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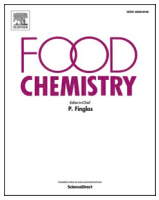

Barley hordein isolates as candidate reference materials for gluten quantitation – a proof-of-concept study

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ABSTRACT

Barley hordeins trigger celiac disease (CeD), but current ELISA methods frequently overestimate gluten from barley because they rely on wheat-based reference materials (RM). To address this, we produced four hordein isolates, namely prolamins, acetonitrile/water-extractable proteins (AWEP), gluten and glutelins, from a blend of seven European and one Canadian barley cultivars. Using SDS-PAGE, HPLC and shotgun proteomics, we confirmed that blending eight cultivars successfully mitigated genetic and environmental variability in protein content and composition. Complementary analyses revealed that the isolates were comparable in protein composition to the respective barley flour fractions, except some divergence in glutelins. These well-characterized hordein isolates are proposed as suitable candidates for a standardized barley RM. Establishing such an RM is essential for accurate gluten quantitation in gluten-free compliance monitoring to reduce the variation of results within and between test kits and thus enhance food safety for consumers with CeD.

1. Introduction

Barley (*Hordeum vulgare*) is the fourth most important cereal worldwide after maize, rice and wheat (Food and Agriculture Organization of the United Nations, 2026). Its agricultural resilience makes it a critical crop for global feed and food security in the face of climate change (Jiang et al., 2025). While approximately 65–70% of barley production is used for animal feed, much of the remaining part is dedicated to malting and brewing, although its high content of β-glucan, tocopherols, phenolic acids and flavonoids make barley an attractive grain for human consumption (Farag et al., 2022). In the context of wheat/gluten-related disorders, barley grain storage proteins, called B-, C-, D- and γ-hordeins, are known to cause celiac disease (CeD) because of their high degree of amino acid sequence homology to the corresponding gluten proteins found in wheat and rye grains (Sollid et al., 2020). Accordingly, barley is listed in the definition of gluten-free foods that are dietary foods consisting of ingredients which do not contain wheat, rye, barley, oats, or their crossbred varieties and the gluten level is 20 mg/kg or below based on the product as sold (Codex Alimentarius Commission, 1979; European Commission, 2014).

Reliable analytical methods to detect residual gluten in supposedly gluten-free foods are important to ensure safe food choices for individuals with CeD. Routine testing is most frequently carried out by ELISA using gluten-specific polyclonal or monoclonal antibodies (mAb) including the R5 (Amnuaycheewa et al., 2022). The R5 mAb mainly targets the epitope QQPFP and its related sequences QQQFP, LQPFP and QLPFP, which are frequently conserved within CeD-immunoreactive peptides (Kahlenberg et al., 2006; Osman et al., 2001). One essential component of commercially available ELISA test kits is the reference material (RM) that is used to convert the absorbance signal to gluten content. Different RMs are used depending on the test kit including Prolamin Working Group (PWG)-gliadin (van Eckert et al., 2006), gluten and wheat protein for sandwich ELISA or partially hydrolysed prolamins from wheat, rye and barley for competitive ELISA (Gessendorfer et al., 2009; Rzychon et al., 2017). As all these RMs are wheat-based, previous studies have shown that hordeins are overestimated by as much as

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approximately 3.5 times (Wehling & Scherf, 2020), 6 times (Lexhaller et al., 2016) and 7–30 times (Kanerva et al., 2006) when applying the standard ELISA procedure. The reason is the high affinity of the R5 mAb to C-hordeins that contain up to 17 repeats of the main QQPFP epitope (Lexhaller et al., 2017). This is why Tanner, Blundell, et al. (2013) collected B-, C- and γ-hordein enriched fractions to optimise the response of an ELISA based on the Skerritt mAb. Huang et al. (2017) proposed using a RM containing 40% of C-hordeins to quantitate gluten in barley-containing foods and 10% of C-hordeins for wheat-containing foods to correct for this overestimation (Huang et al., 2020). While this approach works to make the results for barley and wheat gluten more comparable, the ion-exchange chromatographic purification of C-hordeins from a 40% propanol extract of barley flour is rather time- and labour-intensive. Despite the need for suitable RM to accurately quantitate potential barley gluten contamination in gluten-free products, a specific barley RM remains unavailable and there are no further fundamental comparative studies on barley hordein isolates. Flours of five wheat cultivars (Hajas et al., 2018; Schall et al., 2020), seven rye cultivars (Xhaferaj et al., 2025) and eight barley cultivars (Xhaferaj et al., 2023) were shown to be representative of the wide variety of those grains grown around the world. Blends of these selected cultivars reduced genetic and environmental variability of protein composition compared to single cultivar flours and were suitable to produce rye secalin isolates with better long-term stability compared to flours (Xhaferaj et al., 2025).

Our hypothesis is that blending the selected seven European and one Canadian barley cultivars from a second harvest year will even out the environmental effect on protein composition and produce a reproducible flour as starting material to produce barley hordein isolates. Consequently, we aimed to generate four distinct hordein isolates from the mixture of seven European and one Canadian barley cultivars using varying extraction protocols. To account for environmental variability and ensure a representative hordein profile mostly for European cultivars, the eight barley cultivars selected previously (Xhaferaj et al., 2023) were sourced from a second harvest year. These isolates underwent extensive characterization via complementary gel electrophoretic, chromatographic, mass spectrometric and immunochemical techniques. Providing such well-characterized isolates serves as a critical proof-of-concept toward establishing a standardized barley RM, facilitating more accurate and comparable gluten quantitation to enhance the safety of gluten-free products for consumers with CeD.

2. Materials and methods

2.1. Barley grains and flour preparation

Eight selected barley cultivars (Xhaferaj et al., 2023) were acquired from the harvest year 2022 (Table 1). The selection included seven European and one Canadian cultivars, different row types (two-row vs. six-row) and main uses. The grains of the individual cultivars and the grain mixture (500 g of grains of each of the eight cultivars blended) were milled using a laboratory mill (Cyclotec Mill 991,093, Foss Tecator AB, Höganäs, Sweden). Mixture 1 is the blend of the eight cultivars from the first collection (harvest years 2017 to 2020, depending on the cultivar) and mixture 2 is the blend of the eight cultivars from the second collection (2022). The resulting wholegrain flours were stored in zip-lock bags at 22 °C for at least two weeks.

2.2. Barley hordein isolation

The flour mixture (100 g) was defatted by stirring three times with 250 mL of n-pentane/ethanol (95/5, *v*/v), followed by stirring once with 250 mL of n-pentane at 22 °C for 30 min (Schalk et al., 2017). The suspensions were filtered (Sartorius Stedim Biotech GmbH, Goettingen, Germany) under vacuum in each step. Then, the defatted flour was spread thin, dried for 18 h at room temperature and manually homogenized with a spatula.

Four different barley hordein isolates, namely prolamins, glutelins, gluten and acetonitrile/water-extractable proteins (AWEP), were produced exactly according to Xhaferaj et al. (2025) based on commonly used protocols for plant protein fractionation (Batey et al., 1991; Schalk et al., 2017). In brief, the water- and salt-soluble albumin/globulin fraction was removed three times by mixing the defatted flour with salt solution (400 mmol/L NaCl in 67 mmol/L Na_2PO_4/KH_2PO_4 (pH 7.6)), centrifuging (3750 rcf, 22 °C, 25 min) and discarding the supernatant. The sediment was subsequently used to produce the isolates.

First, the prolamins were extracted three times with aqueous ethanol (60/40, *v*/v), followed by centrifugation and combination of the three supernatants. Second, the glutelins were extracted three times from the sediment after prolamin extraction under reducing conditions at 60 °C and argon atmosphere with 2-propanol/water (50/50, *v*/v) containing 0.1 mol/L Tris-HCl, pH 7.5, 2 mol/L (*w*/*v*) urea and 0.06 mol/L (*w*/*v*) dithiothreitol (DTT). After centrifugation, the respective supernatants were collected and combined.

Third, the gluten isolate was produced in a separate trial by skipping the prolamin extraction and directly using reducing conditions at 60 °C and argon atmosphere with 2-propanol/water (50/50, *v*/v) containing 0.1 mol/L Tris-HCl, pH 7.5, 2 mol/L (*w*/*v*) urea and 0.06 mol/L (*w*/*v*) dithiothreitol (DTT) three times. After centrifugation, the respective supernatants were collected and combined.

Fourth, the AWEP isolate was produced in another separate trial using three times acetonitrile/water (50/50, *v*/v) containing 0.1% formic acid (FA) (*v*/v) as extraction solvent. The suspensions were sonicated once for 30 s, but the two subsequent extraction steps were done

Table 1
General sample information for the eight selected barley cultivars across different years.

Cultivar	Geographical origin	Provider	Row type and main use	Year of collection	Sample code
Celebration	Canada	Carrington Research Extension Center NDSU	Six-row spring, malting	2019	CEL_CAN19
				2022	CEL_CAN22
Coccinel	France	Secobra Saatzucht GmbH	Six-row winter, feed	2020	COC_FRA20
				2022	COC_FRA22
Evelina	Austria	Saatzucht Edelhof	Two-row spring, feed	2020	EVE_AUS20
				2022	EVE_AUS22
Evergreen	Denmark	Nordic Seed	Two-row spring, malting	2020	EVE_DEN20
				2022	EVE_DEN22
GK Judy	Hungary	Cereal Research Non-Profit Ltd.	Two-row winter, malting	2017	GKJ_HUN17
				2022	GKJ_HUN22
Jakobus	Germany	Nordsaat Saatzucht GmbH	Six-row winter, feed	2020	JAK_GER20
				2022	JAK_GER22
Kornelija	Latvia	Institute of Agricultural Resources and Economics	Two-row spring (naked), special foods/feed	2019	KOR_LAT19
				2022	KOR_LAT22
Pixel	France	Secobra Saatzucht GmbH	Six-row winter, feed	2020	PIX_FRA20
				2022	PIX_FRA22

without additional sonication. After centrifugation, the respective supernatants were collected and combined.

The prolamin, glutelin, gluten and AWEP fractions each were concentrated under reduced pressure to remove most of the organic solvent and dialyzed against 0.01 mol/L acetic acid followed by deionized water using a membrane with a molecular weight cut-off of 12–14 kDa (Spectrum Labs Spectra/Por Dialysis Membrane, Thermo Fisher Scientific, Waltham, MA, USA), followed by lyophilization for 48 h at −60 °C and 0.1 mbar. After drying, the isolates were stored at −20 °C until use. For full experimental details, please refer to Xhaferaj et al. (2025).

2.3. Crude protein content

The crude protein content was analyzed according to ICC Standard No. 167 in duplicate for the flours using a Leco FP 528 nitrogen analyzer (Leco Corporation, St. Joseph, MO, USA) and in triplicate for the isolates using a DUMATHERM N Pro nitrogen analyzer (C. Gerhardt, Königswinter, Germany). The conversion factor from nitrogen to protein content was 5.7.

2.4. Protein composition

The protein composition of the flours and isolates was characterized exactly as described in Xhaferaj et al. (2023) and Xhaferaj et al. (2025). Albumins/globulins, prolamins and glutelins were extracted from 100 mg of flour following the three-step procedure, each step involving magnetic stirring, vortexing, centrifugation and adjustment of the final solution volume to 2 mL. The isolates were reconstituted in the respective extraction solution at a concentration of 1 mg/mL. After filtration (0.45 µm Whatman Spartan, Cytiva Europe GmbH, Freiburg im Breisgau, Germany), the solutions were used for HPLC analysis.

2.5. Reversed-phase high-performance liquid chromatography

The total extractable protein content and the distribution of B/γ-, C- and D-hordeins were measured by RP-HPLC using the setup, column, mobile phase, and separation gradients outlined in Xhaferaj et al. (2025). The proteins were detected using UV absorption at 210 nm and their quantities were calculated using external calibration with Prolamin Working Group (PWG)-gliadin (distributed by Arbeitsgemeinschaft Getreideforschung e.V., Detmold, Germany). The hordein types were assigned based on their retention times in the chromatograms of the reduced prolamin and glutelin fractions, respectively, and their percentages relative to the total peak area.

2.6. Gel permeation high-performance liquid chromatography

The relative molecular weight (Mw) distribution of the prolamin, reduced prolamin and glutelin fractions were determined using GP-HPLC. The separation used the exact same apparatus, column, and mobile phase and detection at 210 nm as described by Xhaferaj et al. (2023). The integration limits were set by analyzing proteins with known Mw, namely cytochrome C from horse heart (12.4 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and albumin from bovine serum (66 kDa) (Sigma-Aldrich, Darmstadt, Germany). The following Mw ranges were applied: (1) >66 kDa, (2) 66–29 kDa, (3) 29–12.4 kDa, (4) <12.4 kDa.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The flours (20 mg) or isolates (2 mg) were extracted with 1 mL of the extraction buffer (pH 8.5) overnight under reducing conditions (DTT, 50 mmol/L) as described earlier (Xhaferaj et al., 2025). After shaking for 10 min at 60 °C, the suspensions were centrifuged at 2370 rcf for 5 min at 20 °C. Then, the samples (10 µL) were applied to a NuPAGE 4–12% Bis-Tris protein gradient gel (1.0 mm, 10-well, Invitrogen, Carlsbad, CA, USA) with MOPS running buffer (pH 7.7) including DTT (5 mmol/L) as reducing agent. The gels were developed at 200 V and 115 mA with a run time of 30 min. Staining with Coomassie Brilliant Blue G-250 (3 mmol/L), destaining and fixing was conducted according to Lagrain et al. (2012) and Xhaferaj et al. (2025). A scan of the gels was taken (LAS-3000, Fujifilm, Minato, Tokyo, Japan) and the Mw of the bands was estimated using the marker proteins by the AIDA Image Analysis software V3.27.001 (Elysia-raytest, Angleur, Belgium).

2.8. R5 enzyme-linked immunosorbent assay

The responses of the barley isolates toward the R5 mAb were tested using the RIDASCREEN Gliadin ELISA test kit (R7001, R-Biopharm, Darmstadt, Germany) following the methodology described by Lexhaller et al. (2017) and Xhaferaj et al. (2025). The prolamin isolate was diluted in 60% ethanol, the glutelin and gluten isolates were diluted using the glutelin extraction solution and the AWEP isolate was diluted in acetonitrile/water (1 + 1, v + v) containing 0.1% FA (v + v). The final dilution (1 + 11.5, v + v) was done with the kit sample dilution buffer. The ELISA was carried out according to the manufacturer's instructions to measure at least four serial dilutions of each extract in the working range from 5 to 40 ng/mL. The ELISA absorbances were plotted against the protein concentrations of the isolate solutions determined by RP-HPLC considering the dilution factors. The RP-HPLC concentrations were also plotted against the ELISA concentrations using the kit standard. Calibration curves were calculated using the cubic spline function of the RidaSoft Win Software (R-Biopharm).

2.9. Discovery proteomics of the hordein isolates

2.9.1. Sample preparation

The protein isolates were dissolved in 1 mL of Tris-HCl (0.5 mol/L, pH 8.5)/1-propanol (1 + 1, v + v) and 100 µL of tris(2-carboxyethyl) phosphine (0.05 mol/L, pH 8.5), followed by incubation for 30 min at 60 °C. For alkylation, 100 µL of 2-chloroacetamide (0.5 mol/L, pH 8.5) was added and the samples were shaken for 45 min at 37 °C in the dark. Then, the samples were dried under reduced pressure (40 °C, 6 h, 8 mbar). The reduced and alkylated dried samples were reconstituted in 1 mL of 0.1 mol/L Tris-HCl containing 0.04 mol/L urea (pH 7.8). After trypsin addition (1 + 49, enzyme-to-protein, w + w), the samples were incubated for 18 h at 37 °C in the dark. Trifluoroacetic acid (10 µL) was added to stop the tryptic hydrolysis and the samples were evaporated to dryness. Solid phase extraction using Discovery DSC_{18} columns (100 mg, Sigma Aldrich, MO, USA) was performed for clean-up according to the manufacturer's instructions. The peptides were eluted with acetonitrile/water (40 + 60, v + v) containing 0.1% FA followed by drying and storage at −20 °C until LC-MS/MS analysis (Xhaferaj et al., 2025).

2.9.2. Liquid chromatography tandem mass spectrometry

The samples were reconstituted in 1 mL of acetonitrile/water (2 + 98, v + v) containing 0.1% FA and filtered (0.45 µm). A Vanquish UHPLC system coupled to a Q-Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) was used for discovery proteomics. Peptide separation was achieved on an Aeris PEPTIDE XB-C_{18} column (150 × 2.1 mm, 1.7 µm, Phenomenex, Aschaffenburg, Germany) with water/FA (999 + 1, v + v) (A) and acetonitrile/FA (999 + 1, v + v) (B) as solvents with a flow rate of 0.2 mL/min, a column temperature of 30 °C, an injection volume of 10 µL and a linear gradient: 0 min 5% B, 0.4 min 10% B, 32 min 32% B, 34–37 min 80% B, and 38–45 min 5% B. The Orbitrap settings were positive ionization mode with default charge state of +2, resolution of 70,000, automatic gain control (AGC) target of 3E06 and scan range of m/z 360–1300. Data-dependent acquisition was performed using resolution of 175,000, AGC target of 1E05, maximum injection time of 50 s, TopN of 15, isolation window of m/z 2.4, fixed first mass of m/z 140.0 and

nominal collision energy of 28.

2.9.3. Proteomics data analysis

The raw MS/MS data were searched against the *Triticeae* database from UniprotKB (*Triticeae*, downloaded Feb. 23, 2023, protein entries: 557,477) using the search engine Andromeda implemented in MaxQuant (version 2.2.0.0) (Tyanova et al., 2016). Carbamidomethylation on cysteine was set as a fixed modification, methionine oxidation and protein acetylation at the *N*-terminus as variable modification, trypsin as enzyme with a maximum of two missed cleavage sites and match-between runs was enabled (matching time window 0.7 min, alignment time window 20 min). The peptide and protein false discovery rate was set to 1% each and the resulting peptides were filtered for a minimum length of seven amino acids. Intensity-based absolute quantitation (iBAQ) protein intensities were adjusted via total sum normalization to account for variations in protein injection amounts across samples and to calculate the relative abundance of the protein groups. Three search procedures were used to identify immunoreactive peptides. The first was a search for epitopes recognized by the mAb R5 (Kahlenberg et al., 2006; Osman et al., 2001). The second was a search for CeD-relevant $CD4^+$ T-cell epitopes (Sollid et al., 2020). The third strategy involved searching for complete and partial potentially harmful sequences according to Naegeli et al. (2017), by looking for the sequence QX_1PX_2, where X_1 represents L, Q, F, S or E and X_2 can represent Y, F, A, V or Q.

2.10. Statistics

All determinations were carried out as triplicates, unless otherwise stated. Mean values ($n = 3$) and absolute standard deviations (SD) were calculated in Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) (Tukey's post hoc test, $p < 0.05$) was used to test for significant differences between the samples. All statistical and Pearson's correlation analyses were performed using Origin 2021b software (OriginLab Cooperation, Northampton, MA, USA).

3. Results

3.1. Barley flour protein content and composition

The protein content of the eight selected barley cultivars and the respective flour mixtures ranged from 7.4 g/100 g (PIX_FRA20) to 18.1 g/100 g (KOR_LAT19) (Fig. 1). The mean and median values were 10.4 g/100 g and 9.4 g/100 g, respectively, and lay thus close to the protein content of the flour mixtures (Table S1). The gluten content was between 5.0 g/100 g (PIX_FRA20) and 15.9 g/100 g (KOR_LAT19) and there was a strong correlation between protein and gluten content (Pearson's $r = 0.997$), partially driven by the KOR_LAT samples from both years with exceptionally high protein and gluten content. The mean content of albumins/globulins, prolamins and glutelins was 2.4 g/100 g (range: 2.0–3.1 g/100 g), 4.7 g/100 g (range: 1.8–11.3 g/100 g) and 3.2 g/100 g (range: 2.0–5.6 g/100 g), respectively. Relative to total gluten content, the mean percentages were 59.7% of B/γ-hordeins, 26.3% of C-hordeins and 14.0% of D-hordeins (Table S2).

When comparing the same cultivars each from the two different harvest years, there were significant differences in protein content for five out of eight samples (one-way ANOVA, $p < 0.05$). There were also significant differences in gluten content for six out of eight samples, in B/γ-hordein content for seven samples, C-hordein content for six samples and D-hordein content for only two samples (Table S1). There was also a significant difference in protein content between both mixtures (Fig. 1), mostly because the measured values for mixture 2 were consistently lower for all protein fractions and types compared to the calculated values.

The relative Mw distribution of the prolamin, reduced prolamin and glutelin fractions was also compared among each sample with GP-HPLC within four ranges: >66 kDa, 29–66 kDa, 12.4–29 kDa and < 12.4 kDa

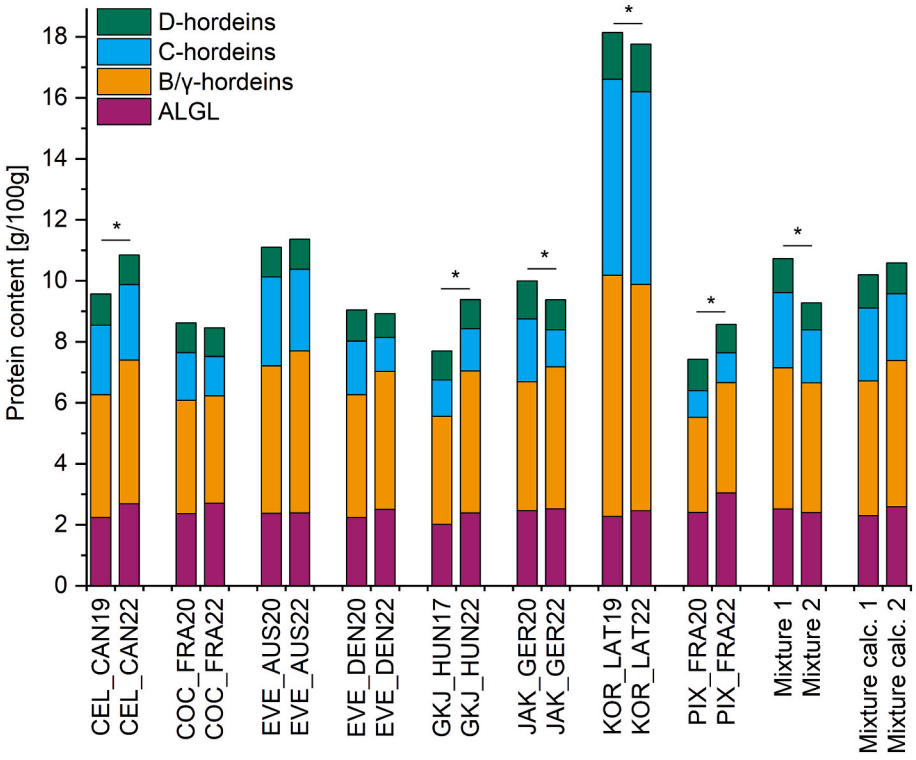

Fig. 1. Protein content of the eight selected barley cultivars and their respective mixtures from two different harvest years. The mixture consists of the selected eight cultivars in equal proportions from the years 2017–2020 (mixture 1, first collection) and 2022 (mixture 2, second collection). Mixture calc. is the calculated composition resulting from the mean values of the eight single flours, respectively. The asterisk indicates a significant difference between the protein content of both years (one-way ANOVA, Tukey's test, $p < 0.05$). ALGL, albumins/globulins.

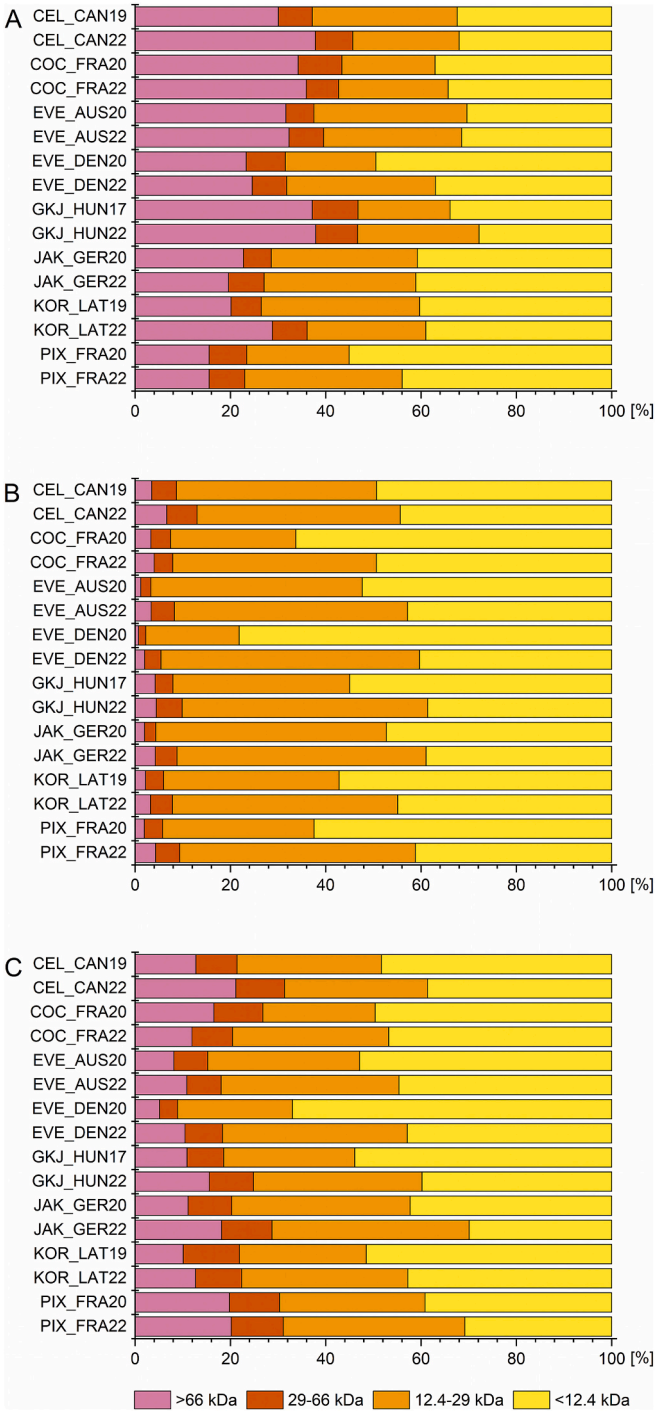

Fig. 2. Molecular weight distribution of the prolamins and glutelins from the eight selected barley cultivars from two different harvest years. Prolamin fraction (A), reduced prolamin fraction (B) and glutelin fraction (C).

(Fig. 2, Table S3). The percentage of prolamins in the 29–66 kDa range was lowest for all samples (mean: 7.5%), whereas the prolamins in the >66 kDa, 12.4–29 kDa and < 12.4 kDa ranges amounted to mean percentages of 28.0%, 26.7% and 37.9%, respectively, with characteristic differences between the cultivars. As expected, reduction of prolamins resulted in a decrease of prolamins in the >66 kDa and 29–66 kDa ranges to means of 3.2% and 4.1% and a concomitant increase of the 12.4–29 kDa and < 12.4 kDa ranges to means of 42.2% and 50.5%. The mean Mw distribution in the glutelin fraction was 13.5% in the >66 kDa range, 8.9% for 29–66 kDa, 32.5% for 12.4–29 kDa and 45.0% for <12.4 kDa. The pattern within the reduced prolamin and glutelin fractions was consistent overall but did not allow a differentiation between the cultivars.

3.2. Yield and protein content of the hordein isolates

The mixture of the eight selected international barley cultivars (mixture 2, harvest year 2022, Table 1) was used to extract the four different isolates, namely prolamins, AWEP, gluten and glutelins. Based on 100 g of flour, the yields were 6.8 g of prolamins, 2.9 g of AWEP, 5.5 g of gluten and 3.9 g of glutelins (Table S4). The crude protein content of the isolates was highest for AWEP (84.0%), followed by prolamins (81.1%), gluten (74.2%) and glutelins (64.0%) with the lowest value.

3.3. Molecular weight distribution of the hordein isolates

SDS-PAGE and GP-HPLC were used to characterize the Mw distribution of the hordein isolates in comparison to the flour mixture (Fig. 3). The four isolates showed similar high-intensity bands on the SDS-PAGE in the 35 to 50 kDa range of B/γ-hordeins. The gluten and glutelin isolates had a weak additional band at about 90 kDa, assigned to D-hordeins. C-hordeins (range of 55 to 70 kDa) were weakly visible in the AWEP, prolamin and gluten isolates, but less clearly compared to the flour (Fig. 3A). The Mw distribution determined by GP-HPLC was similar for the prolamin fraction of the flour mixture and the prolamin and AWEP isolates, with percentages of 34.2–36.4 for the >66 kDa range, 6.0–7.8 for the 29–66 kDa range, 30.1–33.8 for the 12.4–29 kDa range and 23.9–28.6 for the <12.4 kDa range (Fig. 3B, Table S5). The glutelin fraction of the flour mixture and the glutelin isolate were also comparable in their Mw distribution with percentages of 15.0 and 17.9 for the >66 kDa range, 9.3 and 6.2 for the 29–66 kDa range, 36.6 and 39.1 for the 12.4–29 kDa range and 39.1 and 36.8 for the <12.4 kDa range. In comparison to the glutelins, the gluten isolate had a lower percentage of

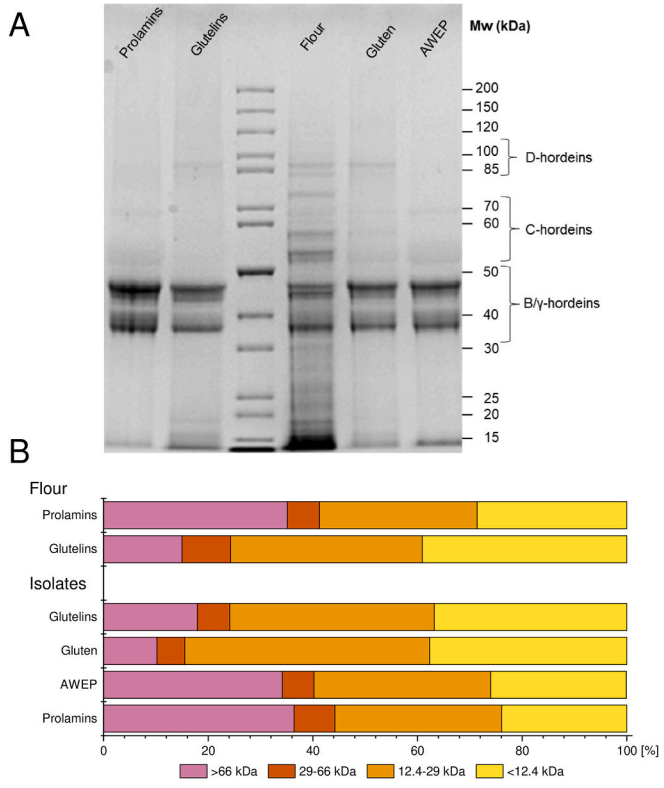

Fig. 3. Molecular weight distribution of the four isolates in comparison to the barley flour mixture analyzed by SDS-PAGE (A) and GP-HPLC (B). AWEP: acetonitrile/water-extractable proteins; Mw: molecular weight.

proteins in the >66 kDa and 29–66 kDa ranges (10.2% and 5.3%), a higher percentage in the 12.4–29 kDa range (46.8%) and a similar percentage in the <12.4 kDa range (37.7%). Both the glutelin and gluten fractions were analyzed under reducing conditions, so that lower percentages of proteins in the range > 29 kDa were expected compared to the prolamin and AWEP fractions that were not reduced. Overall, the Mw distributions of the AWEP and prolamin isolates were similar to each other and to the flour fraction. The same was true for the glutelin isolate and the flour fraction, whereas the gluten isolate shared characteristics of both prolamins and glutelins, as expected.

3.4. Protein distribution of the hordein isolates

The distribution of B/γ-, C- and D-hordeins was analyzed by RP-HPLC to compare the isolates and the flour (Fig. 4, Table S6). The pairwise comparison between the respective isolates and flour fractions showed similar percentages of B/γ-, C- and D-hordeins, although some differences were statistically significant due to low standard deviations of the triplicates. The largest difference was observed for the glutelin fraction, where the isolate had 81% of B/γ-hordeins and 10% of D-hordeins, while the flour fraction had 71% and 20%, respectively. This represents an expected consequence of the fractionation and isolation process itself, because the glutelins are extracted as the last step following removal of albumins/globulins and prolamins. The repeated manual handling steps likely introduce some variation compared to the flour, especially when working on a larger scale.

3.5. R5 ELISA responses to the hordein isolates

The response of the R5 mAb to the four hordein isolates was measured in the sandwich ELISA (Fig. 5) and linear approximations were calculated, where the slope indicates the strength of the response (Table S7). The AWEP and prolamin isolates showed the highest response against the R5 mAb (slope of 5.59 and 4.41), followed by the gluten isolate (slope of 2.65). The glutelin isolate only produced a weak response with absorbances well below the kit standard (PWG-gliadin) and a slope of 0.16.

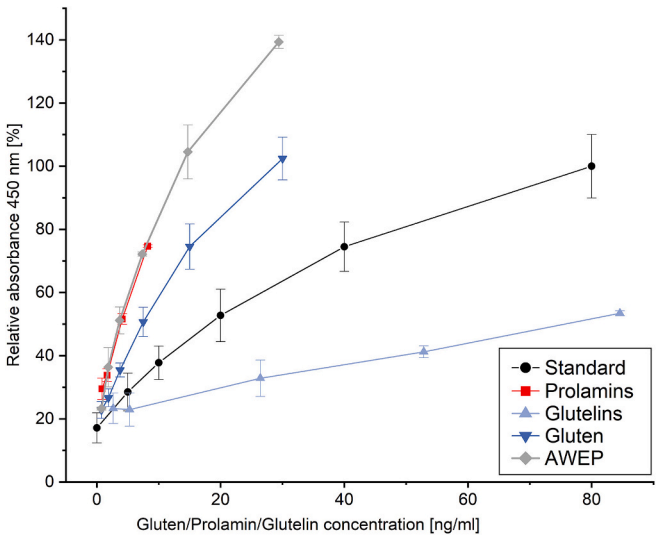

Fig. 5. ELISA responses to the four isolates. R5 sandwich ELISA absorbances (λ = 450 nm) of the test kit standard (PWG-gliadin), the prolamin, glutelin, gluten and acetonitrile/water-extractable protein (AWEP) fractions of barley as a function of the concentrations quantitated by RP-HPLC. Error bars indicate the minimum and the maximum value.

3.6. Proteomic characterization of the hordein isolates

The proteomic analysis of the four barley hordein isolates identified 1246 tryptic peptides across all samples that mapped to 396 protein groups. Of these, 20 protein groups were identified as gluten proteins, of which nine could be assigned to B-, C-, D- and γ-hordeins judging by the fasta header and comparison of identified amino acid sequences to homologous sequences from wheat. Only one protein group (UniProtKB accession: I6TEV8) was assigned to C-hordeins based on one peptide, due to the overall scarcity of tryptic cleavage sites within the sequence (only three at positions 1, 19 and 250). Although trypsin has been used before for proteomics analysis of barley C-hordein antisense lines

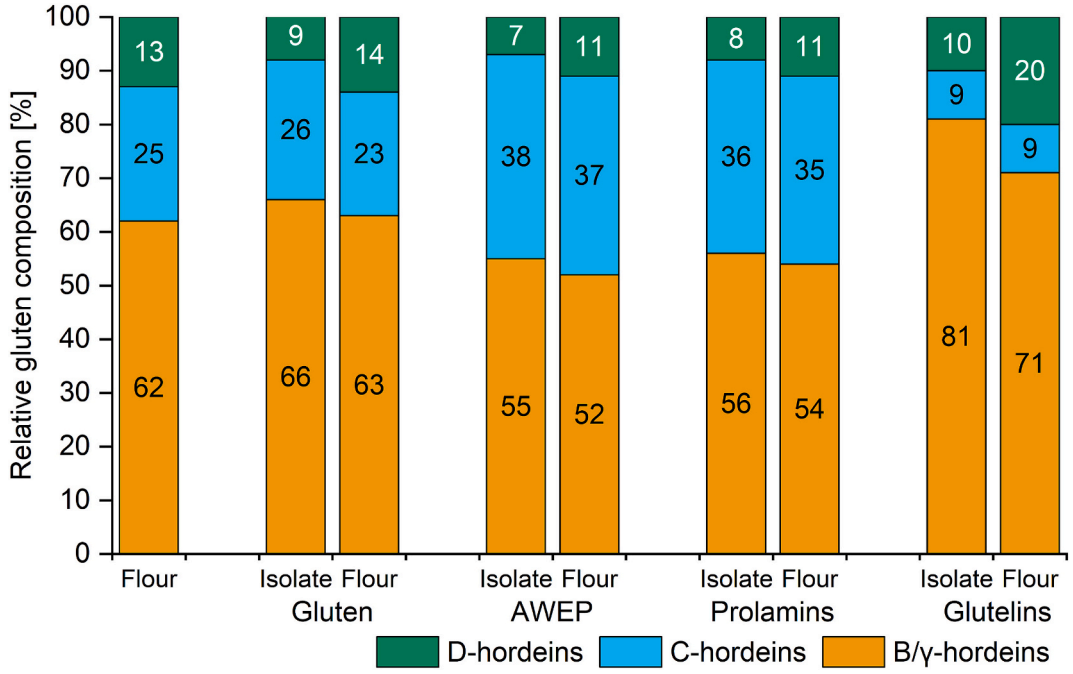

Fig. 4. Hordein composition of the four isolates and the barley flour mixture extracted with the respective solvents. The hordein distribution (%) is given relative to total hordein content analyzed by RP-HPLC. AWEP: acetonitrile/water-extractable proteins.

(Schmidt et al., 2016) and is well suitable to detect B-, D- and γ-hordeins, it is known that chymotrypsin is the better option for C-hordeins (Colgrave et al., 2017). As our analysis was mainly intended to differentiate B- and γ-hordeins and detect potential immunoreactive peptides, we chose trypsin. For D-hordeins, we also identified only one protein group (Q40054), but with a high score (268) based on 10 peptides. Three protein groups were assigned to B-hordeins (I6TMW4, P06470 and I6TEV5, score up to 259) and four to γ-hordeins (P80198, A0A8I6WAD5, I6TMV6 and P17990, score up to 164).

Relative quantitation of the protein groups using the iBAQ algorithm resulted in a C-hordein proportion of less than 0.01% according to expectations due to tryptic hydrolysis, which is why they were not included in Fig. 6. The gluten, AWEP and prolamin isolates showed similar proportions of 63–64% of B-hordeins, 16–22% of γ-hordeins and 14–20% of D-hordeins (Fig. 6 A–C). In comparison, the glutelin isolate was particularly enriched in D-hordeins, resulting in proportions of 47% of B-hordeins, 39% of D-hordeins and 14% of γ-hordeins.

3.7. *Identification of potentially immunoreactive peptides*

Potentially immunoreactive peptides were identified by searching all identified peptides across the four isolates for (i) epitopes recognized by the mAb R5 (Kahlenberg et al., 2006; Osman et al., 2001), (ii) CeD-relevant $CD4^+$ T-cell epitopes (Sollid et al., 2020) and (iii) complete and partial potentially harmful sequences according to Naegeli et al. (2017). Overall, 12 potentially immunoreactive peptides were identified within the nine B-, C-, D- and γ-hordeins (see section 3.6), of which nine were classified as potentially immunoreactive based on the tetrapeptide motifs QLPQ, QQPQ and QLPA (Table 2). The remaining three peptides carried an R5 epitope (QQPFP and QQPYP), but none of the peptides had a full-length CeD-relevant $CD4^+$ T-cell epitope.

4. Discussion

The protein content of seven out of the eight selected barley cultivars ranged from 7.4 to 11.1 g/100 g and 8.5–11.4 g/100 g considering the flours from the first and second collection, respectively. The cultivar Kornelija from Latvia had a very high protein content of 17.8 and 18.1 g/100 g in 2019 and 2022, according to expectations, because it is the only naked (hull-less) cultivar within our selection and dedicated to special food and feed uses. Previous studies have also reported a wide range of values for the protein content of barley flours from 7.7 g/100 g (Schalk et al., 2017), to 9.9–21.8 g/100 g (Olesen et al., 2025), to 10.1–11.5 g /100 g (Zubillaga et al., 2025) and 10.5–21.2 g/100 g (Joestl et al., 2025). Protein content is mainly dependent on nitrogen input, but further information on agronomic practices was not available from the providers. Therefore, one limitation of this study is that protein content cannot be directly linked to cultivation conditions. The target protein content for malting barley is 9.5–12.5% and of the three malting barleys, the cultivar Celebration met this range, whereas the cultivars Evergreen and GK Judy had less protein. The second limitation of this sample set is that it did not include cultivars from other major barley exporting countries including Australia and Argentina due to sourcing constraints. This is why further work beyond the proof-of-concept presented here should build on the results of international diversity genotyping studies to capture as much of the genetic diversity as possible for an RM to be truly representative (Jayakodi et al., 2020).

The hordein composition with 48.5–70.5% of B/γ-hordeins, 17.3–41.3% of C-hordeins and 9.7–20.5% of D-hordeins over all samples agreed well with earlier reports (Lange et al., 2007; Lexhaller et al., 2017; Schalk et al., 2017; Wroblewitz et al., 2014), except that the percentage of D-hordeins was slightly lower with 7.6% (Schalk et al., 2017), 6.1% (Lexhaller et al., 2017) or 4.9% (Lange et al., 2007) than the results reported here. Huang et al. (2017) also reported 16.5–33.1% of C-hordeins, which is well in line with our results. The comparison of protein/gluten content and hordein composition for the same cultivars

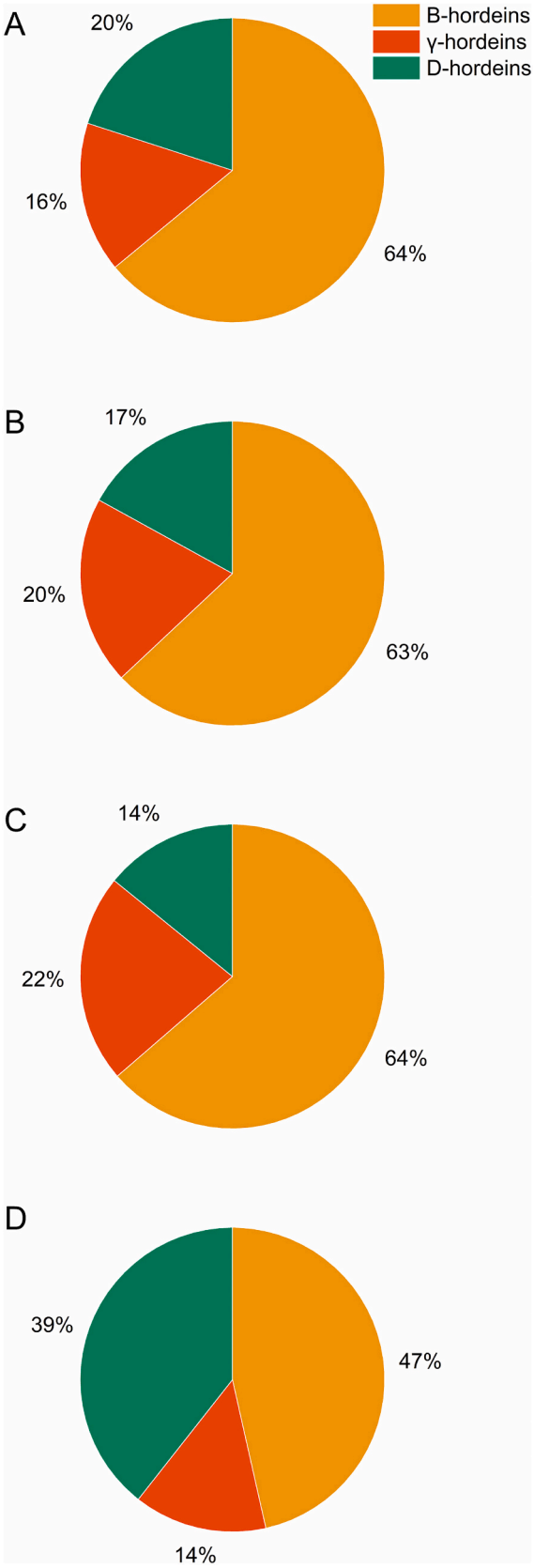

Fig. 6. Hordein composition of the four isolates analyzed by LC-MS/MS. Gluten (A), acetonitrile/water-extractable proteins (B), prolamins (C), and glutelins (D). Please note that the approach did not adequately detect C-hordeins due to tryptic hydrolysis as stated in section 3.6.

Table 2
Potential immunoreactive peptide sequences. The search procedure to identify immunoreactive epitopes within peptides identified by LC-MS/MS uses the following strategies: R5 mAb: The epitopes recognized by the R5 mAb ELISA (Amnuaycheewa et al., 2022; Kahlenberg et al., 2006; Osman et al., 2001), immunoreactive peptides recognized by $CD4^+$ T cells (Sollid et al., 2020) and potentially harmful epitopes selected based on the EFSA search strategy (Q-X_1-P-X_2; X_1 = L, Q, F, S, E; X_2 = Y, F, A, V, Q) (Naegeli et al., 2017). Epitopes are highlighted in bold.

Barley protein group	Protein accession	Amino acid sequence	Characteristics
B-hordeins	I6TMW4	SQMLQQSSCYVLQQQCCQ**QLPQ**IPEQFR	potentially harmful
	I6TMW4	PFPS**QQPFP**QQPPFWQQQPILSK	R5 epitope
	I6TMW4	IARSQMLQQSSCHVLQQQCCQ**QLPQ**IPEQFRHEAIR	potentially harmful
	P06470	YPEQP**QQPFP**WQQPTIQLYLQQQLNPCK	R5 epitope
	P06470	IARSQMLQQSSCHVLQEQCCQ**QLPQ**IPEQFR	potentially harmful
	I6TEV5	QGVQIVQ**QQPQ**PQEVGQCVLVQGRDIVQPQQLAQMEAIR	potentially harmful
	I6TEV5	QGVQIVQ**QQPQ**PQEVGQCVLVQGR	potentially harmful
γ-hordeins	P80198	DYLASLGA**QLPA**AAAGAK	potentially harmful
	A0A8I6WAD5	PFGQYQQPLT**QQPYP**QQQPLAQQQPSIEEQHQLNLCK	R5 epitope
	A0A8I6WAD5	SQMLQQSSCHVLQQQCCQ**QLPQ**IPEQLRHEAVR	potentially harmful
	I6TMV6	SQMLQQSSCHVLQEQCCQ**QLPQ**IPEQFR	potentially harmful
D-hordeins	Q40054	IARSQMLQQSSCYVLQQQCCQ**QLPQ**IPEQFRHEAVR	potentially harmful

from the two collections revealed significant differences due to environmental variability, which is well-known (Prystupa et al., 2021; Wroblewitz et al., 2014). However, the overall picture in terms of hordein composition remained largely similar (Fig. 1). Since these samples were sourced by different providers globally rather than from a controlled trial, specific environmental (including harvest year) effects on hordein composition cannot be further separated.

GP-HPLC provided complementary information to the RP-HPLC results on the relative Mw distribution of the prolamin, reduced prolamin and glutelin fractions. The percentages were according to expectations, with the prolamin fraction showing disulfide-linked B-hordein oligomers in the >66 kDa range, that were not present anymore after reduction (Fig. 2). In contrast to the clear separation of C-hordeins in RP-HPLC, GP-HPLC did not allow a distinct separation of C-hordeins from B/γ-hordeins, due to the low resolution of the separation. The >66 kDa range of the glutelin fraction corresponded to D-hordeins and the percentage of 13.5% in the >66 kDa range corresponded well to the 14% observed by RP-HPLC.

Overall, the results showed differences in barley protein composition, depending both on the cultivar and the collection. As intended, the selected eight cultivars covered a high variability in protein composition (Xhaferaj et al., 2023) and the mixtures from both collections were confirmed to be a representative blend of the eight cultivars and thus a suitable starting material for hordein isolate production.

In terms of yield, the stepwise isolation of prolamins and glutelins produced a higher yield (10.7 g) than the combined one-step extraction of gluten (5.5 g). The acetonitrile/water extraction resulted in the lowest yield overall (2.9 g), likely due to inadequate solubility of hordeins under these conditions. The crude protein content of the prolamin isolate (81.1%) was slightly lower compared to earlier work (87.3% in Schalk et al., 2017 and 83.3% in Gessendorfer et al., 2009), while that of glutelins was comparable (64.0%) versus 62.0% in Schalk et al. (2017).

The pattern on the SDS-PAGE gel (Fig. 3) showed the expected bands at 35 to 50 kDa for B/γ-hordeins, 55 to 70 kDa for C-hordeins and about 90 kDa for D-hordeins (Huang et al., 2017; Lange et al., 2007; Tanner, Colgrave, et al., 2013). It has to be noted that the apparent Mw on SDS-PAGE is higher than expected based on the amino acid sequences for B/γ-hordeins (31.4 kDa/33.2 kDa, P06470/ P80198), C-hordeins (33.1 kDa, I6TEV8) and D-hordeins (72.9 kDa, Q40054), due to the high proline content and repetitive primary structure of hordeins which prevent the formation of compact structures and result in a larger hydrodynamic volume during migration in the gel (Shewry & Tatham, 1990). The Mw distributions of the AWEP and prolamin isolates were congruent with one another and reflected the profile of the flour prolamin fraction. Similarly, the glutelin isolate closely matched the flour glutelin fraction, while the gluten isolate exhibited characteristics of both prolamins and glutelins. C-hordeins were only stained weakly on SDS-PAGE, likely due to poor binding of Coomassie Brilliant Blue G-250, as has been reported before for the homologous ω-secalins (Xhaferaj et al., 2025). RP-HPLC analyses clearly confirmed the presence of C-hordeins in all isolates and showed that all isolates produced on lab-scale had the expected composition based on the analytical characterization of the corresponding fraction directly from the barley flour mixture (Fig. 4).

The response of the R5 mAb to the four hordein isolates followed the order: AWEP ≈ prolamins > gluten > glutelins (Fig. 5). This further confirmed that the prolamin and AWEP isolates were comparable in their composition and the high response of the R5 mAb to prolamins along with a very weak response to glutelins agrees with Lexhaller et al. (2016). Accordingly, the AWEP, prolamin and gluten isolates could be used as RM for ELISA, with the advantage that their production is less laborious compared to the C-hordeins proposed earlier (Huang et al., 2017; Huang et al., 2020). Although we ensured that the final dilution step was always carried out with the test kit buffer for better comparability, solvent effects cannot be excluded as a confounding factor, because the four isolates were reconstituted in their respective extraction solvents. A common RM to compare results within and between ELISA test kits helps to reduce the substantial variation of results for gluten content of the same sample depending on the ELISA (Rzychon et al., 2017). This proof-of-concept study on barley hordein isolate production includes only results from one ELISA test kit, limiting its scope. Further work needs to include more detailed evaluations of ELISA responses across different test kits.

The fundamental characterization of the four barley hordein isolates by shotgun proteomics essentially confirmed the other analyses which showed a similar hordein composition for the AWEP and prolamin isolates. However, due to the poor detection of C-hordeins using trypsin for hydrolysis (Colgrave et al., 2017), the gluten isolate was also like the AWEP and prolamin isolates. Only the glutelin isolate was enriched in D-hordeins compared to the others. One advantage of the proteomics approach is that it allows the differentiation of B- and γ-hordeins, which was not possible using the other analytical techniques. However, C-hordeins were only detected with a very low intensity and based on only one peptide, due to the use of trypsin which produces hardly any MS/MS-suitable peptides, because tryptic cleavage sites are missing within the long repetitive domain of C-hordeins, as has been reported earlier (Colgrave et al., 2017). The number of identified hordeins (one D-hordein, one C-hordein, three B-hordeins, four γ-hordeins) was slightly lower but comparable to the ones described by Olesen et al. (2025). Considering the missing detection of C-hordeins by proteomics, which presents a limitation of this study, the iBAQ proportions of 63–64% of B-hordeins, 16–22% of γ-hordeins and 14–20% of D-hordeins agreed well with those of RP-HPLC. The specific search for potentially immunoreactive peptides only revealed three peptides carrying one R5 epitope (Kahlenberg et al., 2006; Osman et al., 2001) and 12 with potential immunoreactive sequences according to Naegeli et al. (2017). All

peptides with an R5 epitope were assigned to B/γ-hordeins, explaining the strong response of the AWEP, prolamin and gluten isolates in the ELISA. Unexpectedly, no full-length CeD-relevant $CD4^+$ T-cell epitopes (Sollid et al., 2020) were detected, but this may also be due to the use of tryptic cleavage, since other studies focusing on CeD-active peptides frequently use chymotrypsin (Tissen et al., 2026) or combinations of trypsin and chymotrypsin (Bahmani et al., 2021 and references cited therein).

5. Conclusion

This study demonstrated that a blend of seven European and one Canadian barley cultivars provides a starting material to produce barley hordein isolates that are mostly representative of European barleys. While individual cultivars showed high genetic and environmental variability in protein content between harvest years, blending effectively mitigated these changes. Comprehensive characterization via complementary methods confirmed that the prolamin, AWEP and gluten isolates were comparable to the original barley flour fractions in terms of protein composition and Mw distribution, while the glutelin isolate differed slightly. Due to their strong response in the R5 ELISA, the AWEP and prolamin isolates were identified as the most viable candidates for use as RM. Their production is relatively straightforward, making them more practical for large-scale laboratory standardization compared to specific hordein types. While tryptic hydrolysis confirmed the presence of D-, B-, and γ-hordeins in the isolates, the lack of cleavage sites in C-hordeins highlights a limitation in current shotgun proteomics, which can be overcome using alternative peptidases. These well-characterized hordein isolates present a proof-of-concept toward establishing a standardized barley RM to correct the current overestimation of barley gluten in ELISA testing to ensure more accurate labeling and enhance food safety for individuals with CeD.

CRediT authorship contribution statement

Majlinda Xhaferaj: Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gabriella Muskovics:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Zsuzsanna Bugyi:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Sándor Tömösközi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Katharina A. Scherf:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.foodchem.2026.150962.

Data availability

Data will be made available on request.

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