Food Research International 218 (2025) 116910

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Quantitation of amylase/trypsin-inhibitors in barley using targeted LC-MS/MS

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ARTICLE INFO

Keywords:
Allergen
LC-MS/MS
Non-celiac gluten sensitivity (NCGS)
Non-celiac wheat sensitivity (NCWS)
Stable isotope dilution analysis

ABSTRACT

Amylase/trypsin-inhibitors (ATIs) are known allergens and triggers of non-celiac wheat sensitivity. Until now, ATIs were only quantitated in wheat species. We developed and validated a targeted stable isotope dilution analysis LC-MS/MS method to quantitate ten barley-specific ATIs, including one monomeric and one dimeric amylase-inhibitor, four chloroform/methanol-soluble types, three subtilisin/chymotrypsin-inhibitors and one amylase/subtilisin-inhibitor. After successful validation in terms of precision, recovery and limits of detection and quantitation, the method was applied to 181 barley accessions from the Global EcoSeed panel, comprising 113 two-row and 68 six-row barleys of different genetic backgrounds. The overall ATI content was 1.1–5.2 mg/g, corresponding to 0.7–3.6 % of the total protein content with no clear distinction between two-row and six-row barleys. This study is the first to provide insights on the ATI content and composition of barley, which can be used to make low-ATI foods for special dietary needs.

1. Introduction

Cereal grains like corn, wheat, rice and barley are major staple foods worldwide, covering over 60 % of the global food demand. Barley ranks fourth in global production with a harvest volume of 142 million metric tons in the harvest year 2023/24. The European Union is the leading producer worldwide. Barley was one of the first grains to be domesticated and it is cultivated worldwide due to its adaptability to different climates and geographical areas. It is mainly used for animal feed and beer brewing, but also for the production of bread, soup and breakfast cereals (US Department of Agriculture and USDA Foreign Agricultural Service, 2025). As barley and wheat are closely related, barley grain components may also trigger different disorders like respiratory or food allergy, celiac disease and non-celiac wheat sensitivity (NCWS) (Dahal-Koirala et al., 2020; Lindfors et al., 2019; Volta et al., 2019). NCWS is associated with gastrointestinal symptoms like diarrhea, bloating and abdominal pain and extraintestinal symptoms like headache, depression and brain fog (Czaja-Bulsa, 2014; Schuppan et al., 2015). Its prevalence varies from 0.6 % up to 6 % in the Western population. The symptoms disappear on a gluten-reduced or gluten-free diet (DiGiacomo et al., 2013; Molina-Infante et al., 2015). Diagnosis is complicated, as until now, there are no specific biomarkers (Catassi et al., 2013; Catassi et al., 2015; Sapone et al., 2012). Putative triggers for NCWS include fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) and amylase/trypsin-inhibitors (ATIs). Wheat ATIs are known to trigger the innate immune response in humans by activating the toll-like receptor 4 TLR4-MD2-CD14 complex. The following release of proinflammatory chemokines and cytokines in myeloid cells may lead to the symptoms typical of NCWS as well as worsening of other already pre-existing inflammatory reactions (Junker et al., 2012).

ATIs are cysteine-rich proteins from the albumin/globulin fraction of 120–150 amino acids in length with a compact secondary structure due to four to five intramolecular disulfide bonds. They are located in the endosperm of the grains and may be involved in grain maturation and carbohydrate storage, but they function also as protectors from common parasites and pathogens by inhibiting the enzymes α-amylase and/or

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https://doi.org/10.1016/j.foodres.2025.116910
Received 17 March 2025; Received in revised form 15 June 2025; Accepted 19 June 2025
Available online 23 June 2025

trypsin (Finnie et al., 2002; Oda et al., 1997; Payan, 2004; Priya et al., 2013). In wheat, they comprise about 2–4 % of the total protein content (Altenbach et al., 2011). So far, 19 isoforms, classified into four subgroups have been described (Altenbach et al., 2011). The first group is monomeric ATIs, with 0.28 as the most important form. The second group comprises the two homodimeric inhibitors 0.19 and 0.53. The third group contains the heterotetrameric inhibitors CM1, CM2, CM3, CM16 and CM17, originally known as chloroform/methanol-soluble (CM) proteins. The fourth group contains the other inhibitors CMX1/3, CMX2, WASI, WTI and WCI (Altenbach et al., 2011; Geisslitz et al., 2020; Geisslitz et al., 2021; Reig-Otero et al., 2018). Barley (*Hordeum vulgare*) contains ATIs that are similar to those in wheat. Barley grains contain monomeric and dimeric forms with inhibitory activity only against amylase (AI-type) like barley monomeric amylase-inhibitor (BMAI) and barley dimeric amylase-inhibitor (BDAI). For example, there is a resemblance between BDAI and wheat 0.19/0.53 (45.8 % amino acid sequence identity). Furthermore, barley also contains tetrameric CM-types (CMa, CMb, CMc, CMd and CMe) resembling those present in wheat (CM1, CM2, CM3, CM16, CM17, CMX1/3, CMX2), i.e., CMa has identities with CM1 and CM2 (84.1 % each), CMb with CM16 (90.2 %) and CM17 (82.5 %) or CMd (84.5 %) with CM3 (Barber et al., 1986; Geisslitz et al., 2021; Sanchez-Monge et al., 1986). Minor ATI-types include the barley chymotrypsin-inhibitors (CI-types) subtilisin/chymotrypsin-inhibitor 1 A (SCI-1A), subtilisin/chymotrypsin-inhibitor 1B (SCI-1B) and chymotrypsin-inhibitor 2 (CI-2), as well as the amylase/subtilisin-inhibitor (ASI) (Table S1).

LC-MS/MS is used most often to quantitate ATIs, because it is possible to detect multiple ATIs in parallel with high sensitivity and selectivity. It is necessary to select marker peptides representing the different ATI-types. Some ATIs have very similar amino acid sequences, making it difficult to find peptides that are unique for only one protein. Therefore, a careful choice of marker peptides is crucial to establish a selective method (Geisslitz et al., 2021). Stable isotope dilution analysis (SIDA) is often used for the quantitation of complex molecules, for example Alternaria toxins (Gonçalves et al., 2022; Tölgyesi et al., 2021). Furthermore, it is already well-established for ATI quantitation, using stable isotope labeled peptides or concatemers (QconCAT) as internal standards. The analysis of 13 different ATIs in a set of different wheat samples (common wheat, spelt, durum wheat, emmer, einkorn) showed that einkorn had a very low ATI content, whereas spelt and emmer had higher ATI contents than common wheat (Geisslitz et al., 2018; Geisslitz et al., 2020). The quantitative results obtained with QconCAT-assisted data-independent acquisition LC-MS/MS were similar to the results of Geisslitz et al. (2020) showing high correlations of inter-laboratory and cross methodological reproducibility for ATIs CM2, CM3 and CM16 (R^2 = 0.70–0.88) but no correlation for 0.28 (R^2 = 0.01) (Sielaff et al., 2021).

In previous research, the six barley ATIs CMa, CMc, CMd, CMe, BMAI and BDAI were relatively quantitated in twelve different barley cultivars (Bose, Byrne, Howitt, & Colgrave, 2019). However, currently there is no method for the absolute quantitation of barley-specific ATIs. Consequently, the first aim of the current study was to develop an LC-MS/MS method comprising ten different barley ATIs. It includes two AI-types (BMAI, BDAI), four CM-types (CMa, CMc, CMd, CMe), three CI-types (SCI-1A, SCI-1B and CI-2) as well as ASI. The second aim was to use the newly implemented method to analyze the ATI content in a sample set of 181 different barley accessions of the EcoSeed panel (Alqudah et al., 2021; Nagel et al., 2019). The results can be used to get insights, if the genetic background has an influence on the ATI composition of the different barley accessions. Here, we provide the first comprehensive evaluation of ATIs in barley which can be used to identify accessions with either very high or low ATI content.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade or higher and purchased from Sigma-Aldrich (Steinheim, Germany) or VWR Merck (Darmstadt, Germany). Deionized water was generated by the water purification system Arium 611VF (Sartorius, Goettingen, Germany). Trypsin (TPCK treated) was purchased from Sigma-Aldrich. Unlabeled and stable isotope labeled peptides (IS) were synthesized by GenScript (Piscataway, NJ, USA) The labeled peptides contained each either $[^{13}C]_6$- and $[^{15}N]_4$-labeled arginine or $[^{13}C]_6$- and $[^{15}N]_2$-labeled lysine at the C-terminal end.

2.2. Barley grain samples

Barley grain samples (5 g) were provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany). The sample set contained 181 barley accessions of the EcoSeed Panel grown under field conditions in Gatersleben, Germany, and harvested in 2018 (Nagel et al., 2019). The panel contains different genetic backgrounds of accessions comprising 113 two-row and 68 six-row barley accessions, including 103 cultivars, ten breeding lines and 65 landraces. The grains were milled into wholemeal flours using a coffee-mill (Robert Bosch GmbH, Stuttgart, Germany). For method development, the barley cultivar Sandra of harvest year 2013 was used.

2.3. Crude protein content

The crude protein content was determined in triplicate by measuring the nitrogen content in the barley flours with the Dumas combustion method. A Dumatherm Nitrogen analyzer (Gerhardt Instruments, Königswinter, Germany) was used with a factor of 5.71 for calculation of the crude protein content.

2.4. Sample workup

Sample preparation was performed as described previously by Geisslitz et al. (2020) with slight modifications. Preliminary experiments were conducted to optimize the extraction and digestion efficiency as well as to guarantee a sufficient reduction and alkylation of the proteins. The sample workup parameters were also tested and optimized in experiments conducted by Kaemper et al. (2025). Barley wholegrain flour (50 mg) was extracted with ammonium bicarbonate buffer (1 mL, 50 mmol/L, pH 7.8) twice under stirring at 22 °C for 30 min. After each extraction, the samples were centrifuged for 25 min at 3550 rcf and the supernatants were combined. The extracts were evaporated to dryness and the residue was resolubilized in Tris-HCl (320 µL, 0.5 mol/L, pH 8.5) and 1-propanol (320 µL). The isotope labeled standards (50 µL of a mixed solution containing all labeled standards in a concentration of 20 µg/mL each) were then added. Using a tris(2-carboxyethyl)phosphine-solution (TCEP) (50 µL, 0.05 mol/L TCEP in 0.5 mol/L Tris-HCl, pH 8.5), the proteins were reduced for 30 min at 60 °C. Afterwards, the cysteine residues were alkylated with chloroacetamide (CAA) (100 µL, 0.5 mol/L CAA in 0.5 mol/L Tris-HCl, pH 8.5) for 45 min at 37 °C in the dark, followed again by evaporation until dryness. Tryptic digestion (1 mL trypsin solution, enzyme-to-substrate ratio 1:50, 0.04 mol/L urea in 0.1 mol/L Tris-HCl, pH 7.8) was carried out overnight for 18 h at 37 °C in the dark. The reaction was stopped with 4 µL trifluoroacetic acid. The solution was again evaporated to dryness. The residue was dissolved in 1 mL of 0.1 % formic acid (FA) and filtered through a 0.45 µm regenerated cellulose membrane (WICOM, Heppenheim, Germany) prior to the LC-MS/MS measurement.

2.5. Response lines for quantitation

For stock solutions (1 mg/mL), the peptides (P) and internal standard (IS) were solubilized according to the recommendations provided by the manufacturer (solubilized either in deionized water or DMSO) and afterwards stored at −80 °C. Two solutions were prepared from the stock solutions: Solution 1 contained P1–10 and solution 2 included IS1–9, resulting in a final concentration of 100 µg/mL for each peptide and internal standard. An aliquot of solution 1 and 2 was reduced with TCEP and alkylated with CAA according to the workup of the barley wholegrain flour samples. Reduced and alkylated solutions 1 and 2 (concentration: 20 µg/mL for each peptide and internal standard) were mixed in molar ratios n(P)/n(IS) between 0.1 and 9.8 (9 + 1, 4 + 1, 3 + 1, 1 + 1, 1 + 3, 1 + 4 and 1 + 9) for the calculation of response lines for each peptide.

2.6. Targeted LC-MS/MS method

The targeted LC-MS/MS method is based on the wheat ATI quantitation method described earlier (Geisslitz et al., 2020). For peptide separation and quantitation of the 10 different barley ATIs, an UltiMate 3000 HPLC (Dionex, Idstein, Germany) with an Aqua-C18 column (150 × 2 mm, 5 µm, 12.5 nm, Phenomenex, Aschaffenburg, Germany) coupled with a TripleQuadrupole mass spectrometer (MS) (TSQ Vantage, ThermoFischer Scientific, Bremen, Germany) was used. The following conditions were used for HPLC separation (Geisslitz et al., 2020): solvent (A), FA (0.1 %, *v*/v) in water; solvent (B), FA (0.1 %, v/v) in acetonitrile; flow rate 0.2 mL/min; injection volume 10 µL; column temperature 22 °C; gradient 0–18 min 5–30 % (B), 18–20 min 30 % (B), 20–21 min 30–90 % (B), 21–24 min 90 % (B), 24–25 min 90–5 % (B), 25–35 min 5 % (B). For the TripleQuad MS the following parameters were used (Geisslitz et al., 2020): electrospray ionization (ESI) in positive mode; spray voltage 4500 V; declustering voltage −10 V; vaporizing temperature 50 °C; capillary temperature 300 °C; sheath gas pressure 40 au; aux gas pressure 5 au. Selected reaction monitoring (SRM) was used to analyze the transitions from precursor ions to product ions. A summary of the MS parameters for each peptide and its corresponding ATI as well as the experimentally optimized collision energies (CE) for each product ion is displayed in Table 1.

2.7. Data analysis

Peak area integration was performed using Skyline 22.2.0.312 (MacCoss Lab Software, University of Washington, Seattle, WA, USA) with manual correction of the automated peak integration. The peptide to IS ratio for P10 was calculated manually, because IS9 was used for quantitation as the two peptides only differ in one amino acid and have the same retention time. For quantitation, the average peak ratio of the transitions of the five product ions was used for each peptide. Response lines were calculated as linear regression of peak area ratios A(P1-P10)/A(IS1-IS9) against the corresponding molar ratios n(P1-P10)/n(IS1-IS9). The ATI content was calculated from two replicates with single injection. Calculation of the ATI protein content was performed by taking the MW of each ATI (Table S2) and the corresponding peptide, resulting in a specific factor for each ATI. In total, ten different ATIs of four main types (AI-types, CM-types, CI-types and ASI) were quantitated in the sample set. Seven peptides were unique, whereas three peptides matched to two protein accessions each (CMc and CMd) or to 21 protein accessions (CI-2). For these, the highest MW described in the UniProtKB database (https://www.uniprot.org) was used to calculate the protein content.

2.8. Method validation

For method validation, experiments to calculate precision, limit of detection (LOD), limit of quantitation (LOQ) and recovery were performed. Repeatability precision was determined by the same analyst on the same instrument, by working up and analyzing six replicates ($n = 6$) of barley accession Sandra in three different weeks each ($n = 18$). Precision was calculated as Horwitz ratio (HorRat) defined as the coefficient of variation in percent divided by repeatability in percent. The method was considered precise for each peptide with a HorRat in the range of $0.5 \leq x \leq 2.0$ W (Horwitz et al., 1980).

To determine the LOD and LOQ, 50 µL of a mixture of P1–P10 in twelve different concentrations (0.01–40 µg/mL) and 50 µL of a mixture of IS1–IS9 (20 µg/mL each) were added to 50 mg gluten-free wheat starch (Bezgluten, Koniusza, Poland) in triplicate ($n = 3$). The starch was determined to be ATI-free by LC-MS/MS analysis prior to the experiments. Sample workup and analysis was exactly the same as for the barley flours. The samples with the lowest spiked concentration, where the ratios of all five precursor to product ion transitions were still constant in the triplicate and therefore still fulfill the identification criteria were used to determine LOD and LOQ. LOD and LOQ were calculated as three and ten times the standard deviation of that triplicate, respectively (Geisslitz et al., 2018). Recovery was determined by diluting the flour of barley Sandra with analyte-free starch in different ratios, ranging from 20 to 80 % of barley flour. The analyte-free starch and the barley flour (10 g mixes in different flour/starch ratios) were homogenized in an overhead shaker (Stuart rotator drive tube rotator STR4, Cole-Parmer GmbH, Wertheim, Germany) overnight. Sample workup was performed in triplicate ($n = 3$) for each dilution step as previously described for the barley flours. Recovery was determined by calculating the theoretical ATI content before dilution and comparing the results with the quantitated ATI content in 100 % barley flour for each of the ten barley-specific ATIs (Geisslitz et al., 2018).

2.9. Data analysis

Principal component analysis (PCA), cluster analysis, one-way analysis of variance (ANOVA) with Tukey's Test ($p < 0.05$) and boxplots were calculated and plotted with OriginPro (version 2023,

Table 1
Overview of the LC-MS/MS parameters for the quantitation of the ten barley amylase/trypsin-inhibitors (ATIs).

Peptide (P)/ Internal Standard (IS)	ATI	Amino Acid Sequence	Precursor *m/z*		Product Ions	Collision Energy (eV)	Retention Time (min)
			P	IS			
P1/IS1	BMAI	ATVAEVFPGC*R	603.8	608.8	y4/y5/y6/y7/y8	18/19/20/18/20	13.8
P2/IS2	BDAI	DCCQEVANISNEWC*R	970.9	975.9	b5/y3/y6/y8/y9	37/28/27/29/29	16.2
P3/IS3	CMa	SHPDWSVL*K	534.8	538.8	b4/y3/y4/y5/y7	23/18/23/20/16	13.4
P4/IS4	CMc	ELAGISSNC*R	553.8	558.8	y4/y5/y6/y7/y8	18/18/19/18/19	9.6
P5/IS5	CMd	LPEWMTSAELNYPGQPYLA*K	1154.6	1158.6	b3/b4/y3/y5/y8	43/43/41/34/37	20.6
P6/IS6	CMe	TYVVSQICHQGP*R	515.6^{+}	518.9^{+}	y3/y4/y5/y6/y7	20/20/20/19/20	11.4
P7/IS7	ASI	ADANYYVLSAN*R	678.8	683.8	y4/y5/y6/y7/y8	21/20/21/19/20	13.6
P8/IS8	CI-2	TEWPELVG*K	529.8	533.8	b3/y3/y4/y6/y7	23/22/16/18/15	16.1
P9/IS9	SCI-1A	YPEPTEGSIGASSA*K	747.4	751.4	b3/y4/y6/y9/y12	22/30/27/24/23	10.9
P10/IS9	SCI-1B	YPEPTEGSIGASGAK	732.4	751.4	b3/y4/y6/y9/y12	30/24/29/28/25	10.9

*K, lysine ($[^{13}C]_6$, $[^{15}N]_2$); *R, arginine ($[^{13}C]_6$, $[^{15}N]_4$). +Charge state of the precursor was 3+; all charge states were 2+ .

OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Identification of barley-specific ATI marker peptides

To identify possible marker peptides for the quantitation of barley-specific ATIs, the UniProtKB database was searched for known ATI sequences in the proteome of *Hordeum vulgare* (organism ID 4513). The search resulted in 41 known ATIs (nine of which are reviewed proteins) in barley. An in silico digestion of these 41 ATIs with trypsin led to 113 possible peptides, of which 22 were unique in barley. A targeted MS/MS scan for all 113 peptides was then performed with an extract of a mixture of barley flours. Of the 113 peptides, 43 showed a signal with a high intensity (range 10^3–10^6) and many overlapping product ion signals (Table S3), 16 could be identified with a signal intensity of 500 or below or a bad signal quality (non-Gaussian peak shape, few product ions) and 54 peptides had no detectable signals. The 43 peptides with good signal quality also had to be differentiated (Table S4). The selection of marker peptides is crucial for a representative quantitation of the ATI and there are specific criteria that need to be fulfilled in order to guarantee a successful quantitation. The marker peptides should be unique, contain as little cysteine as possible due to possible incomplete alkylation and have a length of 8–26 amino acids. Tryptophan and methionine should be avoided in the sequence, because of possible oxidation. Lastly, the peptides should show an intensity of 10^3–10^6 (Ludwig & Aebersold, 2014). The marker peptides in this study were chosen according to these criteria, which led to a total of 15 possible ATI marker peptides (Table S5). In first experiments, the 15 peptides were acquired and their suitability as marker peptides was assessed. For the ATIs BMAI, BDAI, CMc, CMd and ASI there were two possible marker peptide options each. Due to the more difficult synthesis and the generally poorer reproducibility associated with increasing peptide length, if both peptides had the same signal intensity and quality, the shorter of the two peptides was preferred as marker peptide. This lead in total to ten marker peptides (P1–10) for the quantitation of ATIs in barley. The method comprises different barley ATI including the two AI-types BMAI and BDAI, the four CM-types CMa, CMc, CMd and CMe, the three CI-types SCI-1A, SCI-1B and CI-2, as well as ASI. This method is the first one for an absolute quantitation of ATIs in barley.

3.2. Targeted LC-MS/MS method for barley samples

The first step in developing a targeted LC-MS/MS method was to acquire full scan mass spectra of P1-P10 as well as IS1-IS9 in the 2+ and 3+ charge states of the precursor ion. IS9 was used for the quantitation of both P9 and P10, because they differ only in one amino acid position where serine in P9 is replaced by glycine in P10. The 2+ charge state showed higher signal intensities and was used for quantitation, except for P6/IS6, where the 3+ charge state had a higher signal intensity. Five precursor to product ion transitions were chosen for each peptide and monitored using SRM. Characteristics for the selection were the intensity of the signals, where the 4–5 signals with the highest intensities were chosen, as well as the *m/z* ratio which included at least one transition with an *m/z* higher than the precursor ion for better selectivity. The CE for each transition were optimized experimentally, aiming for the highest signal intensity through varying the CE. The peak area ratios A(P1-P10)/A(IS1-IS9) were plotted against the molar ratios n(P1-P10)/n(IS1-IS9). The response lines for all ten barley ATIs were linear with $R^2 > 0.98$. The response lines were stable over the measurement period and met all the criteria for a robust measurement with reproducible results.

Bose et al. (2019) first reported results on the ATI composition in barley using peak areas of unlabeled marker peptides determined by LC-MS/MS. In accordance with our study, they identified marker peptides for the four CM-types CMa, CMc, CMd and CMe and the two AI-types BMAI and BDAI. They used the same unique marker peptides as in our study for BMAI, BDAI, CMa and CMc. For CMd and CMe different marker peptides were used, most likely because of different properties of the LC-MS/MS systems. It was not possible to use unique peptides for all ten ATIs, because the barley protein database is not complete in UniProtKB and it also contains redundant sequences. The marker peptides of the three ATIs CMc, CMd and CI-2 were not unique and present in two, two and 21 ATIs of the same type, respectively. These ATI sequences showed minimal differences in their amino acid composition, respectively, leading to slightly different MW.

3.3. Method validation

Precision, LOD, LOQ and recovery were determined (Table 2). The HorRat was used to determine the precision. For a method to be considered precise, the HorRat has to be in a range of $0.5 \leq \text{HoR} \leq 2.0$. The developed method was precise for all ten peptides ($0.6 \leq \text{HorRat} \leq 2.0$). Precision was further calculated by measuring the same barley flour in sixfold determination on three different days and calculating the standard deviation for each ATI. The standard deviation was between 0.6 % and 3.1 %, which also indicates a high precision of the method.

Regarding LOD and LOQ, all peptides were detected with high sensitivity resulting in LODs between 0.001 μg/g and 0.23 μg/g and LOQs between 0.004 μg/g and 0.77 μg/g. For the proteins, the LODs were between 0.01 μg/g and 2.15 μg/g and the LOQs were between 0.04 μg/g and 7.18 μg/g.

The method showed recoveries between 97 % and 116 % for all ATIs except CI-2 (154 %). The higher recovery for that ATI can be explained by its concentration in barley flour, which is just above the LOQ and may therefore be affected by the noise of the measurement. The good recoveries of the method indicate that the same amount of protein is extracted in the flour as well as in the diluted barley flour sample (65 % barley flour in ATI-free wheat starch).

As ATIs are cysteine-rich proteins, it was not possible to use peptides without cysteine. During sample workup, a reduction and alkylation step was performed, to ensure reproducible detection of the peptides containing cysteine residues. The most important aspects were a high signal intensity and low matrix interferences, which was given for all ten barley ATIs, even if the content was near the LOD. The precision of the method was comparable, the recoveries and LODs were slightly better than in the SIDA method reported by Geisslitz et al. (2020), which had recoveries of 93.1–150.4 % and LODs of 0.44–31.13 μg/g for wheat ATIs. Sielaff et al. (2021) also quantitated wheat ATIs using QconCAT, with comparable validation results.

3.4. Quantitation of ten barley-specific ATIs in the EcoSeed Panel

The ATI content in a sample set of 181 different barley accessions from the EcoSeed Panel was analyzed by SIDA. The set comprised 113 two-row and 68 six-row barley genotypes originating from 23 countries and grown together under the same conditions in Gatersleben, Germany. It included 103 cultivars, 13 breeding lines and 65 landraces (Alqudah et al., 2021; Nagel et al., 2019). More detailed information on the different samples used for analysis are provided in Table S6.

According to the population structure of the EcoSeed panel, the sample set was clustered into four groups. Q1 contains six-row Ethiopian landraces (36 samples), Q2 contains two-row German cultivars (46 samples), Q3 contains all six-row cultivars (22 samples), Q4 contains the other two-row cultivars (35 samples) and one group with the remaining samples (n.g., 42 samples) (Fig. 1). Potential significant differences between the groups were tested by one-way ANOVA (Tukey's test, $p < 0.05$) in the following. The samples of the EcoSeed panel contained 4 μg/g (LOQ) to 0.63 mg/g of BMAI, with no significant differences between the five groups. BDAI had the highest content of all ATI-types over all five groups with 0.29 μg/g (LOD) to 2.32 mg/g. Groups Q1 and Q3, containing six-row cultivars and six-row Ethiopian landraces had a significantly lower BDAI content compared to Q2, the two-row German

Table 2
Precision, limit of detection (LOD), limit of quantitation (LOQ) and recovery of amylase/trypsin-inhibitor (ATI) peptides and proteins in barley flour.

Peptide	ATI	Amino acid sequence	UniProtKB Accession	Precision		Recovery in 65 % flour (%)	Sensitivity peptide (µg/g)		Sensitivity protein (µg/g)	
				HOR	RSD (%)		LOD	LOQ	LOD	LOQ
P1	BMAI	ATVAEVFPGCR	P16968	0.57	1.31	114	0.17	0.55	1.23	4.09
P2	BDAI	DCCQEVANISNEWCR	P13691	0.69	2.38	101	0.02	0.08	0.29	0.97
P3	CMa	SHPDWSVLK	P28041	0.59	2.10	101	0.18	0.58	2.15	7.18
P4	CMc	ELAGISSNCR	E7BB45	1.24	2.77	108	0.02	0.06	0.26	0.86
P5	CMd	LPEWMTSAELNYPGQPYLAK	P11643	0.83	3.14	97	0.23	0.77	1.62	5.40
P6	CMe	TYVVSQICHQGPR	P01086	0.66	2.69	98	0.14	0.48	1.32	4.39
P7	ASI	ADANYYVLSANR	P07596	0.71	1.16	113	0.004	0.013	0.06	0.19
P8	CI-2	TEWPELVGK	A8V3Q1	1.54	0.79	154	0.001	0.004	0.01	0.04
P9	SCI-1A	YPEPTEGSIGASSAK	P16062	2.00	1.91	114	0.05	0.17	0.31	1.02
P10	SCI-1B	YPEPTEGSIGASGAK	P16063	0.95	0.60	116	0.06	0.21	0.38	1.27

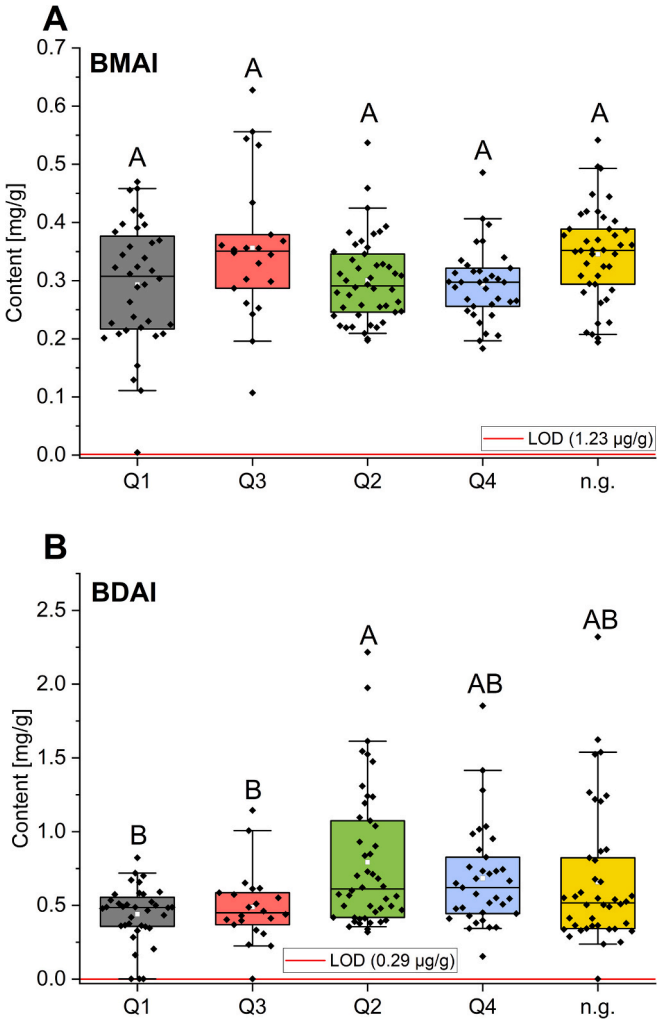

Fig. 1. Content of BMAI (A) and BDAI (B) in 181 barley accessions from the EcoSeed panel. The box represents the 25th and 75th percentiles, the square in the box is the mean and the line the median. The diamond shaped points are the data points for each of the 181 samples. The whiskers include all data points from 5 % to 95 %. The data are sorted by the five major clusters defined by Nagel et al. (2019). Q1 are the six-row Ethiopian Landraces ($n = 36$), Q3 are six-row cultivars from other countries ($n = 22$), Q2 are two-row German cultivars ($n = 46$), Q4 are other two-row cultivars ($n = 35$) and n.g. are the remaining samples of the set not fitting into the other groups ($n = 42$). Boxes with different capital letters indicate significant differences between the groups (one-way ANOVA with Tukey's test, $p < 0.05$).

cultivars. There was no significant difference between Q4, n.g. and the other three groups.

The content of CMe (1.3 µg/g (LOD)–0.94 mg/g) and CMd (0.10 mg/g–0.82 mg/g) was similar, followed by CMa with a slightly lower content (0.10 mg/g–0.71 mg/g) and CMc with a lower content (0.26 µg/g (LOD)–0.31 mg/g) (Fig. 2). For CMa, the two-row German cultivars (Q2) had a significantly higher content than the six-row cultivars (Q3) and n.g., but there was no significant difference to the groups Q1 and Q4. For CMc, Q2 had a significantly higher content than n.g., but these two groups did not differ significantly from the groups Q1, Q3 and Q4. For CMd, Q1 had a significantly higher content than n.g., but there was no significant difference to the groups Q2, Q3 and Q4. For CMe, the groups containing two-row cultivars (Q2, Q4) had a similar content and it was significantly higher than in the cultivars of Q3. Q1 and n.g. showed no significant difference to Q2, Q3 and Q4.

ASI (0.041 mg/g–0.34 mg/g), CI-2 (0.033 mg/g–0.30 mg/g), SCI-1A (0.061 mg/g–0.51 mg/g) and SCI-1B (0.023 mg/g–0.27 mg/g) were present in a low content (Fig. 3). SCI-1A and SCI-1B showed very similar results. For SCI-1A, its content in cultivars of Q3 was significantly higher than in accessions of Q4 and n.g., whereas Q1 and Q2 were not different from these groups. For SCI-1B, the content in n.g. accessions was significantly lower than that of Q1 and Q3, which were both six-row barleys, but not different from Q2 and Q4, both containing two-row barleys. Cultivars of Q1 also had a significantly higher content than those of Q4, but there was no difference to Q2 and Q3. For CI-2, group Q3 had a significantly higher content compared to Q2 and n.g. Q1 and Q4 showed no significant difference to these three groups. For ASI, cultivars of group Q3 had a significantly higher content than all other groups. Groups Q1 and n.g. were similar to each other, as well as to Q4 regarding the content of ASI, but significantly lower than Q3 and Q2.

The total protein content in the EcoSeed panel showed high variability in a range between 104.6 mg/g and 212.2 mg/g (Fig. 4 A). There was no significant difference in the protein content of the different groups. The overall ATI content (the sum of the ten ATI-types) in the samples was in a range between 1.1 mg/g and 5.2 mg/g. The total ATI content in the cultivars belonging to Q2 was significantly higher than that of n.g. accessions, but both groups did not significantly differ from Q1, Q3 and Q4 (Fig. 4 B). The share of the sum of ATIs was 0.7–3.6 % of the total protein content with no significant difference in the five groups (Fig. 4 C). It was thus slightly lower than the 3.7–4.5 % in common wheat determined by Geisslitz et al. (2020), but mostly higher than the 0.2–1.6 % in different wheat species reported by Call et al. (2020).

3.5. ATI distribution in barley

Fig. 5 A shows the mean distribution of the different ATI-types over all samples. BDAI was the most abundant ATI with an overall mean of 21.6 % in the samples. It also had the overall widest range from 11.8 % to 31.4 %. The CM-types CMa, CMd and CMe all had similar shares between 13.0 % and 14.6 %, whereas CMc had a lower share with 4.9 %.

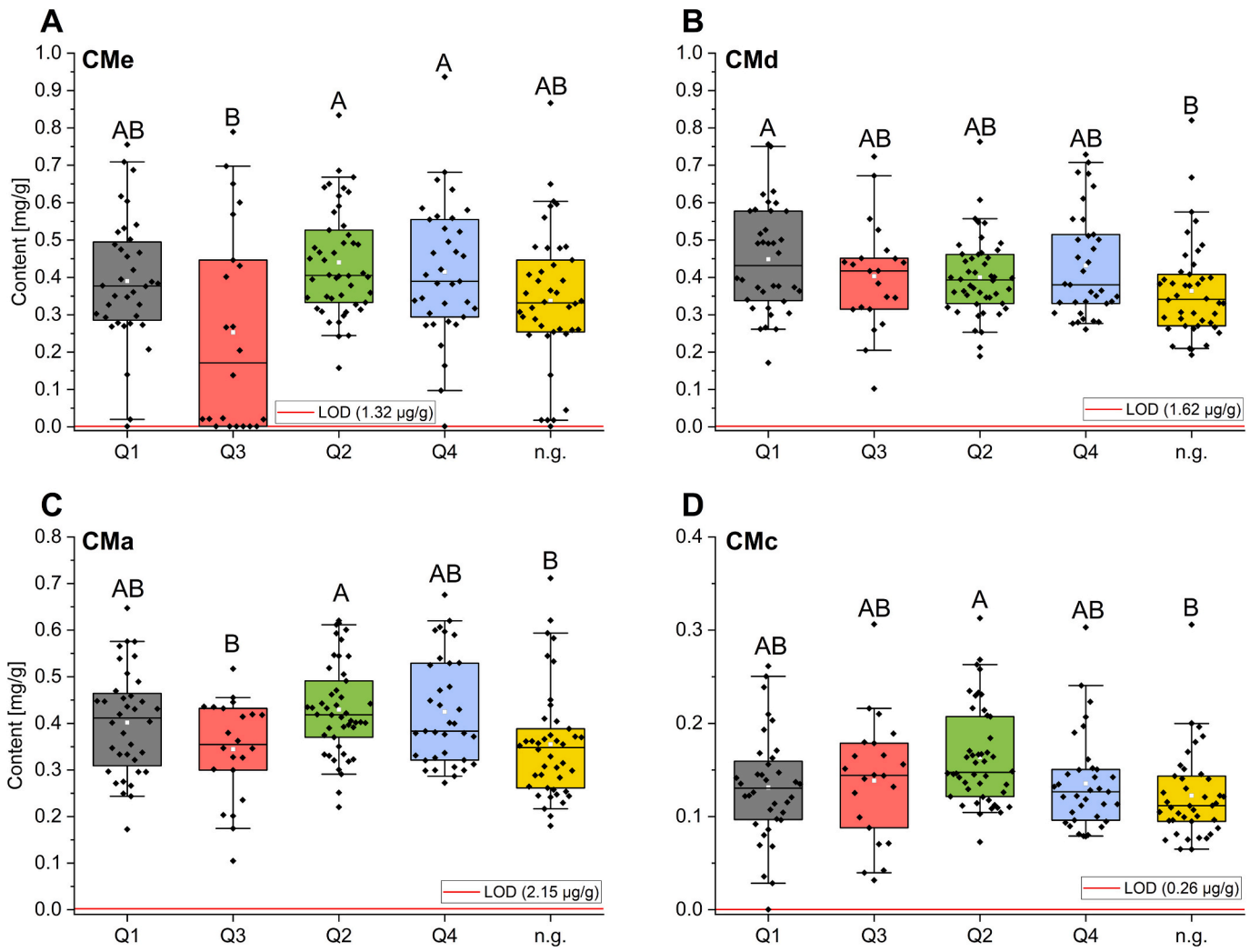

Fig. 2. Content of CMe (A), CMd (B), CMa (C) and CMc (D) in 181 barley accessions from the EcoSeed panel. The data are displayed and sorted as in Fig. 1.

BMAI had a mean share of 11.6 %, the CI-types had shares between 3.4 % and 7.1 % and ASI had a share of 3.8 %.

The ATI composition of the barley accessions differed between the five groups (Fig. 5 B). In Q1 and Q3, the share of BDAI was 16.2 % and 18.2 %, whereas in Q2, Q4 and n.g. it was higher with 25.0 %, 23.2 % and 23.2 %, respectively. For that ATI, Q1 was significantly different from Q2, Q4 and n.g., whereas Q3 was significantly different from Q2. Further, in Q3 the share of CMe (8.2 %) was significantly lower than in the other groups, where it had a share between 12.3 % and 14.3 %. The SCI-1A share was significantly higher in Q1 and Q3 (8.3–9.2 %) than in the other three groups (6.1–6.6 %). In total, CM-types accounted for the highest share with 44.6–50.7 %, followed by the AI-types with 27.2–36.9 % and the CI-types with 12.5–18.4 % and the ASI with 4.9–8.6 %.

From the perspective of food allergen detection, it is currently not yet clear how to select specific target proteins for an estimation of the allergenic potential of barley-based foods and beverages, as little is known on the allergenic potential of barley-specific ATIs. In wheat, 0.19 and CM3 have shown a high bioactivity (Zevallos et al., 2017). As mentioned, BDAI and CMd are similar to 0.19 and CM3, which makes it likely that those two barley ATIs also have a high bioactivity. The allergen database lists BMAI as a known allergen from barley (WHO/IUIS Allergen Nomenclature, 2021). Those three ATIs, therefore, might be used as target proteins in the future. However, further research on their bioactivity and allergenic potential is needed.

3.6. Grouping of barley accessions by content of ATIs

A PCA based on the content of the different ATIs in each of the 181 barley accessions was performed to see whether the ATIs allow a separation into the five groups (Q1–4, n.g.) according to Nagel et al. (2019). The scree plot indicated that the first three principal components need to be considered and these accounted for an overall variation of 68.2 %. All variables pointed onto the positive side of PC1 (Fig. 6 A). Regarding PC2, the CI-types and ASI contributed to the positive side, whereas the AI- and CM-types had loadings onto the negative side. PC3 allowed a separation between ASI and the CM-types, which were located on the negative side, while the AI- and CI-types were on the positive side (Fig. 6 B). In general, structurally similar ATI-types showed into the same directions in the loading plots. There was no clustering into the five different groups defined by Nagel et al. (2019), which indicates that the content of the ten ATIs did not allow a discrimination between the five groups. Most of the samples were located towards the middle of the plots, with only some outstanding samples.

The outstanding samples in PC1/PC2 were four samples from group Q1, one sample from Q2, two samples from Q3 and one sample from each Q4 and n.g. (Fig. 6 A). HOR4519 (Q3) had the lowest total ATI content of all accessions with very low contents of AI- and CM-types. HOR5879 had the lowest total ATI content within Q1 with contents of the AI-types, CMc and CMe below LOD and LOQ. HOR6573 and HOR6382 (both Q1) had very low contents of the AI-types and CMc or

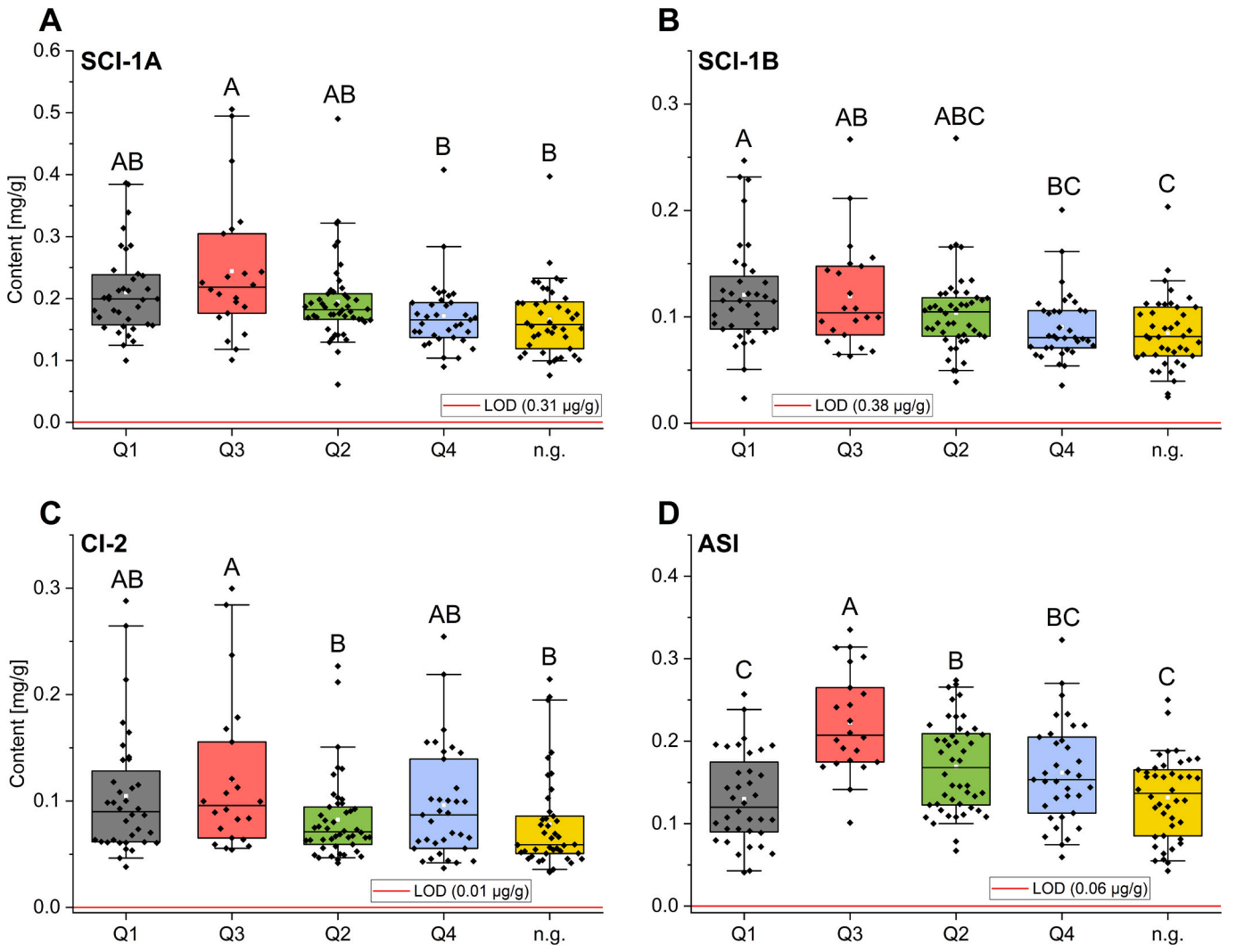

Fig. 3. Content of SCI-1A (A), SCI-1B (B), CI-2 (C) and ASI (D) in 181 barley accessions from the EcoSeed panel. The data are displayed and sorted as in Fig. 1.

even below LOD. HOR2410 (Q3) had very high contents of all CI-types, but the lowest content of the two AI-types within the Q3 group. HOR2192 (Q2) had the highest content of the CI-types among the samples in the Q2 group. HOR6193 (Q1) was the sample with the highest content of total ATI, of CMa, of SCI-1A and of SCI-1B and showed further high contents of CMc, CMd, CMe and CI-2 within Q1. HOR2202 (n.g.) had the overall highest total ATI content, the highest content of CMa and CMd and also showed a very high content of the AI-types and CMc and CMe. HOR2496 (Q4) had the highest total ATI content and that of BDAI, as well as a high content of BMAI, CMa and CMc, of within Q4.

The evaluation regarding PC2/PC3 enabled further insights into the sample set (Fig. 6 B). The outstanding samples in PC1/PC2, more precisely HOR5879, HOR6573, HOR6382 and HOR6193 from group Q1, HOR2192 from Q2, HOR4519 and HOR2410 from Q3, HOR2496 from Q4 and HOR2202 from n.g. were also outstanding in PC2/PC3 except for HOR6193 and HOR4519. In addition to the barley accessions mentioned before, two samples of Q1, two samples of Q2, one sample of Q3, two samples of Q4 and three samples of n.g. were also outstanding. HOR6974 (n.g.) showed a relatively high content of AI-types, and this was especially true for the BDAI content in HOR2098 and HOR2171 from Q2. HOR2858 and HOR2901 (both n.g.) were also similar to each other, having the highest BDAI content and high contents of BMAI, CI-2, SCI-1A and SCI-1B within the group of not group accession. HOR1736 (Q3) was located on the positive side of PC2 and PC3, due to a low content of CMa and CMe or even below the LOD. HOR6118 (Q1) had high contents of SCI-1A and SCI-1B, but a low content of BDAI resulting into a loading onto the positive side of PC2 and the negative side of PC3. HOR5780 (Q1) had a low content of the CI-types, resulting in loadings on the negative side of PC2 and PC3. HOR2094 and HOR2102 (both Q4) were very similar to each other and had loadings onto the negative sides of PC2 and PC3. HOR2102 had high contents of CMa, CMc, CMd and ASI, but low contents of SCI-1B and CI-2, while HOR2094 had a similar content of BMAI, CMa, CMc, CMd, SCI-1B and CI-2 compared to HOR2102.

3.7. Comparison of barley and wheat ATIs

Barley grains contain ATIs similar to those in wheat, occurring in monomeric, dimeric and tetrameric forms (Carbonero & García-Olmedo, 1999; Sanchez-Monge et al., 1986). BMAI is similar to 0.28, BDAI is similar to 0.19 and 0.53, CMa is similar to CM1 and CM2 and CMd is similar to CM3. The quantitative results in the studies by Geisslitz et al. (2018) and Geisslitz et al. (2020) were obtained with the same LC-MS/MS instrument as the one used in this study, which allows a good comparability of the results. The LODs for the barley-specific marker peptides of 0.001 µg/g to 0.23 µg/g were lower compared to those of wheat, which were between 0.05 µg/g and 3.92 µg/g (Geisslitz et al., 2020). The quantitative results were in the same range for BMAI and 0.28. In barley, the BMAI content was between 0.004 mg/g (LOQ) and 0.63 mg/g, whereas in wheat the content of 0.28 was between 0.31 mg/

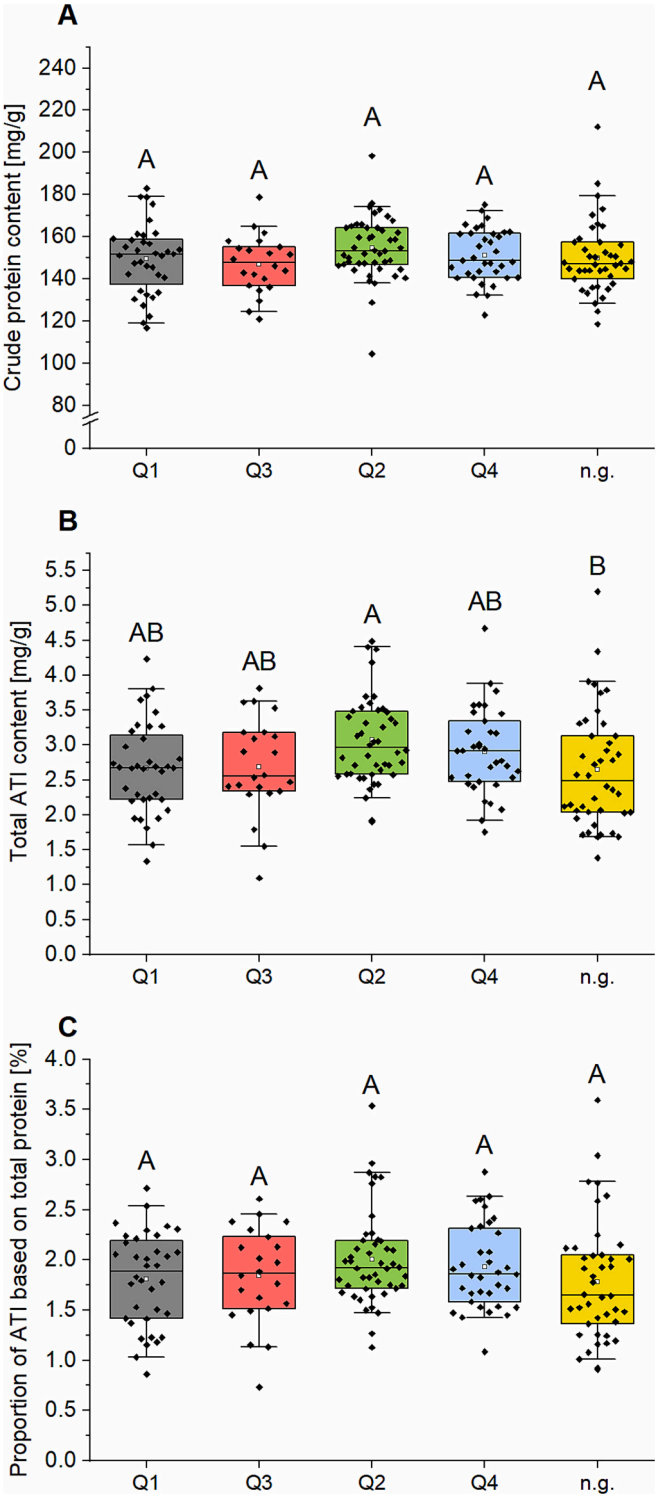

Fig. 4. Content of crude protein (A) and total ATI (B) and the proportion of ATI based on the total protein content (C) in 181 barley accessions from the EcoSeed panel. The data are displayed and sorted as in Fig. 1.

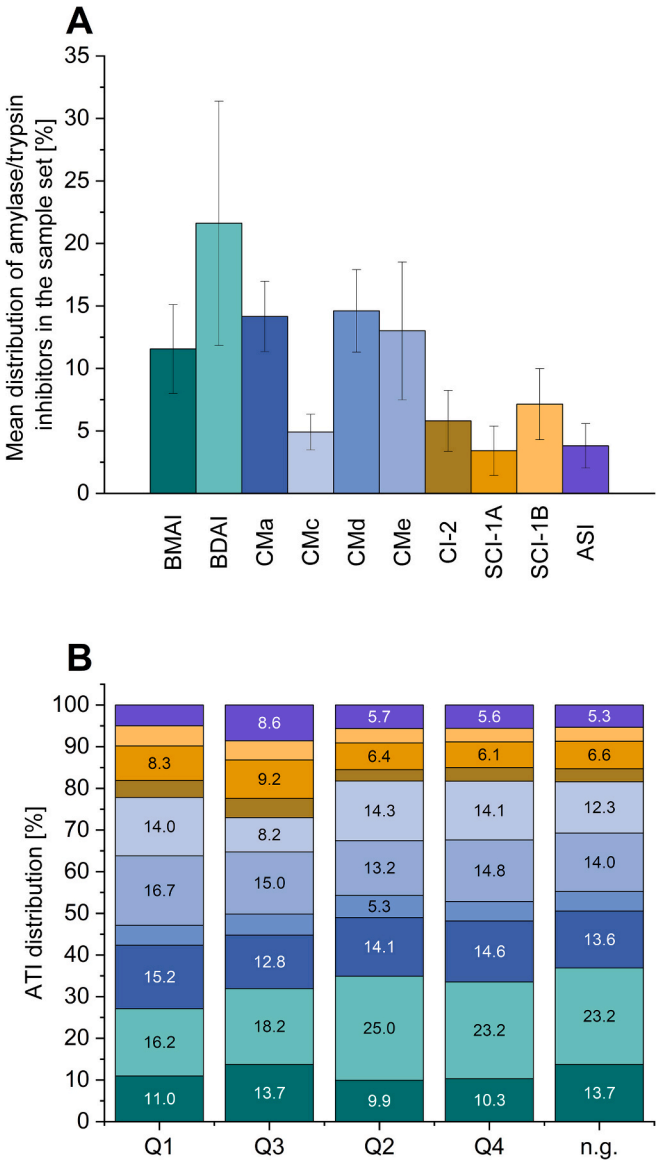

Fig. 5. Mean ATI distribution in 181 barley accessions of the EcoSeed Panel (A) and in the five groups (B). The data are sorted by the four major clusters defined by Nagel et al. (2019) in B. Q1 are the six-row Ethiopian Landraces ($n = 36$), Q3 are six-row cultivars from other countries ($n = 22$), Q2 are two-row German cultivars ($n = 46$), Q4 are other two-row cultivars ($n = 35$) and n.g. are the remaining samples of the set not fitting into the other groups ($n = 42$). A was made based on Altenbach et al. (2011). Values lower than 5.0 % are not displayed in panel B.

g and 0.41 mg/g (Geisslitz et al., 2020). The results of BDAI (0.29 µg/g to 2.32 mg/g) were comparable to 0.19 (0.71 mg/g to 1.13 mg/g) and 0.53 (0.15 mg/g to 0.24 mg/g) (Geisslitz et al., 2020) and were also comparable, although the content in barley was slightly higher as the sum of 0.19 and 0.53. CMa (0.10 mg/g to 0.71 mg/g) was comparable to the sum of CM1 (0.17 mg/g to 0.23 mg/g) and CM2 (0.30 mg/g to 0.41 mg/g) (Geisslitz et al., 2020). The content of CMd (0.10 mg/g to 0.82 mg/g) was comparable to wheat CM3 (0.64 mg/g to 0.91 mg/g) (Geisslitz et al., 2020), but CMd showed a greater range.

Regarding Fig. 5 A, Altenbach et al. (2011) did a similar evaluation for the US bread wheat Butte 86 based on the data from the proteomic analysis of Dupont et al. (2011). The monomeric 0.28 wheat proteins had a share in the same range (12.5 %) as the structurally similar BMAI in barley (11.6 %). The same applies to dimeric 0.19 and 0.53 in wheat (25 %), which are structurally similar to BDAI (21.6 %), wheat CM1 and CM2 (17.5 %), which are similar to barley CMa (14.2 %) as well as wheat CM3 (12.5 %), which is similar to CMd (14.6 %). This comparison shows that structurally similar ATIs in barley and wheat have a similar proportion in the total ATI content.

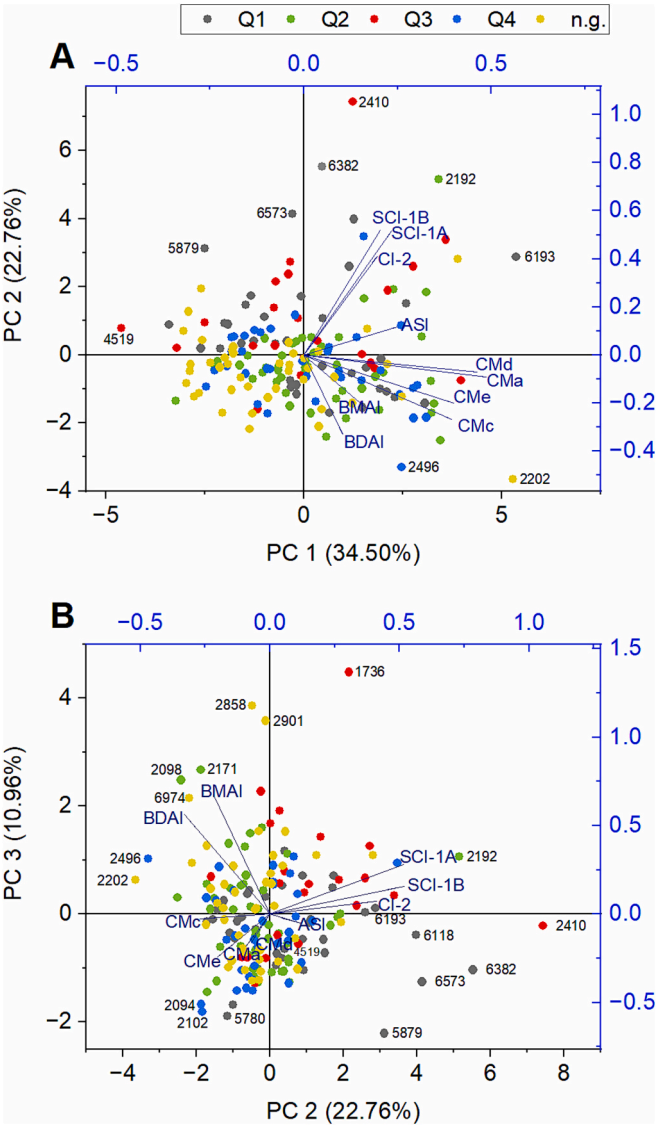

Fig. 6. Principal component analysis biplot based on the content of ten ATIs in 181 barley accessions of the EcoSeed panel considering PC1 and PC2 (A) and PC2 and PC3 (B). Q1 (grey) are the six-row Ethiopian Landraces (n = 36), Q2 (green) are two-row German cultivars (n = 46), Q3 (red) are six-row cultivars from other countries (n = 22), Q4 (blue) are other two-row cultivars (n = 35) and n.g. (yellow) are the remaining samples of the set not fitting into the other groups (n = 42). The groups were defined by Nagel et al. (2019). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

In conclusion, we identified ten barley-specific marker peptides to quantitate BMAI, BDAI, four CM-types, three CI-types and ASI in barley flours. The targeted LC-MS/MS method combined with stable isotope dilution analysis is the first one to provide the absolute content of these ATI-types. The analysis of 181 barley accessions from the EcoSeed panel, comprising 113 two-row and 68 six-row barleys of different genetic backgrounds revealed no clear distinction between two-row and six-row barleys based on the ATI content and composition. The three accessions with the lowest overall total ATI content were HOR4519 (six-row cultivar originally from Eritrea), HOR1255 (six-row landrace originally from Greece) and HOR5879 (six-row landrace originally from Ethiopia). Further work will investigate whether these accessions have a low ATI content independent of environmental factors, which is important for breeding. The newly developed method will also be applied to studying the ATI content in barley-based foods, such as beer. The results will provide the basis to develop better tolerable foods for patients with allergy and NCWS.

CRediT authorship contribution statement

Sarah Joestl: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Dalia Z. Alomari:** Writing – review & editing, Resources, Formal analysis. **Ahmad M. Alqudah:** Writing – review & editing, Resources, Formal analysis. **Andreas Börner:** Writing – review & editing, Resources, Conceptualization. **Sabrina Geisslitz:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Katharina A. Scherf:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Funding

Co-funded by the European Union (ERC, GLUTENOMICS, 101040437). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them.

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.

Acknowledgement

The authors thank Alexandra Axthelm and Sami Kaviani-Nejad (Leibniz-LSB@TUM) for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.116910.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. Mass spectrometry data are publicly available on Panorama Public https://panoramaweb.org/barley-ati.url.

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