICC No. 207), falling number (ICC No. 107/1), sedimentation value (ICC No. 116/1), and wet gluten quantity and gluten index (ICC No. 155). Grain protein content, kernel hardness, and sedimentation value were measured on ground grain, whereas falling number was determined on flour. Water absorption was assessed using a Promylograph T3 (Labortechnik Egger, Neumarkt, Austria) according to the manufacturer's protocol. Milling properties were evaluated as farina and flour yield with a Bühler MLU-202 laboratory mill (Bühler AG, Uzwil, Switzerland) according to a standard German milling procedure. Loaf volume was measured by rapeseed displacement in a standardized

Table 1
Targeted LC-MS/MS parameters for the quantification of eleven gluten marker peptides.

Peptide/ Internal Standard	Amino acid sequence	Unique[a]	Gluten protein type	Leading (razor) protein UniProtKB ID	Precursor *m/z*		Product ions	CE	RT [min]
					P	IS			
P1/IS1	CQAIHNVVHAIILHQQQQQ*R	Group	α-Gliadin 1	R9XW75	606.1^{4+}	608.6^{4+}	y9/y8/y7/y6/y5/y4/y3/b3	27	8.6
P2/IS2	NLALETLPAMCNVYIPPYCTIAPVGI*FGTN	No	α-Gliadin 2	R9XUM8	1103.9^{3+}	1107.2^{3+}	y15/y9/y4/y3/b3/b4/b5/b6/b7	25	18.1
P3/IS3	SLVLQTLPSMCNVVVPPECSIM*R	No	γ-Gliadin	H9BFB6	898.8^{3+}	902.1^{3+}	y10/y9/y8/y7/y6/y5	25	14.7
P4/IS4	SVNVPLY*R	Protein	LMW-GS 1	A0A0S2GJQ0	474.3	479.3	y6/y5/y4/y3/b3/b4	25	8.2
P5/IS5	TLPTMCSVNVPLYSSITSAPLGVGS*R	Group	LMW-GS 2	P93790	1354.2	1359.2	y17/y16/y14/y13/y10/y7/y3/b8	25	14.7
P6/IS6	VFLQQQCIPVAMQ*R	No	LMW-GS 3	R4JAQ1	859.5	864.5	y12/y11/y10/y9/y8/y7/y6/y4/b3	25	10.8
P7/IS7	GQQGYYPTSLQQPGQGQQGYYPTSLQHTGQ*R	Group	HMW-GS 1	Q0Q5D3	1151.9^{3+}	1155.2^{3+}	y13/y11/y10/y8/y6/y5/y4/b6	29	9.4
P8/IS8	EGEASEQLQCE*R	Protein	HMW-GS 2	A0A060MZP1	718.3	723.3	y10/y9/y8/y7/y6/y5/y4/y3/b3/b4	25	5.1
P9/IS9	AQQPATQLPTVC*R	No	HMW-GS 3	Q6RX92	735.4	740.4	y11/y10/y9/y8/y7/y6/y5/y4/b3	25	7.3
P10/IS10	ELQESSLEAC*R	No	HMW-GS 4	Q6RX92	661.3	666.3	y9/y8/y7/y6/y4/y3/b3/b4	25	6.5
P11/IS11	GGSFYPGETTPPQQLQQ*R	No	HMW-GS 5	B1B520	996.0	1001.0	y13/y12/y10/y9/y8/y7/y3/b4	27	8.7

[a] "Protein" denotes that the peptide is unique to a single protein; "group" denotes that it is unique to a protein group; *R: arginine ($[^{13}C]_6$, $[^{15}N]_4$); *F: phenylalanine ($[^{13}C]_9$ $[^{15}N]$); CE: collision energy (normalized); RT: retention time; 3^+ precursors were 3^+; 4^+ precursors were 4^+; all other ones 2^+; P, peptide; IS, internal standard; LMW-GS: low-molecular-weight glutenin subunit; HMW-GS: high-molecular-weight glutenin subunit.

baking test (Arbeitsgemeinschaft Getreideforschung, 2016). Grain protein content and grain hardness were measured for each plot. For all other quality traits and for proteomic analysis, grain samples from all replications were pooled in equal proportions for each genotype.

2.4. Sample preparation for targeted LC-MS/MS and SIDA

Protein extraction was performed in a single step using a solution consisting of 50 % (*v*/v) 1-propanol in 2 mol/L urea and 0.1 mol/L tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5). Dithiothreitol (DTT; 10 mg/mL, *w/v*) was added immediately before extraction. Flour (50 mg) was extracted in triplicate using 1 mL of extraction solution and the suspensions were vortexed for 5 min, sonicated for 5 min at 22 °C, and incubated in a thermomixer at 1500 rpm for 30 min at 60 °C. The tubes were centrifuged (21,380 rcf, 15 min, RT) and 800 µL of the supernatant were evaporated to dryness (800 Pa, 4–6 h, 40 °C). Protein extracts were reconstituted in 300 µL 0.5 mol/L Tris–HCl (pH 8.5) and 300 µL 1-propanol, then spiked with 50 µL of an internal standard mixture containing IS1–IS11 (10–120 µL). The concentration of each IS in the solution was tuned, based on preliminary measurements, to match expected peptide levels and target a 1:1 analyte:IS ratio. For reduction, 100 µL of 0.05 mol/L tris(2-carboxyethyl) phosphine (TCEP) in 0.5 mol/L Tris-HCl (pH 8.5) was added. The samples were incubated in a thermomixer (1000 rpm, 30 min, 60 °C). For alkylation, 100 µL of 0.05 mol/L 2-chloroacetamide (CAA) in 0.5 mol/L Tris-HCl (pH 8.5) was added followed by renewed incubation (1000 rpm, 45 min, 37 °C). The samples were evaporated to dryness and redissolved in 800 µL of 0.1 mol/L Tris-HCl (pH 7.8) and 0.04 mol/L urea with addition of trypsin at an enzyme-to-substrate ratio of 1:25. The samples were incubated in the dark (200 rpm, 37 °C, 18 h). The digestion was stopped by heating for 5 min at 95 °C. The peptides were purified directly with solid phase extraction (SPE) using Discovery DSC-18 SPE 96-well plates with a bed weight of 100 mg/well (Supelco, Sigma-Aldrich). Wells were activated with 2 mL of methanol and equilibrated with 2 mL of 80 % (*v*/v) acetonitrile (ACN) and 0.1 % (v/v) formic acid (FA) in water. Afterwards, a washing step was performed with 3 mL of 2 % (v/v) ACN and 0.1 % (v/v) FA in water. The extracts were loaded onto the wells and allowed to drip through without vacuum. They were then washed with 5 mL of 2 % (v/v) ACN and 0.1 % (v/v) FA in water. Peptides were eluted without vacuum with 2 × 0.5 mL of 40 % (v/v) ACN and 0.1 % (v/v) FA in water. The eluates were collected and evaporated to dryness (800 Pa, 4–6 h, 40 °C).

2.5. Targeted LC-MS/MS and SIDA

The SPE purified samples were reconstituted in 1 mL of 2 % ACN and 0.1 % FA and measured on a Vanquish U-HPLC (Thermo Fisher Scientific) coupled to an Orbitrap Q Exactive plus (Thermo Fisher Scientific). The peptides were separated on an Aeris PEPTIDE XB-C18 (1.7 µm, 10 nm, 150 mm × 2.1 mm) column (Phenomenex) at a flow rate of 0.2 mL/min. The solvents were 0.1 % FA in water (A) and 0.1 % FA in ACN (B). The linear gradient was: 0–1 min, 2–10 % B; 1–13 min, 10–30 % B; 13–16 min, 30–40 % B; 16–18 min, 40–60 % B; 18–20 min, 60–80 % B; 20–22 min, 80 % B; 22–23 min, 80–2 % B; 23–30 min, 2 % B. The injection volume was 10 µL and the column temperature 30 °C. The ESI source was operated in positive mode with an ion spray voltage of 3.0 kV, sheath gas flow rate of 35 and auxiliary gas flow rate of 10. No sweep gas was used. The capillary temperature was set to 350 °C and the S-lens level to 60. Data were acquired in parallel reaction monitoring (PRM) mode. MS2 parameters were set as follows: resolution: 17,500, AGC target: 5e5, maximum IT: 50 ms, isolation window: *m/z* 2.4. An isolation list with the *m/z* of the peptides and internal standards was added (Table 1). The collision energy for each peptide was optimized in preliminary experiments. The general method runtime was 30 min.

2.6. Response lines

The response lines were prepared as described earlier (Geisslitz et al., 2018; Geisslitz et al., 2020). Two solutions (100 µg/mL of each peptide), solution 1 with P1 − P11 and solution 2 with IS1 − IS11, were prepared from the stock solutions. An aliquot of each solution 1 and 2 was reduced with TCEP and alkylated with CAA as described for the flour samples. The alkylated solutions 1 and 2 (20 µg/mL of each peptide) were mixed in molar ratios n(P)/n(IS) between 9.1 and 0.1 (9 + 1, 4 + 1, 3 + 1, 1 + 1, 1 + 3, 1 + 4, and 1 + 9) for calibration.

Across the full sample set, the response line was measured 31 times. To ensure comparability, we removed outliers and averaged the response curves across peptides, then used this averaged response to calculate the peptide content, respectively. This choice increased deviation for unstable peptides but provided uniform results. For the stable peptides, the mean closely matched the measured responses of each day. The slopes of all response lines were between 0.6 and 1.3, while the intercepts were close to 0.0 and R^2 between 0.997 and 1.000. The purities of peptides indicated by the manufacturer were taken into account.

2.7. Data analysis of SIDA and protein quantification

Peak area integration was performed using Skyline (version 24.1.0.199, 6a0775ef83) (MacCoss Lab Software, University of Washington, Seattle, WA, USA) (MacLean et al., 2010). The data are publicly available on Panorama Public (Sharma et al., 2018). The mean peak area ratios of the 5–8 transitions (precursor–product ion pairs for each peptide) were used for quantification. These constant transition ratios additionally served as an identification criterion for the peptides. Response curves were generated by linear regression of peak area ratios A(P1–P11)/A(IS1–IS11) against the corresponding molar ratios n(P1–P11)/n(IS1–IS11). All quantifications were based on three technical replicates with one injection each. The content of each gluten protein group was calculated by multiplying the peptide content with the respective conversion factor ($M_{protein}/M_{peptide}$), using the leading razor protein molecular mass without the signal peptide. The leading razor protein assignments were taken from the MaxQuant results of the untargeted runs (see Section 2.9).

2.8. Method validation

Repeatability precision was evaluated using six replicates ($n = 6$) of a wheat flour mixture composed of the eight parental lines of the BMWpop and RGT Reform. Intermediate precision was assessed by analyzing a total of 18 replicates on three different days (n = 6 per day; same operator, same instrument) (AOAC International, 2013). Recovery was determined according to Geisslitz et al. (Geisslitz et al., 2020) as the ratio of the peptide content measured in the wheat flour mixture to that of the corresponding sample in which the flour mixture had been diluted 1:1 with gluten-free wheat starch. The limit of detection (LOD) and limit of quantification (LOQ) were determined according to Geisslitz et al. (Geisslitz et al., 2020). The unlabeled (P1–P11) and labeled (IS1–IS11) peptides were spiked at eight concentration levels (2, 1.5, 1.25, 1.00, 0.75, 0.5, 0.25, and 0.1 µg/mL) into 50 mg of gluten-free wheat starch as an analyte-free matrix ($n = 3$). Sample preparation and LC–MS/MS analysis were carried out as described above. LOD and LOQ were calculated from the lowest concentration level fulfilling the identification criterion, i.e., consistent transition ratios. LOD was defined as three times and LOQ as ten times the standard deviation of this lowest valid concentration point (Magnusson & Ornemark, 2014).

2.9. Untargeted LC-MS/MS and identification of gluten marker peptides

The eight parental wheat lines of the BMWpop and the comparison standard RGT Reform were prepared as described in 2.4, just without adding the internal standard mixture. Samples were analyzed in an untargeted manner using UHPLC and nanoLC to allow comparison with SIDA data.

For UHPLC measurements, the purified samples were reconstituted in 1 mL of 2 % ACN and 0.1 % FA. LC–MS/MS analyses were carried out on a Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Peptides were separated on an Aeris PEPTIDE XB-C18 LC column (1.7 µm, 10 nm, 150 mm × 2.1 mm; Phenomenex) at a flow rate of 0.2 mL/min (injection volume: 10 µL, column temperature 30 °C). Mobile phases consisted of (A) 0.1 % FA in water and (B) 0.1 % FA in ACN. The linear gradient was programmed as follows: 0 min, 2 % B; 1.5 min, 10 % B; 23 min, 30 % B; 28 min, 40 % B; 31 min, 60 % B; 34–37 min, 80 % B; and 38–45 min, 2 % B. ESI source parameters were: spray voltage, 3.0 kV; sheath gas, 35; auxiliary gas, 10; no sweep gas was applied. The capillary temperature was maintained at 350 °C, and the S-lens level was set to 60. Data were acquired in data-dependent acquisition (DDA) mode with positive ESI polarity. Full MS scans were recorded with the following settings: resolution, 70,000; AGC target, 3e6; maximum IT, 50 ms; scan range, *m/z* 360–1300. dd-MS2 scans were performed with: resolution, 17,500; AGC target, 1e5; maximum IT, 100 ms; TopN, 10; isolation window, *m/z* 2.4; fixed first mass, *m/z* 140.0; normalized collision energy, 28; and dynamic exclusion, 20 s. The total run time was 45 min.

For nanoLC-measurements, the purified samples were reconstituted in 1 mL of a solution containing 2 % ACN and 0.1 % FA and further diluted 1:10. LC–MS/MS analyses were carried out on a U3000 nanoLC system (Thermo Fisher Scientific) coupled to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Peptide separation was performed on a bioZen XB-C18 nanoLC column (2.6 µm, 250 mm × 0.075 mm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.3 µL/min. The mobile phase consisted of 0.1 % FA in water (A) and 0.1 % FA in ACN (B). The linear gradient was as follows: 0–5 min, 2 % B; 5–6 min, 2–5 % B; 6–45 min, 5–20 % B; 45–60 min, 20–33 % B; 60–62 min, 33–100 % B; 62–65 min, 100 % B; 65–66 min, 100–2 % B; 66–80 min, 2 % B. The injection volume was 10 µL and the column temperature 40 °C. The loading pump operated in isocratic mode at 100 % A with a flow rate of 8.0 µL/min. The spray voltage of the nanoFlex electrospray ionization (ESI) source was 2.8 kV and the S-level 60. DDA was performed with the following MS1 scan parameters: resolution: 140,000, automated gain control (AGC) target: 3e6, maximum injection time (IT): 50 ms, scan range: *m/z* 300–1300. dd-MS2 scan parameters were: resolution: 17,500, AGC target: 1e5, maximum IT: 50 ms, TopN: 10, isolation window: *m/z* 2.4, fixed first mass: *m/z* 120.0, normalized collision energy: 28, dynamic exclusion: 45 s. The total run time was 80 min.

Peptide and protein identification was done in MaxQuant (Version 2.4.9.0) with the integrated Andromeda search engine (Geisslitz et al., 2020; Tyanova et al., 2016). The MS/MS raw data were searched against a wheat protein database from UniProtKB (fasta file for organism_id [4565], downloaded on Sept. 28, 2023, 151,978 entries). Oxidation of methionine and *N*-terminal protein acetylation were specified as variable modifications and carbamidomethylation on cysteines as fixed modification. Trypsin was set as enzyme and match between runs was enabled. The default settings of MaxQuant were used for the remaining parameters.

2.10. Statistical analysis

Data analysis and statistics were performed with Excel, version 2016 (Microsoft, Redmond, WA, USA), OriginPro 2023 (OriginLab Corporation, Northampton, MA, USA) and R, version 4.5.1 (R Core Team, 2025).

Adjusted mean values for grain protein content and grain hardness across replicated plots were calculated using a linear mixed model as described in Geyer et al. (Geyer et al., 2022). The label-free quantification (LFQ) data, already normalized by MaxQuant, were subjected to standardized preprocessing prior to statistical analysis. Protein groups annotated as potential contaminants were removed and only protein

groups reliably identified in at least two of the three replicates were retained. For comparison of the targeted and untargeted results, the LFQ intensities of protein groups containing the leading razor protein were used. If the peptide was also present in other proteins of other protein groups, this was not considered, because MaxQuant uses the leading razor protein of a protein group exclusively to quantify this group.

Pearson correlation coefficients (r) between peptide content (and peptide combinations) and baking quality traits were calculated in R, with significance set at $p \leq 0.05$. Classification was based on the absolute value of the correlation coefficient (|r|) and divided into equal intervals: very weak ($|r| < 0.2$), weak ($0.2 \leq |r| < 0.4$), moderate ($0.4 \leq |r| < 0.6$), strong ($0.6 \leq |r| < 0.8$), and very strong ($|r| \geq 0.8$). Principal component analysis (PCA) was calculated in R. Protein and quality tables were inner-joined by SampleID and standardized; protein variables were log1p-transformed. Variables with >20 % missing values were removed, remaining missing values were median-imputed, and constants dropped. PCA (proteins, quality traits, and combined) was run using the R package FactoMineR (Lê et al., 2008). We exported eigenvalues, scores, loadings, contributions, and $\cos^2$, mapped variable labels back to original names, and visualized scree, PC1–PC2 scores, and variable plots via R packages factoextra (Kassambara & Mundt, 2020) and ggplot2 (Wickham, 2016).

3. Results & discussion

3.1. Identification of potential gluten marker peptides

Flour protein extracts from nine wheat genotypes were measured using nanoLC-MS/MS in DDA mode to identify potential marker peptides for gluten after tryptic digestion (Kaemper et al., 2025). Only gluten peptides without missed cleavages identified by MaxQuant (Tyanova et al., 2016) were screened by PRM measurements. In total, there were 262 candidate peptides (**Table S1**), of which 72 belonged to α-gliadins, 61 to γ-gliadins, 76 to LMW-GS and 53 to HMW-GS.

Selecting proteotypic, unique marker peptides is critical for absolute quantification (Calderon-Celis et al., 2018; Ludwig & Aebersold, 2015). Gluten proteins have highly repetitive and glutamine/proline-rich sequences (Scherf, 2023; Shewry & Belton, 2024), which limits the generation of such peptides after tryptic digestion. About 35 % of the 262 candidate peptides were longer than 26 amino acids, the majority contained Cys, ≈16 % had *N*-terminal Gln, and ≈9 % of α/γ-gliadin peptides had *N*-terminal carbamidomethylated Cys and, therefore, did not meet the criteria that are typically applied to select marker peptides (Ludwig & Aebersold, 2015). Further, *N*-/*C*-terminal peptides are typically avoided (Ludwig & Aebersold, 2015), but gluten sequences vary most at the *N*-/*C*-termini (Scherf, 2023; Shewry & Belton, 2024).

This is why the final choice of gluten marker peptides for targeted analysis was made based on their consistent detectability only (Table 1), with the limitation that some peptides map to multiple proteins (**Table S2**). Accordingly, these peptides showed reproducible tryptic cleavage, good MS/MS response, with high signals (>1e6) and stable transitions (at least five) as well as similar intensities in repeated independent analyses of the nine samples indicating their stability during the entire workflow. P8 is *N*-terminal, but unique to a single protein. P9 and P10 share the same leading razor protein and overlap in 10 protein assignments, with P9 mapping to 23 proteins (13 unique) and P10 to 51 (41 unique). Thus, targeted quantification of P9 vs. P10 yields different contents and provides information on the non-overlapping protein subsets. Altogether, this again highlights the major challenge in wheat proteomics: due to the high sequence homology, peptides are frequently assigned to large and multiple gluten protein groups (as indicated in Table 1). This is a limitation to subsequent data interpretation and quantification, as the results can no longer be unambiguously traced back to a single protein.

3.2. Method development of targeted LC-MS/MS SIDA

The development of the targeted LC-MS/MS SIDA method was based on Geisslitz et al. (Geisslitz et al., 2018; Geisslitz et al., 2020). First, the precursor ion charge state with the highest intensity was identified, which was 2+ for most peptides, but 3+ for P2, P3 and P7, and 4+ for P1. Then, five to eight stable precursor-to-product ion transitions were selected including at least one product ion with a higher *m/z* than the precursor ion. Finally, the collision energies were experimentally optimized to achieve the highest signal intensity for each product ion (Table 1).

To evaluate the stability of the labeled and unlabeled synthetic peptides and the peptides in the sample, the response lines and the wheat genotype RGT Reform were repeatedly measured over a 15-week period. In total, the response lines were measured 15 times at least in duplicate while RGT Reform was reprocessed and measured 31 times in triplicate (**Fig. S1-S2)**. The responses for P1/IS1, P4/IS4, P5/IS5, P6/IS6, and P8/IS8 were not stable, with increasing ratios, rapidly for P1/IS1 and P8/IS8, and progressively over 15 weeks for P4/IS4, P5/IS5, and P6/IS6. This indicates preferential degradation of the labeled peptide. Likely causes include the alkylated Cys in P1 and *N*-terminal Glu in P8 (pyroglutamate formation) (Reimer et al., 2011). By contrast, P10/IS10 (also *N*-terminal Glu) remained stable over 15 weeks (relative standard deviation (RSD) <5 %). The instability of IS4/IS5/IS6 (LMW-GS; 26/16/8 amino acids) has no obvious cause. The 31 measurements of RGT Reform showed that most peptides in the sample were stable with an RSD <5 % over time, except for two that had higher RSD (P4: 19 % and P11: 12 %). Given these higher standard deviations, quantitative interpretation of these peptides requires caution and their evaluation is inherently more challenging. Nevertheless, subsequent analyses (Section 3.7) demonstrated that these peptides did not exert a measurable influence on any of the baking quality parameters, also indicating that the overall conclusions of this study remain robust and unaffected by their instability.

3.3. Method validation

Precision, LOD, LOQ and recovery were determined using a mix of nine wheat lines (Table 2). Repeatability was good and lay between 1.4 % and 3.9 % for nine peptides with corresponding $HorRat_r$ between 0.5 and 2.0. The intermediate precision was also good (1.8–4.6 %). Only P4 and P11 showed repeatability values of 15.4 % and 7.5 %, respectively. The corresponding $HorRat_r$ were 4.9 and 3.0 and thus above the maximum value of 2.0 for good repeatability. Since P4 is present only at low concentrations in all samples and therefore also in the mix, this could be the reason for the high deviation. The reason for P11 is not quite clear but might be its susceptibility to oxidation due to four glutamine residues. The LODs for the corresponding proteins of the marker peptides were between 0.2 and 5.5 μg/g and the LOQs between 0.8 and 18.4 μg/g (Table 2). These results highlight the sensitivity of the SIDA method and are also in line with previous studies for gluten peptides (Schalk et al., 2018) and wheat amylase/trypsin-inhibitor (ATI) peptides (Geisslitz et al., 2020; Jahn et al., 2025). Recovery values between 98.9 % and 122.5 % were obtained for 10 out of 11 gluten proteins. The extraction of both diluted and undiluted wheat flours therefore achieved similar results. Only P4 showed a high recovery of 177.8 %, likely due to its low concentration.

3.4. Comparison of SIDA with untargeted UHPLC-MS/MS and nanoLC-MS/MS

The eight parental wheat genotypes of the BMWpop (Stadlmeier et al., 2018) and RGT Reform were investigated with the final LC-MS/MS SIDA method (Fig. 1) in comparison to the relative LFQ protein proportions determined by untargeted proteomics in DDA mode using either UHPLC or nanoLC coupled to the Orbitrap MS/MS system. The

Table 2
Validation parameters of the targeted LC-MS/MS method for the quantification of eleven gluten peptides.

No.	Gluten protein type	Repeatability[a]	$HorRat_r$	Intermediate[a]	LOD[b]	LOQ[b]	Recovery[c]
		[%]		[%]	[µg/g]	[µg/g]	[%]
P1	α-gliadin 1	2.8	1.1	2.9	1.5	5.0	109.5
P2	α-gliadin 2	3.0	1.2	2.9	5.5	18.4	111.7
P3	γ-gliadin	2.1	0.9	2.2	1.1	3.7	98.9
P4	LMW-GS 1	15.4	4.9	18.6	0.2	0.8	177.8
P5	LMW-GS 2	2.8	0.8	3.4	1.8	6.1	122.5
P6	LMW-GS 3	2.0	0.8	2.1	2.4	8.0	110.9
P7	HMW-GS 1	3.9	1.4	4.6	3.8	12.8	119.2
P8	HMW-GS 2	2.1	0.9	2.4	4.5	15.0	104.8
P9	HMW-GS 3	1.4	0.6	1.8	4.1	13.7	102.2
P10	HMW-GS 4	1.9	0.9	1.9	1.8	5.9	100.3
P11	HMW-GS 5	7.5	3.0	8.4	4.5	15.1	117.8

[a] Precision in common wheat flours: repeatability: six replicates on one day (n = 6), $HorRat_r$: ratio calculated of the found and calculated value for RSD_r, acceptable values range between 0.5 and 2; intermediate: six replicates on three days ($n = 18$).
[b] limit of detection (LOD) of peptides in gluten-free wheat starch, LOQ: limit of quantification.
[c] recovery in wheat flour diluted with gluten-free wheat starch (1 + 1); RSD_r: relative standard deviation of repeatability; LMW-GS: low-molecular-weight glutenin subunit; HMW-GS: high-molecular-weight glutenin subunit.

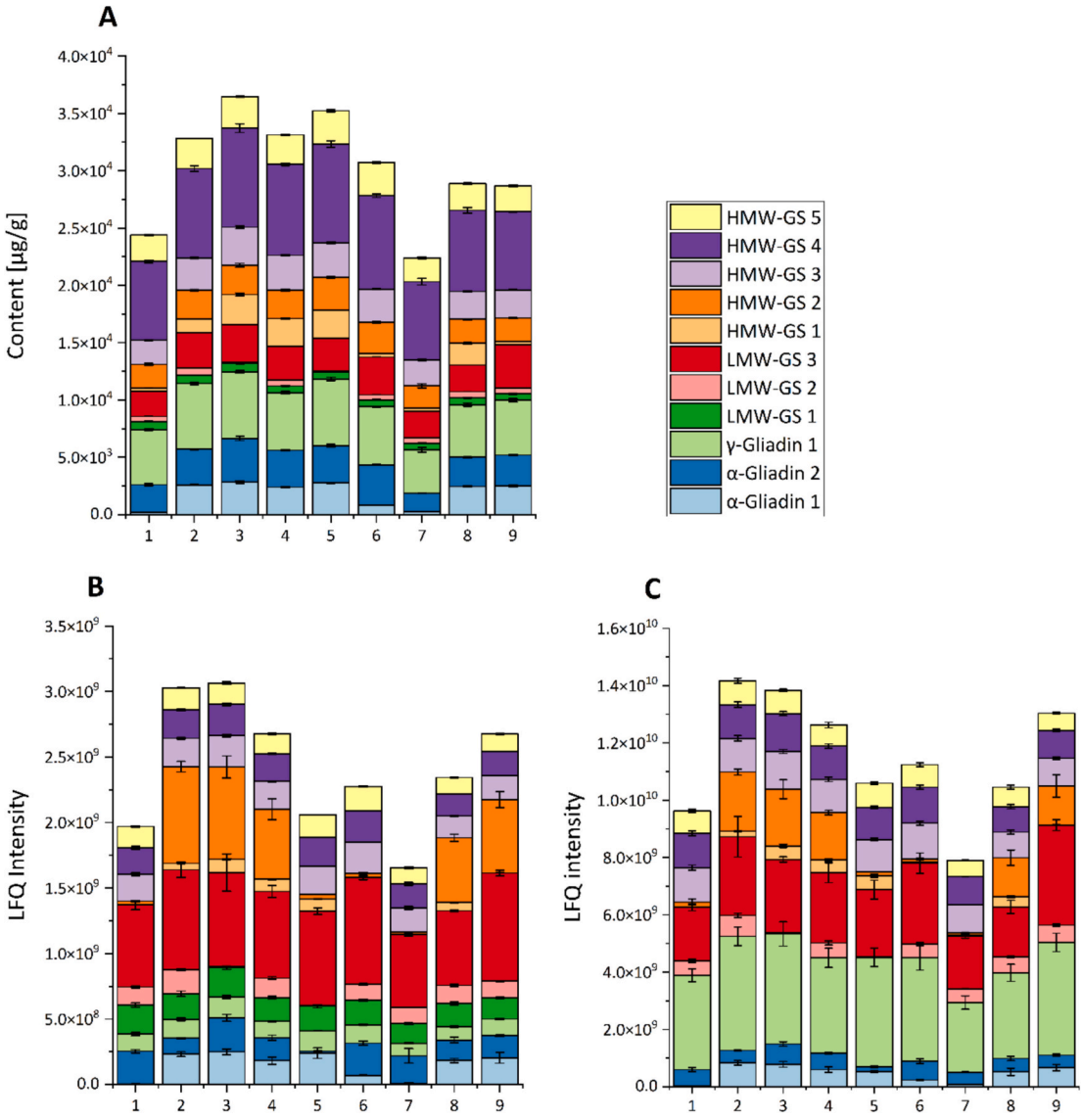

Fig. 1. Comparison of the distribution of wheat gluten protein types in nine wheat lines analyzed by SIDA (A), UHPLC-MS/MS (B) and nanoLC-MS/MS (C). 1: Ambition; 2: FIRL3565; 3: Bussard; 4: Event; 5: Format; 6: Julius; 7: BAYP4535; 8: Potenzial; 9: RGT Reform; LMW-GS: low-molecular-weight glutenin subunit; HMW-GS: high-molecular-weight glutenin subunit.

pattern across methods for the sum of gluten proteins was similar for wheat lines 1–4 and 6–9, with only small differences. While both LFQ datasets looked similar for line 5, the absolute data showed a higher intensity compared to the other lines. When looking at the individual protein quantities/intensities, there were clear differences between relative and absolute data, as well as between the LFQ data (**Fig. S3**).

In summary, the analyzed protein abundances partially agreed between the three methods (Fig. 2). Looking at the average values across the nine wheat lines, this applied to α-gliadin 1, α-gliadin 2, LMW-GS 2, HMW-GS 1, HMW-GS 3 and HMW-GS 5, although these also showed small deviations. Remarkably different abundances were found for γ-gliadin, LMW-GS 1, LMW-GS 3, HMW-GS 2 and HMW-GS 4. Overall, the results for α-gliadins were largely consistent across the methods, with slightly lower abundances detected by nanoLC-MS/MS. By contrast, γ-gliadin estimates diverged markedly with UHPLC-MS/MS resulting in the lowest abundances, SIDA in intermediate, and nanoLC-MS/MS in the highest abundances. This points to substantial LFQ variability. LMW-GS also showed method-dependent discrepancies. LMW-GS 1 was often missed by nanoLC-MS/MS, detected at low levels by SIDA, and slightly higher by UHPLC-MS/MS. The results for LMW-GS 2 were similar in the two LFQ datasets but consistently lower by SIDA. Notably, it was absent in lines 3 and 5 across all methods. LMW-GS 3 showed the largest disparity with very high abundances by UHPLC-MS/MS and high by nanoLC-MS/MS, but much lower by SIDA.

In contrast to LMW-GS, HMW-GS generally showed higher abundances by SIDA than by LFQ. HMW-GS 1 was low by LFQ but higher (and wheat line dependent) by SIDA. HMW-GS 2 was uniform across lines by SIDA but highly variable by LFQ (low in wheat lines 1, 5, 6 and 7, but high in 2, 3, 4, 8 and 9). HMW-GS 3 and HMW-GS 5 were similar across methods. HMW-GS 4 showed the largest discrepancy: the LFQ results were the same as for HMW-GS 3 because both share the same leading razor protein, whereas SIDA reported substantially higher levels. P10 (HMW-GS 4) occurs in nine protein groups/51 proteins, while P9 (HMW-GS 3) is present in 2 groups/23 proteins.

Differences between methods likely arise because SIDA quantification of each protein group relied on a single marker peptide. If other peptides of the same group have better MS behavior, LFQ will capture them, yielding higher relative signals. Single-peptide SIDA is also vulnerable to peptide loss (PTMs, incomplete digestion, instability) (Calderon-Celis et al., 2018; Hoofnagle et al., 2016), explaining the lower SIDA values for LMW-GS 2 and 3. Using multiple peptides per group would increase robustness, as shown for ATI in wheat species (Geisslitz et al., 2020). Another likely cause for method discrepancies is protein misassignment in untargeted data due to high sequence homology (e.g., UniProt IDs Q6RX92 vs. Q6RX93 for HMW-GS differ by just one out of 658 amino acids). γ-gliadins and some LMW-GS are also highly similar, which is why LMW-GS peptides may be wrongly attributed to γ-gliadins, e.g., for LMW-GS 3. This reflects a core challenge in bottom-up untargeted proteomics of wheat flour: repetitive, near-identical sequences and poor tryptic cleavage (sequence coverage often <30 %), which renders peptide-to-protein assignment uncertain (Dupont et al., 2011; Ferranti et al., 2007).

LFQ results differed markedly between HPLC systems. LMW-GS 1 was not detected by nanoLC-MS/MS but present by UHPLC-MS/MS and SIDA, implicating insufficient nanoLC separation of peptide P4. For γ-gliadin 1, SIDA results were between the two untargeted runs, again highlighting chromatographic effects. Differential peptide separation can lead to divergent LFQ intensities across systems, showing the limitations of label-free approaches that remain less robust and reproducible than label-based workflows (Calderon-Celis et al., 2018).

SIDA is more precise, accurate, and sensitive than relative methods (Ludwig & Aebersold, 2015). For example, the results for HMW-GS 2 were similar across lines by SIDA but highly variable by LFQ, pointing to protein misassignment issues. The much higher abundances for HMW-GS 4 (and other proteins) in SIDA likely reflect superior PRM selectivity for P10. Consistent with this, Geisslitz et al. (Geisslitz et al., 2022) found good cross-method agreement for abundant ATI but divergence for low-abundance targets (Geisslitz et al., 2022). Except for P4 and P5, all SIDA marker peptides were highly abundant, making low signal an unlikely cause of the discrepancies between the methods.

There are several studies investigating the wheat proteome (Afzal et al., 2021; Duncan et al., 2017; Vincent et al., 2022), and targeted approaches have traced wheat in non-wheat matrices using wheat-specific peptides (Colgrave et al., 2015; Martinez-Esteso et al., 2016). Gluten protein types have also been quantitatively determined in the literature using HPLC (Wieser et al., 1998; Xhaferaj & Scherf, 2024), with these studies consistently showing that gliadins constitute a larger proportion of total gluten proteins than glutenins. However, relative MS comparisons based on different peptidases have yielded divergent relative proportions of the gluten protein types (Kaemper et al., 2025). In contrast to these approaches, we absolutely quantify individual gluten protein groups by SIDA, employing one isotope-labeled peptide per protein group. Moreover, we focused primarily on the HMW-GS, as they are strongly associated with baking quality, and identified five peptides suitable for absolute quantification, whereas for the other protein types only one to three peptides were chosen. Consequently, the absolute amounts of individual proteins or protein groups reported here do not represent the overall gluten protein composition in wheat. To our knowledge, this is the first SIDA-based LC–MS/MS method targeting specific gluten peptides/proteins.

3.5. *Content of gluten proteins in the BMWpop*

The proteins in the 403 lines comprising the BMWpop, its parent lines, and RGT Reform were quantified using the developed LC–MS/MS SIDA method. As the BMWpop is a MAGIC population, the genetic composition of each progeny line results from recombination among the parental lines. Therefore, the progeny lines are expected to express the same peptides/proteins as those selected here, enabling consistent and comparable protein quantification across the population. Overall, most

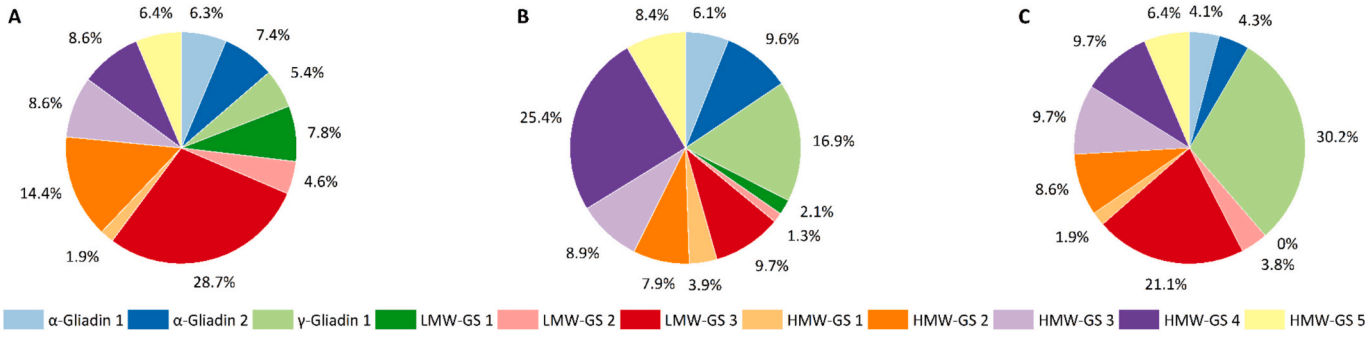

Fig. 2. Comparison of the average percentages of wheat gluten proteins determined in the parental lines of BMWpop and RGT Reform by UHPLC-MS/MS (A), SIDA (B) and nanoLC-MS/MS (C).

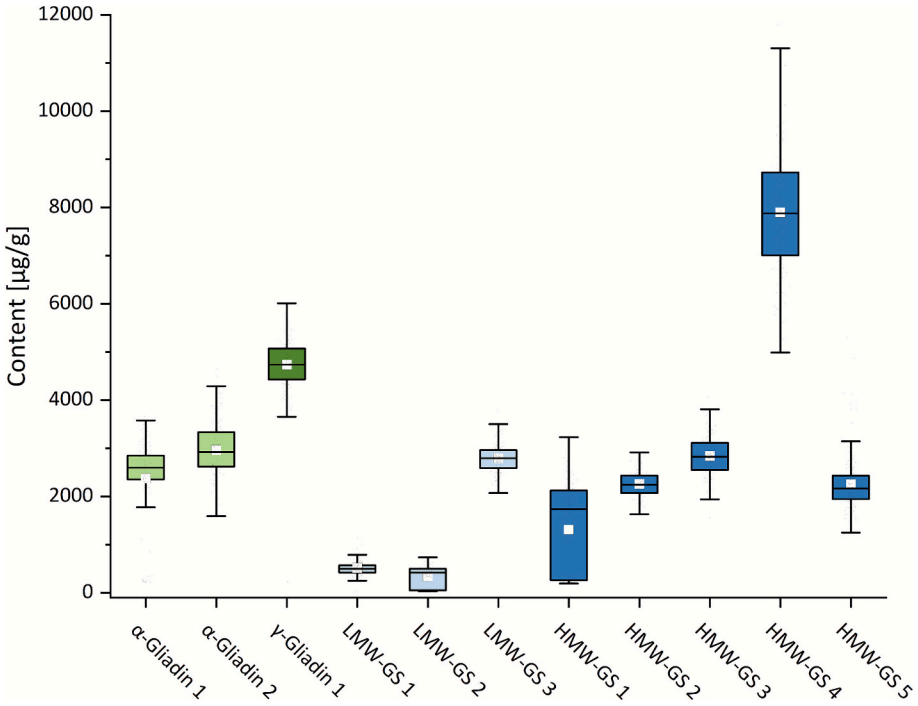

Fig. 3. Content of the eleven wheat gluten protein types in 403 wheat lines comprising the BMWpop, its parental lines and RGT Reform following absolute quantification. HMW-GS: high-molecular-weight glutenin subunit; LMW-GS: low-molecular-weight glutenin subunit.

proteins were present at similar concentrations (2000–4000 µg/g, Fig. 3). LMW-GS 1 and 2 stood out due to their comparatively low levels (<500 µg/g), whereas HMW-GS 4 was detected with higher levels (5000–11,000 µg/g) with substantial variability across the wheat lines.

The genetic assignment of α- and γ-gliadins as well as LMW-GS using UniProtKB was challenging, as discussed above, due to redundant and inconsistent entries. In contrast, more information was available for the HMW-GS (Table S3), because the databases are better annotated. The HMW glutenin alleles of the BMWpop were determined using single-nucleotide polymorphism markers published by Ravel et al., with the parental wheat lines shown in **Table S4** (Ravel et al., 2020). Genetic assignments were made as follows: HMW-GS 1 corresponds to Dy10, HMW-GS 2 to Ax2*, HMW-GS 3 to Dy10, Dy12, and By8, HMW-GS 4 to Dy10, Dy12, By8, By9, and 1Ay, and HMW-GS 5 to Dx2 and Dx5. The groups HMW-GS 1, 3, 4, and 5 showed partial overlap, which resulted from the peptide selection strategy for targeted analysis, where MS detectability was prioritized. Notably, HMW-GS 4 includes most y-type glutenins.

3.6. Relationship between gluten protein quantities and quality parameters

A combined principal component analysis (PCA) was performed using SIDA results and quality parameters of the 403 genotypes, namely gluten index, loaf volume, falling number, farina yield, grain hardness, water absorption, grain protein content, sedimentation value, flour yield, and wet gluten content (Fig. 4). PC1 (28.5 %) and PC2 (11.7 %) together captured 40.2 % of the variance in the dataset. The loadings plot indicated that HMW-GS 2–4, sedimentation value, and grain protein content are nearly collinear and load primarily on PC1 (Fig. 4A). Loaf volume, water absorption, and grain hardness pointed in the same general direction but were closer to the origin, indicating smaller contributions. Notably, HMW-GS 1 (Dy10) aligned with loaf volume yet was separated from the other HMW-GS subunits (2–4). By contrast, wet gluten content and gluten index pointed in opposite directions on PC2, indicating an inverse relationship between them. Farina yield and falling number were only weakly represented on the PC1–PC2 plane.

The scores plot revealed a near-symmetric, center-weighted distribution of the 403 wheat lines, with density tapering toward the periphery (Fig. 4B). The parental lines showed the same pattern, being distributed across all quadrants. In particular, Ambition (395) and BAYP4535 (401) projected far into quadrant III along PC1 and differed markedly from the remaining parents, whereas the reference standard RGT Reform was located near the origin. The 25 outermost samples were concentrated mainly in quadrants III and IV, indicating weaker alignment with the PC1 gradient associated with baking quality and protein content traits. However, lines 51, 156, 210, and 289 were located far to the right along PC1, consistent with elevated HMW-GS abundance. This makes their genetic profiles potentially valuable for future breeding efforts.

3.7. Correlations between gluten protein quantities and quality parameters

Considering the evidence from the PCA, we conducted correlation analyses between gluten protein quantities and quality parameters (Fig. 5). Most correlation coefficients fell within the very weak ($|r| < 0.2$) or weak range ($0.2 \leq |r| < 0.4$). Moderate correlations ($0.4 \leq |r| < 0.6$) were also observed, while strong correlations ($0.6 \leq |r| < 0.8$) were rare and very strong correlations ($|r| \geq 0.8$) were not found. Correlations of $|r| \geq 0.4$ were considered potentially relevant, as they may indicate a measurable influence of individual proteins on technological properties (Cohen, 2013). In the following, all significant correlations ($p \leq 0.05$) were analyzed to distinguish protein groups with statistically relevant associations from those without significant results.

Most gluten proteins showed weak to moderate correlations with baking quality parameters, except for LMW-GS 1 and 2, which displayed almost none, likely due to their low content (Fig. 3). The strongest overall associations were observed for grain protein content, sedimentation value, and wet gluten content with α-gliadin 2, γ-gliadin 1, LMW-GS 3, and HMW-GS 2–4. Wet gluten content was moderately correlated with γ-gliadin 1 (0.50), α-gliadin 2 (0.58), LMW-GS 3 (0.40), and HMW-GS 4 (0.43). Sedimentation value was strongly correlated with HMW-GS 3 (0.61) and moderately with α-gliadin 2 (0.42), γ-gliadin 1 (0.54),

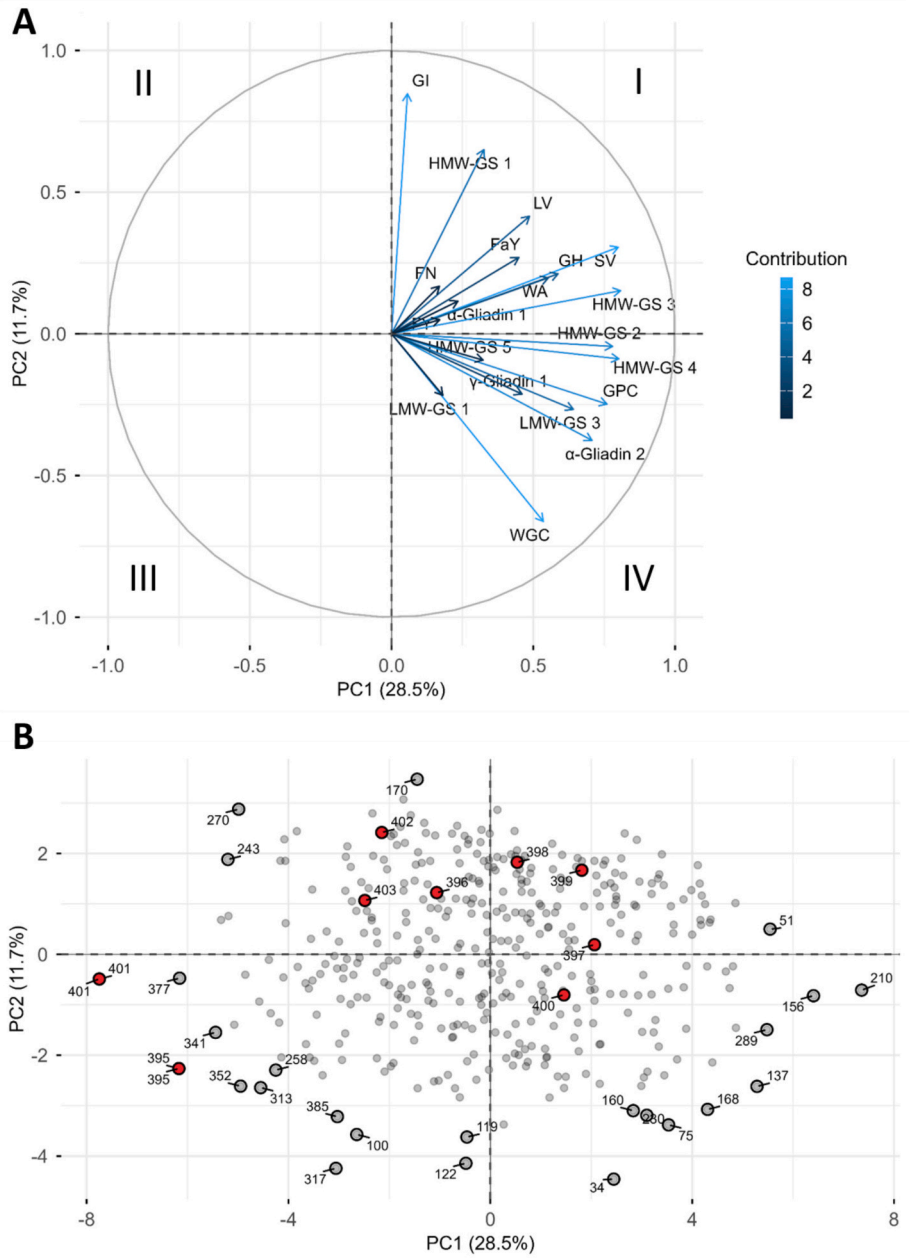

Fig. 4. Principal component analysis of baking quality parameters and absolute protein content in the BMWpop, its parental lines and RGT Reform. (A) Loadings plot; (B) scores plot. FaY, farina yield; FN, falling number; FY, flour yield; GH, grain hardness; GI, gluten index; GPC, grain protein content; HMW-GS, high-molecular-weight glutenin subunit; LMW-GS, low-molecular-weight glutenin subunit; LV, loaf volume; SV, sedimentation value; WA, water absorption; WGC, wet gluten content. The 25 outermost wheat lines (relative to the origin) are highlighted and labeled. Parental lines and comparison standard RGT Reform are highlighted in red and coded as follows: 395: Ambition, 396: FIRL3565, 397: Bussard, 398: Event, 399: Format, 400: Julius, 401: BAYP4535, 402: Potenzial, 403: RGT Reform. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LMW-GS 3 (0.44), and HMW-GS 2/4 (0.48/0.48). Grain protein content was strongly correlated with α-gliadin 2 (0.61), γ-gliadin 1 (0.64), and HMW-GS 3/4 (0.61/0.60), and moderately with LMW-GS 3 (0.56) and HMW-GS 2 (0.51). For the gluten index, HMW-GS 1 showed the highest correlation (0.52; moderate), whereas the other protein fractions were only weakly to very weakly correlated. Moderate correlations were found only for loaf volume with HMW-GS 1 (0.41) and HMW-GS 3 (0.40). Correlations involving farina yield, grain hardness, and water absorption were weak to very weak; while those with falling number and flour yield were not significant. The only significant negative correlations, wet gluten content with HMW-GS 1 and gluten index with α-gliadin 2, were also weak.

Among α-gliadins, α-gliadin 2 (54 proteins) showed moderate correlations, whereas α-gliadin 1 (three proteins) showed only weak ones. Alignments revealed minimal divergence and co-clustering, offering no sequence-based explanation. α-Gliadins can enhance baking quality, as removal of the 6D α-gliadin locus worsened technological traits (Gabriel et al., 2017; Noma et al., 2023). However, in another study, targeted *Gli-D2* silencing (−63 % α-gliadins, replaced by avenins) strengthened dough with only a slight decrease in loaf volume (van den Broeck et al., 2011). Li et al. linked α-gliadins encoded on chromosome 6D, which often contain an odd number of cysteines, to improved gluten quality, likely via structural effects in their unique domains (Li et al., 2014). Most α-gliadin 1/2 proteins cannot be chromosome-mapped, so a D-genome effect cannot be confirmed.

γ-gliadin 1 likewise displayed moderate associations. Within the 34

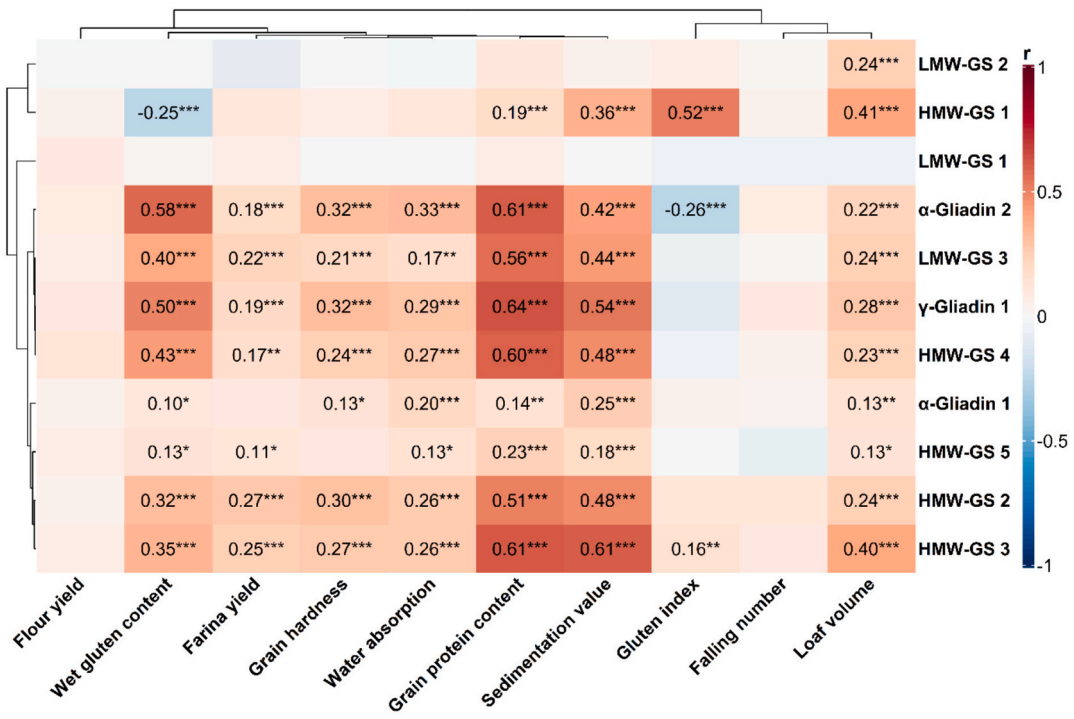

Fig. 5. Correlation matrix of absolute gluten protein contents and baking quality parameters of 403 wheat lines comprising the BMWpop, its parental lines and RGT Reform. Values show Pearson's r; asterisks indicate Benjamini–Hochberg false discovery rate adjusted *p*-values: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$; LMW-GS: low-molecular-weight glutenin subunit; HMW-GS: high-molecular-weight glutenin subunit.

proteins in this group, three have seven cysteine residues and the others eight, precluding a clear attribution of differences in baking quality to the number of cysteine residues. One allele of *Gli-γ1-1D* linked to enhanced baking quality was discovered by Liu et al. (Liu et al., 2023). Zhou et al. found that supplementation with the γ-gliadin fragment *TaGli-γ-2.1* improved gluten dough characteristics (Zhou et al., 2022). Sherman et al. further showed that a particular *Gli-B1* allele can improve the baking quality of durum wheat (Sherman et al., 2018). According to the database analysis, most proteins in the γ-gliadin-1 group cannot be mapped to a specific chromosome; therefore, no specific gene can be inferred and comparison with the literature is not feasible.

LMW-GS 1/2 exhibited few significant correlations, probably owing to their low concentrations, while LMW-GS 3 displayed moderate associations. LMW-GS 1/2 are m-type, whereas all 72 proteins in LMW-GS 3 are i-type with an *N*-terminal isoleucine and eight cysteines (Ferrante et al., 2007), pointing to a beneficial effect of i-type LMW-GS. Earlier findings are inconclusive, because the addition of i-type LMW-GS weakened durum wheat dough, but had little effect in bread wheat (Ferrante et al., 2006), and nine-cysteine i-types were linked to poorer quality (Huang et al., 2021). LMW-GS 3 comprised only eight-cysteine i-types, aligning with the model of six intra- and two intermolecular disulfide bonds that strengthen gluten (D'Ovidio & Masci, 2004). Fully resolving the exact effects requires complete protein identifications with precise allele assignments, which remain incomplete despite wheat genome sequencing successes (The International Wheat Genome Sequencing Consortium, 2018). The complexity of the hexaploid, multigenomic structure of wheat complicates gene–protein assignment, particularly for gliadins, which are encoded by large, partly redundant gene families (e.g., 47 α-gliadin genes producing 26 proteins (Huo et al., 2018); 52 gliadin genes producing 25 α-, γ-, and ω-gliadins (Wang et al., 2017)). By contrast, LMW-GS alleles have been more thoroughly characterized for their relevance to baking quality (Guzmán et al., 2022). Systematically integrating genetic metadata into public protein databases would strengthen links between proteomic, genetic, and functional data.

HMW-GS 1 (Dy10) aligned with indicators of better baking performance but showed a slight opposing trend for wet gluten content. HMW-GS 3 (Dy10, Dy12, By8) matched Dy10 for loaf volume, implying that Dy12/By8 add little for loaf volume beyond Dy10. HMW-GS 4 (most y-types) was weakly related to loaf volume but more clearly to sedimentation value and grain protein content, suggesting functional diversity among y-types. HMW-GS 2 (Ax2*) showed moderate links to protein content and sedimentation value but weak associations to loaf volume. HMW-GS 5 (Dx2, Dx5) had similar modest correlations, but none with loaf volume. HMW-GS Ax2* and Dx5 + Dy10 are commonly tied to stronger gluten network formation and higher loaf volume, in contrast to Dx2 + Dy12 which is linked to poorer quality (Guzmán et al., 2022). Overall, correlations to loaf volume were weak at most, including HMW-GS Dy10, which is generally regarded as particularly influential. However, HMW-GS 1 (Dy10) exerted the greatest influence, even though it was detected in comparatively low content (up to 3200 µg/g, Fig. 3). Indeed, it has been demonstrated that the strongest positive effect on baking quality occurs when HMW-GS of both x- and y-types are present in equal proportions, in contrast to doughs enriched with only one type (Anderson & Bekes, 2011). In this study, y-type HMW-GS appeared to contribute more to baking quality, whereas x-types showed no substantial individual effect. Although the two types are associated, studies indicate that Glu-1 genes of both types are regulated by different transcription factors: x-type genes are mainly controlled by Myb factors, whereas y-type genes are regulated by other transcription factors during the late grain-filling stage (Makai et al., 2015). Further research focuses on additional regulatory elements and promoter regions to achieve a more comprehensive understanding of HMW-GS expression control (Éva et al., 2023; Guo et al., 2015). Dx2/Dx5 could not be distinguished in the targeted data. Molecular markers diagnostic for Glu-D1 allowed the BMWpop lines to be grouped according to their allelic variants (Dx2, $n = 168$; Dx5, $n = 226$). Both groups were correlated separately with the baking quality parameters; however, all correlation coefficients were below 0.4 (**Fig. S4**). Dx2 correlated slightly more than Dx5 which is contrary to reports favoring Dx5 for its extra cysteine (Li et al., 2020;

Shewry et al., 2003). This may indicate that the extra cysteine might also hinder polymerization (Wieser, 2007). Given the low correlations overall, firm conclusions cannot be drawn.

Correlations based on all pairwise protein combinations (as a sum of both) modestly increased associations (**Fig. S5**). The correlation coefficients were highest for grain protein content (0.66–0.73), followed by sedimentation value and wet gluten content (0.58–0.67) and the gluten index (0.52–0.61). The associations to loaf volume remained moderate (0.41–0.50), while those to grain hardness and water absorption were low (0.33–0.36) and even lower for farina yield. Those with falling number/flour yield were not significant. Across quality traits, the top ten pairwise correlations spanned 42 distinct pairs, 35 of which included at least one HMW-GS. The correlations were slightly stronger than those based on one protein, but summation also obscures, e.g., inverse contents.

Correlations based on all possible combinations of the eleven proteins (as sum), showed that the top ten multiple-protein correlations per quality trait were highest for wet gluten content and grain protein content (both 0.79), sedimentation value (0.73) and gluten index (0.72), and moderate for loaf volume (0.62). Grain hardness reached 0.42, water absorption 0.39 and farina yield 0.37, whereas falling number and flour yield resulted in no significant correlations (**Fig. S6**). Notably, the top-scoring combinations always summed $\geq$8 proteins, indicating that the strongest associations emerge only when most proteins are considered. For loaf volume, this also points to contributions from proteins outside the panel and/or non-protein factors. Thanhaeuser et al. reported that gliadin, glutenin and glutenin macropolymer content were more strongly correlated with loaf volume than crude protein content, suggesting these protein fractions may be more reliable predictors (Thanhaeuser et al., 2014). In contrast, a study on 82 wheat lines reported no significant relationship between individual gluten protein types and loaf volume (Schuster et al., 2022). Our findings reinforce that baking quality is inherently complex and cannot be captured by a single or only a few determinants, as also concluded by Schuster et al.

Unique peptide markers per protein are essential for targeted quantification. Given the cost of labeled peptides, proteins most strongly associated with the quality traits of interest should be prioritized. The trade-off is that only selected peptides are measured rather than the broadest possible set per protein. At peptide level, a distinction between Dx2 and Dx5 is needed rather than relying on genetic information. Recent work by Geisslitz & America shows that Dx2/Dx5-specific peptides can be identified (Geisslitz & America, 2025). Extending robust, unique markers to LMW-GS and gliadins remains an open task. Targeted proteomics provides specific, precise peptide quantification. Using stable-isotope–labeled internal standards, SIDA corrects losses and matrix effects and is considered the gold standard for absolute quantification (Ludwig & Aebersold, 2015).

4. Conclusions

We successfully developed and established a targeted SIDA workflow for eleven gluten protein groups, enabling robust, absolute quantification and detailed mapping of protein–quality trait associations. Individual groups were at best modest predictors of baking quality, whereas multi-protein combinations strengthened associations — underscoring that baking quality is a complex, multivariate trait. Data-integration approaches using machine learning could fuse heterogeneous omics and process data to improve quality prediction (Lullien-Pellerin, 2024). Comparison of the untargeted and targeted datasets revealed some differences, possibly due to distinct identification/assignment strategies, different HPLC-Systems and MS instrument acquisition methods. Yet the two approaches are complementary and, taken together, enhance our understanding of the wheat proteome. Future work should expand sequence-specific peptide markers to further align targeted and untargeted findings. A major limitation is the lack of unambiguous mapping of proteins to specific gene loci. Curated reference databases with consistent genetic annotation and standardized nomenclature are needed to enable robust genetic interpretation and cross-study comparability.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the first author employed ChatGPT (OpenAI, 2025) to assist with language refinement and grammatical corrections, as well as to support R coding during data analysis. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Christine Kaemper: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Manuel Geyer:** Writing – review & editing, Resources, Project administration. **Lorenz Hartl:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Sabrina Geisslitz:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.118228.

Data availability

Mass spectrometry data are publicly available on Panorama Public (https://panoramaweb.org/jn823F.url) with the ProteomeXChange ID PXD072549. All detailed values can be found within the Supplementary Material.

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