

Elevated CO₂ induces cultivar-dependent changes in wheat proteins linked to wheat-related disorders and baking quality

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ABSTRACT

Rising atmospheric CO₂ concentrations may alter the protein composition of wheat and thereby affect grain quality. Ten wheat cultivars were treated with elevated CO₂ concentration (eCO₂, 546 ppm) and compared to ambient CO₂ concentration (aCO₂, 450 ppm) in the field to assess changes in amylase/trypsin-inhibitors (ATI), puroindolins (PIN), and gluten proteins using targeted LC-MS/MS and stable isotope dilution analysis. ATI content was higher under eCO₂ than under aCO₂, whereas PIN, gluten proteins, and selected celiac disease-active peptides were lower. The response to eCO₂ was strongly genotype-dependant. The lower gluten protein content under eCO₂ was associated with reduced baking quality. While the observed changes in ATI and selected celiac disease-active peptides may be relevant for wheat-related disorders, their biological and immunological significance remains to be established. The findings highlight the importance of identifying cultivars with low levels of ATI and celiac disease-active peptides while maintaining good baking quality under rising CO₂.

1. Introduction

The global atmospheric CO₂ concentration has increased from ≈340 ppm in 1980 to ≈425 ppm in 2024 (Lan et al., 2025). Depending on the future global socioeconomic pathway, it is simulated that the atmospheric CO₂ concentration will increase to ≈600 ppm until 2050 (Arias et al., 2021). Plants will be confronted with higher CO₂ concentrations, leading, for example, to changes in the protein composition of wheat (*Triticum aestivum* L.), one of the most important staple foods worldwide.

Several studies investigated the effect of elevated CO₂ (eCO₂) concentration (526–800 ppm) compared to ambient CO₂ (aCO₂) concentration (at that time: 375–400 ppm) on the protein composition of wheat mostly using Free-Air Carbon Dioxide Enrichment (FACE) experiments. With few exceptions, most studies included only one cultivar, predominantly from Germany or Australia. However, it has been shown that the changes in the protein composition are dependent on the cultivar (Arachchige et al., 2017; Verrillo et al., 2017; Wang et al., 2022) and it

seems that there are cultivars which are more sensitive to eCO₂ than others.

However, FACE experiments and subsequent characterizations are expensive, labor-intensive, and time-consuming. Therefore, previous studies have allocated resources either to the comparison of different cultivars (Arachchige et al., 2017; Wang et al., 2022) or to the investigation of other factors such as different temperatures (Zhang et al., 2019), different nitrogen fertilization levels (Dier et al., 2020; Pan et al., 2023; Wroblewitz et al., 2014) or different cultivation years (Dier et al., 2020; Högy, Zörb, et al., 2009; Pan et al., 2023; Verrillo et al., 2017).

Only a few FACE studies have provided detailed peptide- or protein-level characterizations relevant to wheat protein functionality and baking quality. Absolute quantitation of peptides and their corresponding proteins can be achieved by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and stable isotope dilution analysis (SIDA). The current study focused on specific marker peptides for

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different major groups of wheat proteins, including amylase/trypsin-inhibitors (ATI), puroindolins (PIN) and gluten proteins. Within gluten proteins, ω -, α - and γ -gliadins and low- (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS) were of interest. The absolute quantitation of these proteins covered the two faces of wheat (Wieser et al., 2020): Proteins essential for baking quality and proteins that trigger wheat-related disorders.

The baking quality of wheat is determined by numerous parameters including protein content, protein composition, kernel hardness, dough properties, gluten aggregation and bread volume (Shevkani et al., 2024). Absolute LC-MS/MS quantitation of gluten proteins can be used to estimate the baking quality, especially since the content of HMW-GS is correlated with bread volume (Kaemper et al., 2026). Further, PIN are responsible for grain hardness, which in turn influences wheat milling properties (Giroux & Morris, 1998).

Wheat-related disorders include celiac disease (CD), wheat allergy and non-celiac wheat sensitivity (NCWS) (Scherf, 2025). Gluten proteins contain CD-active epitopes consisting of at least nine amino acids (Sollid et al., 2020), which are recognized by antigen-presenting cells, leading to damage of the intestinal mucosa. Both gluten proteins and ATI trigger wheat allergy. Further, ATI trigger NCWS and they are involved in the worsening of other pre-existing disorders. More information on CD, wheat allergy and NCWS is summarized in numerous reviews (e.g., Scherf (2025), Singla et al. (2024), Kumar et al. (2024)). ATI can be analyzed by absolute LC-MS/MS and SIDA (Jahn et al., 2025). There are also methods to absolutely quantitate peptides containing CD-active epitopes (Prandi et al., 2014; Pronin et al., 2021; Xu et al., 2025), including one of the most immunodominant gluten peptide, the so-called 33mer with three overlapping CD-active epitopes (Schalk et al., 2017).

Even though several studies are available on the effect of eCO₂ on the protein composition of wheat, very little is known about the impact on ATI. Only one study identified a single ATI (0.19) and showed that eCO₂ increased the amount of 0.19 compared to aCO₂, but the change was dependent on the cultivar (Arachchige et al., 2017). Studies investigating changes in gluten protein composition have reported contradictory results. Some studies reported that there was no effect on gliadins and glutenins when comparing eCO₂ and aCO₂ (Dier et al., 2020; Pan et al., 2023; Wang et al., 2022; Wroblewitz et al., 2014). However, other studies report a decrease of glutenins and their subunits (Fernando et al., 2015; Högy, Zörb, et al., 2009; Soba et al., 2019; Zhang et al., 2019) or an increase (Arachchige et al., 2017; Verrillo et al., 2017) not compensating for different crude protein levels. Studies on the effect of eCO₂ on kernel hardness have also yielded contradictory results, as eCO₂ increased kernel hardness compared to aCO₂ (Fernando, Panozzo, Tausz, Norton, Fitzgerald, et al., 2014; Fernando, Panozzo, Tausz, Norton, Neumann, et al., 2014), but no effect was seen in two other studies (Blandino et al., 2020; Panozzo et al., 2014).

The current study aimed to elucidate the effect of eCO₂ compared to aCO₂ on proteins involved in wheat-related disorders (ATI and CD-active peptides) and important for baking quality (PIN and gluten proteins) in ten cultivars grown in a FACE experiment in 2023. For this, a targeted LC-MS/MS SIDA method was developed to absolutely quantitate twelve ATI, two PIN and 21 gluten protein peptides, of which ten have a CD-active epitope. The study also sought to identify cultivars that are more or less sensitive to eCO₂ and to investigate changes in proteins associated with wheat-related disorders and baking quality at the molecular level.

2. Materials and methods

2.1. Grain samples

Ten winter wheat cultivars (Apostel, Asory, Campesino, Foxx, Hyvega, Informer, KWS Emerick, LG Initial, Moschus and RGT Reform) were grown at Campus Klein-Altendorf, Rheinbach, Germany, by

Forschungszentrum Jülich GmbH during the 2022/2023 season. For analysis of the CO₂ enrichment data, daytime hours between 9 and 17 h were used as a proxy for the FACE system's operating period, approximating the phase of active photosynthesis and CO₂ enrichment. The CO₂ enrichment period commenced on January 6th and was maintained until plant senescence on July 9th, 2023. During this period, aCO₂ averaged 450 ppm and eCO₂ 546 ppm (daily means, 9–17 h). CO₂ concentrations in the elevated treatment were maintained at or above the target threshold of 550 ppm for 53% of the analyzed enrichment period (Appendix B). The FACE experiment and the agricultural practices were the same as reported in Knopf et al. (2024). The experiment followed a randomized complete block design with the two CO₂ treatments as blocks. The experiment comprised one independently controlled eCO₂ FACE ring and one spatially separated ambient CO₂ control ring. Within each ring, each cultivar was represented by three randomized field plots, which were harvested and analyzed separately. These plots were used as biological replicates in the statistical analyses. The cultivars were grown in plots sized 2.2 × 3.0 m with a sowing density of 330 kernels per m² and a row distance of 0.11 m. We performed a core harvest of 2.0 m × 1.5 m. A total of 155 kg N/ha liquid nitrogen fertilizers were applied in two doses of 60 kg and 95 kg per ha on March 24th and May 15th 2023. The field was managed following standard agricultural practices for the region and was monitored regularly to prevent damage from pests and pathogens.

Grain samples from the three replicates were not pooled. Grains were first cleaned using a 2.2 mm sieve and then milled using a Bühler MLU-202 laboratory mill (Bühler AG, Uzwil, Switzerland), resulting in flours of type 550 according to the German flour classification system (ash content of 0.51% to 0.63% based on dry matter).

2.2. Grain protein content and baking quality parameters

Quality traits were determined following standard methods of the International Association for Cereal Science and Technology (ICC): Grain protein content (in % of dry mass) by near-infrared reflectance spectroscopy (NIRS; ICC No. 159), kernel hardness by NIRS (ICC No. 207), falling number (in s; ICC No. 107/1), sedimentation volume (in mL; ICC No. 116/1), and wet gluten quantity (in %) and gluten index (ICC No. 155). Grain protein content, kernel hardness, and sedimentation volume were measured on ground grain, whereas falling number was determined on flour. Loaf volume was measured by rapeseed displacement in a standardized baking test (Arbeitsgemeinschaft Getreideforschung e.V., 2016).

2.3. Selection of marker peptides

All peptides and internal standards were purchased from GenScript Biotech (Netherlands) B.V. (Rijswijk, Netherlands) and stock solutions were prepared with water at a concentration of 1 mg/mL (Table S1). The same 20 peptides as described in Jahn et al. (2025) were used to quantitate ATI. Two peptides each were used for the quantitation of PIN-A and PIN-B as described in Vogel (2019).

For the quantitation of CD-active peptides in ω -gliadins, one peptide (ω -CD-P1) from the study of Norwig et al. (2024) was chosen based on the high number of epitope sequences (four). For the quantitation of CD-active peptides in α -gliadins, one peptide from Xu et al. (2025) (α -CD-P1), one peptide from Prandi et al. (2014) (α -CD-P2), the so-called 33mer from Schalk et al. (2017) (α -CD-P3) and one peptide from Schalk et al. (2018) (α -CD-P4) were chosen. For the quantitation of CD-active peptides in γ -gliadins, one peptide from Xu et al. (2025) (γ -CD-P1) and one peptide from Pronin et al. (2021) (γ -CD-P2) were chosen. For the quantitation of CD-active peptides in LMW-GS and HMW-GS, two and one peptides, respectively, were selected from Norwig et al. (2024) based on the short length and the consistent presence in common wheat (LMW-CD-P1, LMW-CD-P2 and HMW-CD-P1). The same eleven peptides related to baking quality as described in Kaemper et al. (2026) were

included to quantitate two α -gliadins (α -BQ-P1 and α -BQ-P2), one γ -gliadin (γ -BQ-P1), three LMW-GS (LMW-BQ-P1, LMW-BQ-P2 and LMW-BQ-P3) and five HMW-GS (HMW-BQ-P1 to HMW-BQ-P5).

2.4. Sample preparation for targeted LC-MS/MS

Sample preparation was based on Jahn et al. (2025), Norwig et al. (2024) and Kaemper et al. (2026).

2.4.1. Extraction

Proteins were extracted with a two-step protocol in duplicates. Flour (50 mg) was stirred twice with ammonium bicarbonate (Abic) solution (0.5 mL, 50 mmol/L, pH 7.8) for 30 min at 22 °C. After every extraction step, the suspensions were centrifuged for 25 min at 3750g, and the supernatants were combined, which yielded the Abic extract. Subsequently, the residue was stirred twice with gluten extraction solution (0.5 mL, 50% (v/v) 1-propanol in water, 2 mol/L urea, 0.05 mol/L TRIS-HCl, pH 7.5, 1% (w/v) dithiothreitol) for 30 min at 60 °C. The suspensions were centrifuged again for 25 min at 3750g and the supernatants combined, which yielded the gluten extract. The Abic and gluten extracts were evaporated to dryness in a rotational vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) at 8 mbar and 40 °C.

2.4.2. Reduction and alkylation

The Abic extract was dissolved in Tris-HCl (320 μ L, 0.5 mol/L, pH 8.5) and 1-propanol (320 μ L), and a mixture of the 20 and four internal standards for ATI and PIN, respectively, was added in amounts that were optimized to be within the range of the response lines. The gluten extracts were dissolved in Tris-HCl (500 μ L, 0.5 mol/L, pH 8.5) and 1-propanol (500 μ L), and after intense vortexing, the solution was divided into two equal solutions: Gluten-T and gluten-CT. The eleven internal standards for baking quality as well as the four PIN internal standards were added to gluten-T and the ten internal standards with CD-active epitope to gluten-CT (Table S1). Tris(2-carboxyethyl)phosphine (TCEP) (50 μ L, 0.05 mol/L TCEP in 0.5 mol/L Tris-HCl, pH 8.5) was added followed by incubation for 30 min at 60 °C. Then, chloroacetamide (CAA) (100 μ L, 0.5 mol/L CAA in 0.5 mol/L Tris-HCl, pH 8.5) was added and the samples were incubated for 45 min at 37 °C in the dark. The solution was evaporated to dryness in a rotational vacuum concentrator at 8 mbar and 40 °C.

2.4.3. Digestion

The Abic extract and the gluten-T extract were hydrolyzed with trypsin (0.5 mL, enzyme-to-substrate ratio 1:30, 0.04 mol/L urea in 0.1 mol/L Tris-HCl, pH 7.8), and the gluten-CT extract with chymotrypsin and trypsin (0.5 mL, enzyme-to-substrate ratio 1:50 each, 0.04 mol/L urea in 0.1 mol/L Tris-HCl, pH 7.8). Digestion was performed for 18 h at 37 °C in the dark and the reaction was stopped with 5 μ L trifluoroacetic acid. The samples were directly cleaned up by solid phase extraction (SPE).

2.4.4. Sample clean-up

Discovery DSC-18 96-well SPE plates (100 mg) were used for sample clean-up. Activation was done twice with methanol (0.75 mL), equilibration twice with 80% acetonitrile in water containing 0.1% formic acid (0.75 mL) and washing twice with 2% acetonitrile in water containing 0.1% formic acid (0.75 mL). The samples were loaded. The sample tube was rinsed with 2% acetonitrile in water containing 0.1% formic acid (0.75 mL), and the rinse was also loaded. After the sample dropped through, the sample eluate was loaded again to enhance peptide binding. Washing was done five times with 2% acetonitrile in water containing 0.1% formic acid (0.75 mL) and elution twice with 50% acetonitrile in water containing 0.1% formic acid (0.5 mL). The eluate was evaporated to dryness in a rotational vacuum concentrator at 8 mbar and 40 °C. The dried sample was stored at -20 °C until LC-MS/MS

measurement. The samples were dissolved in 2% acetonitrile in water containing 0.1% formic acid (0.5 mL) and analyzed by targeted LC-MS/MS.

2.5. Response lines

Three response lines were prepared. Response line 1 contained ATI and PIN peptides and internal standards, response line 2 CD-active peptides and internal standards, and response line 3 peptides and internal standards of the other gluten proteins. For each response line, two solutions (100 μ L of each peptide), one solution containing only peptides and the other one only internal standards, were prepared from the stock solutions. An aliquot of each solution (peptide concentration: 25 μ g/mL) was reduced with TCEP and alkylated with CAA as described for grain samples. Alkylated solutions 1 and 2 (0.5 μ g/mL of each peptide) were mixed in molar ratios n(P)/n(IS) (9 + 1, 7 + 1, 5 + 1, 3 + 1, 1 + 1, 1 + 3, 1 + 5, 1 + 7, and 1 + 9) for calibration.

2.6. Targeted LC-MS/MS

An ExionLC (Sciex, Darmstadt, Germany) was connected to a 6500+ QTrap (Sciex). The peptides were separated on a bioZen PC-C18 peptide column (100 mm $\times$ 2.1 mm, 1.6 μ m, Phenomenex, Aschaffenburg, Germany) using a flow rate of 0.35 mL/min (CD-active peptides) or 0.4 mL/min (ATI, PIN and other gluten peptides) with solvent A, 0.1% formic acid in water and solvent B, 0.1% formic acid in acetonitrile, and a column temperature of 40 °C. The gradient for ATI, PIN and gluten peptides was: 0–6.5 min 5–15% B, 6.5–10 min 15–30% B, 10–11 min 30–100% B, 11–12.5 min 100% B, 12.5–13 min 100–5% B, 13–15 min 5% B. The gradient for CD-active peptides was: 0–2 min 2% B, 2–5 min 2–12% B, 5–20 min 12–24% B, 20–26 min 24–33% B, 26–27 min 33–100% B, 27–32 min 100% B, 32–33 min 100–2% B, 33–40 min 2% B. The injection volume was 10 μ L for samples and response lines. The MS was operated in electrospray ionization (ESI) positive mode. The ion spray voltage was 5500 eV, and the source temperature was 450 °C. Nitrogen served as nebulizing gas at a level of 55, as heating gas at a level of 65 and as curtain gas at a level of 45. Peptide fragmentation was achieved using nitrogen in the collision cell. Instrument control and data acquisition were performed with Analyst (1.6.3, Sciex).

2.7. Optimization of multiple reaction monitoring parameters

The peptide sequences were included in Skyline (version 24.1.0.199) (Pino et al., 2020). Carbamidomethylation on cysteines was specified as fixed modification. Precursor and product ion masses were set to monoisotopic, and the collision energy and declustering potential were predicted for SCIEX. The filter was set for precursor charges as 2+, 3+, 4+, and 5+, for ion charges as 1+ and 2+, for ion types as y and b, and product ion selection was set from ion 2 to the last ion. Transition lists with a maximum of 250 transitions were exported from Skyline and imported into Analyst. A mixture of the synthetic, alkylated peptides with equal concentrations (1 μ g/mL per peptide) was scanned and the MS raw files were loaded into Skyline. The precursor m/z with the highest peak area was selected. Next, the four most intensive product ions were selected. The declustering potential was optimized by varying between 50 and 110 V. The declustering potential with the highest peak area was. Next, the collision energy was optimized by varying between -12 and +12 eV around the calculated optimal collision energy in Skyline in steps of 3 eV. Again, the collision energy with the highest peak area was chosen. The final parameters of MS detection of peptides and internal standards are summarized in Table S1.

2.8. Data analysis of SIDA

The MS raw data were imported into Skyline and the automatic peak integration was manually verified and adjusted if necessary to match the

start and end of the peak. Averaged peak ratios from the four transitions were used, whereas the ratios between the four transitions were confirmed to be the same for the response lines and the samples ($\pm 5\%$) as indicator for correct identification and threshold for limit of quantitation. Response lines were plotted by linear regression of the peak area ratios $A(\text{peptide})/A(\text{internal standard})$ against the molar ratios $n(\text{peptide})/n(\text{internal standard})$ (Jahn et al., 2025). Each biological replicate (field plot) was analyzed in duplicate, resulting in two technical replicates per biological replicate. Consequently, six individual measurements were obtained per cultivar and treatment (three biological $\times$ two technical replicates, $n = 6$). The validation parameters for the peptides are already reported in Jahn et al. (2025), Vogel (2019) and Kaemper et al. (2026). In general, ATI was evaluated based on Geisslitz et al. (2020) and Jahn et al. (2025). The same peptides as in Geisslitz et al. (2020) were used to calculate the respective ATI content, except ATI-P5, which was used to calculate the content of 0.19 (Jahn et al., 2025). PIN peptides were monitored in both Abic and gluten-T extracts and the content was summed for PIN-A and PIN-B. PIN-A was expressed as PIN-A-P1 and PIN-B-P1 due the better detectability compared to PIN-A-P2 and PIN-B-P2, respectively (Vogel, 2019). Consequently, the total PIN content was based on these two peptides. The calculation of the content of gluten proteins was based on Kaemper et al. (2026) except for the calculation step converting peptide content into protein content. The selection of the UniProtKB identifier was based on following criteria: 1) The number of UniProtKB identifiers should be as low as possible, what means that the identifier should contain as much peptides as possible. 2) If reviewed proteins were available, this UniProtKB identifier was preferred. More information on the selection can be found in the Supplementary Material (Sequence Alignments 1–4 in Appendix A). Sequence alignments were performed with the “Align” tool within UniProtKB using the Clustal Omega program (Sievers & Higgins, 2018). The total gluten content was calculated as the sum of all proteins, regardless of whether they were present in more than one identifier to account for isoforms. In addition, peptides with CD-active epitope were expressed as peptide content.

2.9. Statistical analysis

Outliers of $n = 6$ (three biological with two technical replicates each) were identified with the Grubbs' test on a significance level of $p \leq 0.05$ in OriginPro, version 2023 (OriginLab Corporation, Northampton, MA, USA). For the comparison between $a\text{CO}_2$ and $e\text{CO}_2$, technical replicates were averaged before statistical analysis. Across all ten cultivars, this resulted in $n = 30$ observations per treatment based on three biological replicates per cultivar ($n = 60$ in total). For cultivar-specific comparisons and calculation of percentage changes all six individual measurements (three biological and two technical replicates) were retained and analyzed ($n = 6$). Two-sample t -tests with Welch correction (equal variance was not assumed) comparing $a\text{CO}_2$ and $e\text{CO}_2$ ($n = 30$ per treatment) were performed in OriginPro, version 2023. To account for multiple hypothesis testing in these treatment's comparison, p -values were adjusted using the Benjamini–Hochberg procedure to control the false discovery rate. Results from these comparisons were considered statistically significant at an adjusted p -value ≤ 0.03 (Appendix B). Cultivar-specific comparison ($n = 6$) were evaluated using unadjusted p -values. Two-way analysis of variance (ANOVA) with the factors genotype (ten cultivars), treatment ($a\text{CO}_2$ and $e\text{CO}_2$) and the interaction between genotype and treatment were performed in OriginPro, version 2023. Partial eta squared (η_p^2) was calculated using the app “effect size for ANOVA” for Origin Pro, version 2025. Correlation analysis was performed in OriginPro, version 2023, using the application “correlation plot” with Pearson as correlation type.

3. Results and discussion

3.1. Effect of $e\text{CO}_2$ on ATI

Twelve individual ATI (0.19, 0.28, 0.53, CM1, CM2, CM3, CM16, CM17, WASI, CMX1/2/3, WCI and WTI) were quantitated and summed up, which corresponded to the total ATI content. Further, total ATI was expressed as proportion of grain protein content (Fig. 1, Table S2 and S3). The total ATI content was significantly higher ($+5.7\%$) in cultivars grown under $e\text{CO}_2$ compared to cultivars grown under $a\text{CO}_2$. Because the grain protein content was significantly lower (-4.7%) under $e\text{CO}_2$ than under $a\text{CO}_2$, the difference in the total ATI proportion based on grain protein was even more pronounced ($+10.6\%$).

Overall, the decrease in grain protein content of -4.7% under $e\text{CO}_2$ is in line with the literature (Dier et al., 2020; Högy et al., 2013; Högy, Wieser, et al., 2009); however, the decrease was dependent on the cultivar. One cultivar, Hyvega, yielded even a higher grain protein content ($+2.7\%$) under $e\text{CO}_2$, while the decrease varied between -1.9% and -10.3% for the other nine cultivars. The cultivar dependence is also in line with literature (Arachchige et al., 2017; Wang et al., 2022). Only one cultivar, Foxx, showed a lower total ATI content under $e\text{CO}_2$ than under $a\text{CO}_2$ (-1.9%), but the increase was between $+1.7\%$ and $+11.3\%$ for the other nine cultivars. This means that grains accumulated more ATI under $e\text{CO}_2$ than under $a\text{CO}_2$.

The two ATI 0.19 and CM3 are the most abundant and most bioactive ones (Geisslitz et al., 2023; Zevallos et al., 2017), and the high abundance was confirmed here (Fig. 2). For eight of the twelve individual ATI, significant differences were found between cultivars grown under $a\text{CO}_2$ and $e\text{CO}_2$ (Table S4–S15): The content of CM3 ($+15.2\%$), CM17 ($+6.6\%$), CM16 ($+9.4\%$), CM2 ($+5.7\%$), CMX1/2/3 ($+11.9\%$), WCI ($+10.2\%$) and CM1 ($+5.5\%$) was significantly higher in cultivars grown under $e\text{CO}_2$ compared to $a\text{CO}_2$.

Considering the ATI proportion, the differences between $e\text{CO}_2$ and $a\text{CO}_2$ were again more pronounced (Fig. S2). The proportion of 0.19 ($+4.9\%$), CM3 ($+20.9\%$), CM17 ($+12.0\%$), CM16 ($+14.9\%$), CM2 ($+11.1\%$), CMX1/2/3 ($+17.3\%$), WCI ($+15.6\%$), CM1 ($+11.0\%$) and WASI ($+8.1\%$) was significantly higher in cultivars grown under $e\text{CO}_2$ compared to $a\text{CO}_2$.

The ATI distribution and the content of individual ATI are in accordance with the literature and comparable to modern common wheat cultivars (Geisslitz et al., 2023; Jahn et al., 2025). It is already known that modern cultivars can contain relatively high amounts of WTI, although others contain very low quantities (Jahn et al., 2025).

3.2. Effect of $e\text{CO}_2$ on PIN

The content (-23.0%) and the proportion (-19.0%) of PIN-A, as well as of total PIN (-17.6% and -13.5% , respectively), were significantly lower under $e\text{CO}_2$ compared to $a\text{CO}_2$ (Fig. 3).

As there are hardly any targeted LC-MS/MS SIDA methods to detect and quantitate PIN in wheat, our study describes the first method for the quantitation of PIN. Overall, the content of PIN-A (218–275 $\mu\text{g/g}$), PIN-B (47–262 $\mu\text{g/g}$) and total PIN (276–537 $\mu\text{g/g}$) (Table S16–S18) are in the same range as described by Vogel (2019) (PIN-A: 118–202 $\mu\text{g/g}$; PIN-B: 36–230 $\mu\text{g/g}$; total PIN: 181–357 $\mu\text{g/g}$).

3.3. Effect of $e\text{CO}_2$ on peptides with CD-active epitope

In total, ten gluten peptides with CD-active epitope were quantitated by SIDA (Table 1). The absolute content of the individual peptides with CD-active epitope showed partially large variations between the cultivars (Fig. 4, Table S19–S28). This was especially true for ω -CD-P1, which varied between 3.2 $\mu\text{g/g}$ and 106.3 $\mu\text{g/g}$, α -CD-P1 (31.9–251.0 $\mu\text{g/g}$) and LMW-P2 (1.7–29.3 $\mu\text{g/g}$). In general, with the exception of HMW-CD-P1, the mean absolute content was lower in $e\text{CO}_2$ than in $a\text{CO}_2$. The difference between both groups was significant for ω -CD-P1

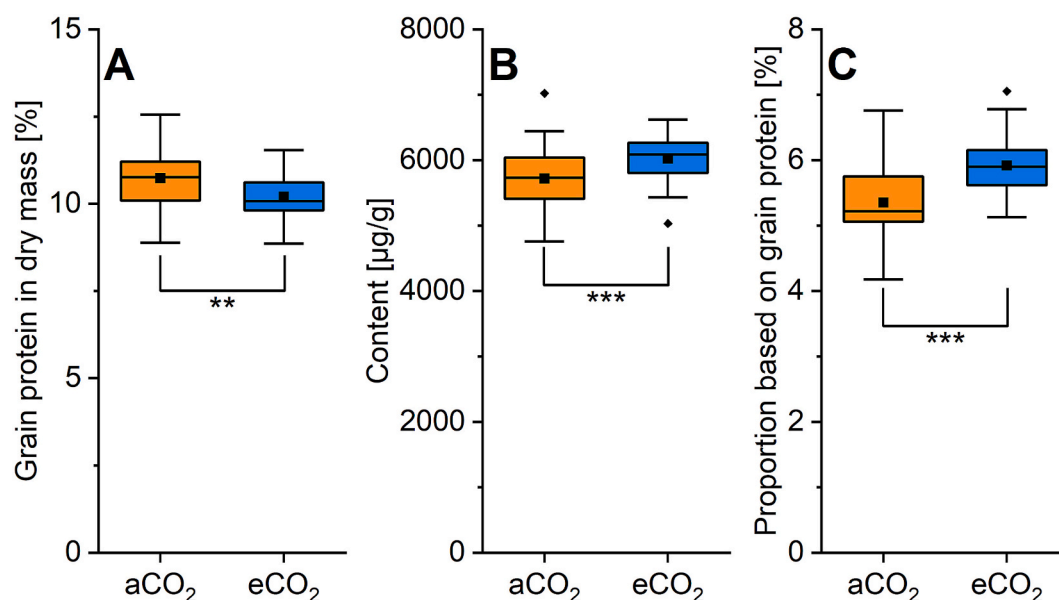


Fig. 1. Protein content and total ATI content in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration ($n = 30$ per treatment): A, Grain protein content in dry mass; B, absolute total ATI content (sum of twelve individual ATI); C, proportion of total ATI based on grain protein in dry mass. Boxes represent 25% and 75% percentiles, the whiskers represent the minimum and maximum, the points outside the whiskers are outliers, the line in the box is the median, and the point is the mean. Significant differences are marked with asterisks based on Benjamini-Hochberg-adjusted p -values (two-sample t -test, ** $p < 0.01$; *** $p < 0.001$).

(−19.7%), α-CD-P2 (−14.0%), α-CD-P3 (−11.4%), α-CD-P4 (−9.4%), γ-CD-P1 (−29.1%), γ-CD-P2 (−6.2%), LMW-CD-P1 (−41.3%) and LMW-CD-P2 (−26.1%). LMW-CD-P2 was not detectable in Asory and Campesino under both conditions and whereas LMW-CD-P1 was absent in KWS Emerick and Moschus only under eCO₂.

Because the grain protein content was lower under eCO₂ than under aCO₂, the differences in the proportion of the CD-active peptides between aCO₂ and eCO₂ were less pronounced compared to the absolute content (Fig. S2). Overall, again, the mean was lower under eCO₂ than under aCO₂ with the exception of HMW-CD-P1 (+14.8%). The difference between both groups was significant only for α-CD-P2 (−9.4%), γ-CD-P1 (−24.8%), and LMW-CD-P1 (−34.7%).

In general, we revealed a lower content of α-CD-P3 (33-mer, 15.4–73.9 µg/g) and α-CD-P4 (23.6–58.0 µg/g) compared to Pronin et al. (2021) (α-CD-P3: 79.6–634.6 µg/g; α-CD-P4: 55.4–234 µg/g) and to Schalk et al. (2017) (α-CD-P3: 90.9–602.6 µg/g). Even though the values were all determined by SIDA, possible reasons for these discrepancies are the use of different extraction methods. The content of α-CD-P1 (27.8–258.8 µg/g) and γ-CD-P2 (39.6–78.7 µg/g) were well comparable with Pronin et al. (2021) (α-CD-P1: 191–960.8 µg/g; γ-CD-P2: 12.5–54 µg/g) and Schalk et al. (2018) (α-CD-P1: 137.2 µg/g), even though we quantitated these peptides by SIDA and not via calibration using another heavy labeled peptide as in those earlier reports. Even though α-CD-P1 and γ-CD-P1 are included in the SIDA study by Xu et al. (2025), those and our results are not comparable, because we analyzed flour and they analyzed extracted Osborne fractions. Nevertheless, it proves that these two peptides are important, well detectable by MS, and should be included in methods characterizing CD-active peptides. The peptides ω-CD-P1, LMW-CD-P1, LMW-CD-P2 and HMW-CD-P1 were analyzed for the first time using LC-MS/MS SIDA, but were already included before in a comparative study on different wheat species using label free quantitation (Norwig et al., 2024).

Overall, we conclude that increasing CO₂ levels will not increase the absolute content of immunoreactive peptides, nor their proportion, even when accounting for the reduced grain protein content under eCO₂.

3.4. Effect of eCO₂ on gluten proteins related to baking quality

Eleven peptides representing two α-gliadins, one γ-gliadin, three LMW-GS and five HMW-GS were quantitated by SIDA to evaluate the effect of eCO₂ on proteins related to baking quality according to Kaemper et al. (2026) (Table S29–S39). In addition, the protein content for the ten peptides with CD-active epitope were calculated (Table S40–S49), because these proteins are not only important for CD, but may play an important role for baking quality as well. The content of these 21 gluten proteins was summed up corresponding to the total content (Fig. 5, Table S50).

In general, with the exception of HMW-GS 2 and HMW-GS 6, the mean absolute gluten content of types, subunits as well of total (−18.7%) was lower under eCO₂ than under aCO₂. The difference between both groups was significant for ω-gliadin 1 (−19.7%), γ-gliadin 1 (−16.4%), γ-gliadin 2 (−29.1%), γ-gliadin 3 (−6.2%), α-gliadin 1 (−33.8%), α-gliadin 4 (−14.0%), α-gliadin 5 (−11.4%), α-gliadin 6 (−9.4%), LMW-GS 1 (−9.1%), LMW-GS 2 (−63.6%), LMW-GS 4 (−63.6%), LMW-GS 5 (−63.6%), HMW-GS 3 (−6.9%), HMW-GS 4 (−14.3%) and HMW-GS 5 (−30.5%).

Because the grain protein content was lower under eCO₂ than under aCO₂, the differences in the proportion between aCO₂ and eCO₂ were less pronounced compared to the absolute content (Fig. S3). The lower protein content led also to a higher proportion of HMW-GS 2 (+5.2%) and HMW-GS 6 under eCO₂ compared to aCO₂. In contrast, the mean was again lower under eCO₂ than under aCO₂ for the other gluten proteins. The difference between both groups was significant only for γ-gliadin 1 (−12.1%), γ-gliadin 2 (−24.8%), α-gliadin 1 (−29.7%), α-gliadin 4 (−9.4%), LMW-GS 2 (−61.4%), LMW-GS 4 (−34.7%), HMW-GS 4 (−9.9%), and HMW-GS 5 (−26.9%). The proportion of the total content was also significantly lower under eCO₂ compared to aCO₂ (−14.3%).

Kaemper et al. (2026) also used LC-MS/MS SIDA to investigate eleven gluten protein types (α-gliadin 1 and 2, γ-gliadin 1, LMW-GS 1, 2 and 3, and HMW-GS 1, 2, 3, 4 and 5) in nine wheat lines, including RGT Reform. In general, our results revealed slightly lower contents compared to the literature, particularly for α-gliadin 1 (here: 68.4–2463.6 µg/g vs. 225.0–2841.3 µg/g), α-gliadin 2 (here:

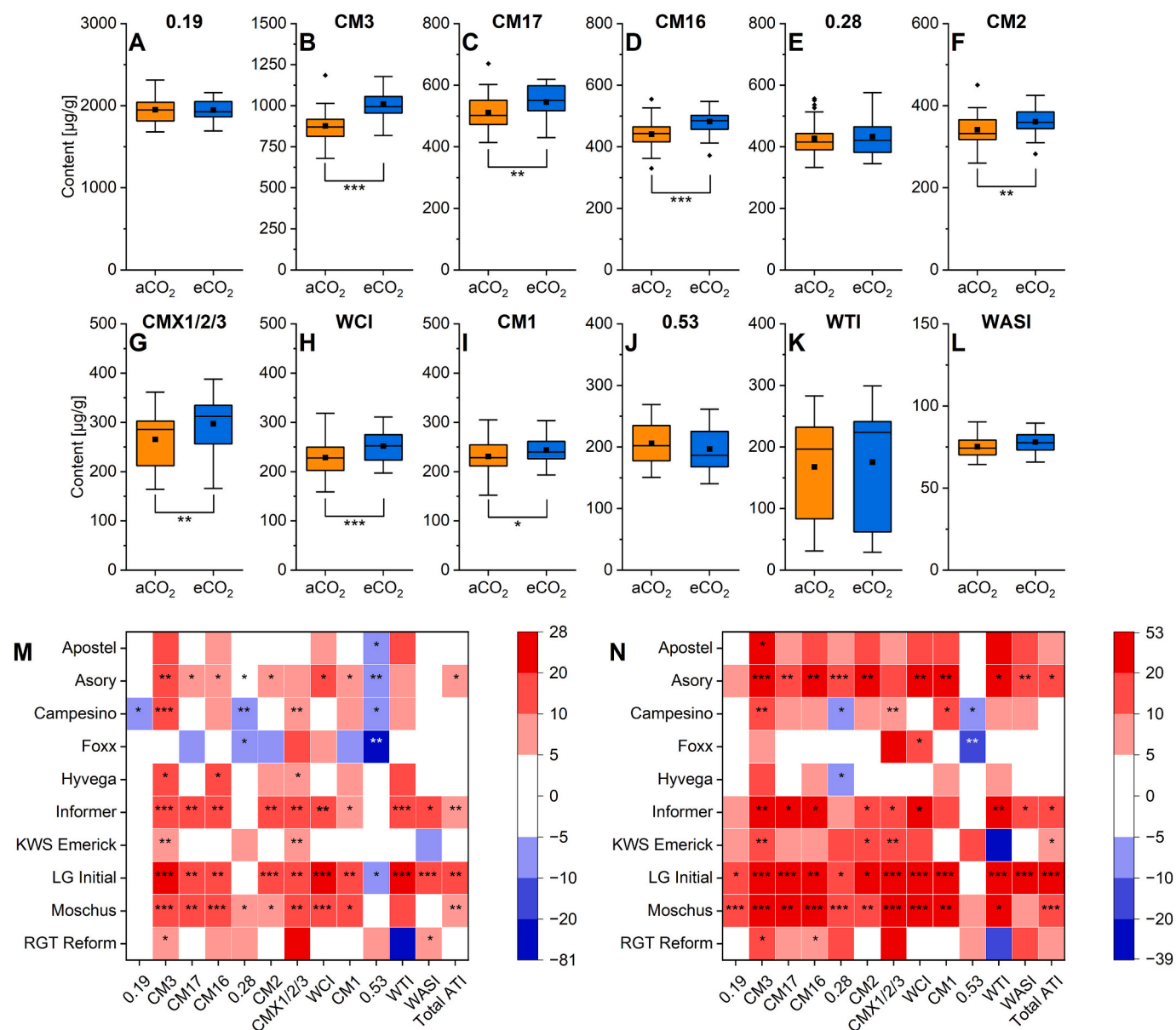


Fig. 2. Absolute content of individual ATI in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration ($n = 30$ per treatment): A, 0.19; B, CM3; C, CM17; D, CM16; E, 0.28; F, CM2; G, CMX1/2/3; H, WCI; I, CM1; J, 0.53; K, WTI; L, WASI. Data are displayed as in Fig. 1. Significant differences are marked with asterisks based on Benjamini-Hochberg-adjusted p -values (two-sample t -test, * $p \leq 0.03$; ** $p < 0.01$; *** $p < 0.001$). Percentage change per cultivar ($n = 6$): M, content; N, proportion based on grain protein. Red color represents a higher content or proportion of each ATI under eCO₂ than aCO₂, and blue color represents a lower content or proportion. Significant differences are marked with asterisks (two-sample t -test, * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1182.2–2833.0 µg/g vs. 1589.0–3816.4 µg/g), γ -gliadin 1 (here: 3723.1–5185.8 µg/g vs. 3808.3–5820.1 µg/g), LMW-GS 3 (here: 90.4–2941.9 µg/g vs. 2200.8–3778.0 µg/g), HMW-GS 2 (here: 1915.7–2597.8 µg/g vs. 1957.4–2867.4 µg/g), HMW-GS 3 (here: 1717.9–2498.4 µg/g vs. 2101.8–3316.9 µg/g) and HMW-GS 4 (here: 4933.0–7047.2 µg/g vs. 6817.4–8645.0 µg/g). In contrast, we obtained higher amounts for LMW-GS 1 (here: 1592.9–1952.4 µg/g vs. 571.5–747.3 µg/g) and LMW-GS 2 (here: 457.6–1024.1 µg/g vs. 62.6–647.3 µg/g). The most pronounced difference was found for HMW-GS 5; we detected 7620.4–11,502.2 µg/g, while in literature 2077.3–2910.3 µg/g was reported. Possible reasons for these discrepancies include the use of different extraction methods (e.g., the separation step after gluten extraction in gluten-T and gluten-CT) and the analysis of different cultivars grown under varying environmental conditions. Direct quantitative comparisons between studies should

therefore be interpreted with caution, as differences in wheat germ-plasm, sample preparation, extraction procedures, and analytical methodologies can substantially affect the reported values.

Overall, we conclude that eCO₂ altered the gluten protein composition, which is in contrast to other studies in which no change was reported in German and Chinese cultivars (Dier et al., 2020; Wang et al., 2022; Wroblewitz et al., 2014). The decrease of gluten protein types under eCO₂ was dependent on the gliadin type and glutenin subunit as well as on the cultivar. The decrease of ω -, α - and γ -gliadins, as well of LMW-GS is in agreement with literature (Arachchige et al., 2017; Högy et al., 2013; Wieser et al., 2008; Zhang et al., 2019). Based on our results, we agree with both statements that glutenins and their subunits decrease (Fernando et al., 2015; Högy, Zörb, et al., 2009; Soba et al., 2019; Zhang et al., 2019) or increase (Arachchige et al., 2017; Verrillo et al., 2017), because the content of HMW-GS 2 and HMW-GS 6 was higher, that of

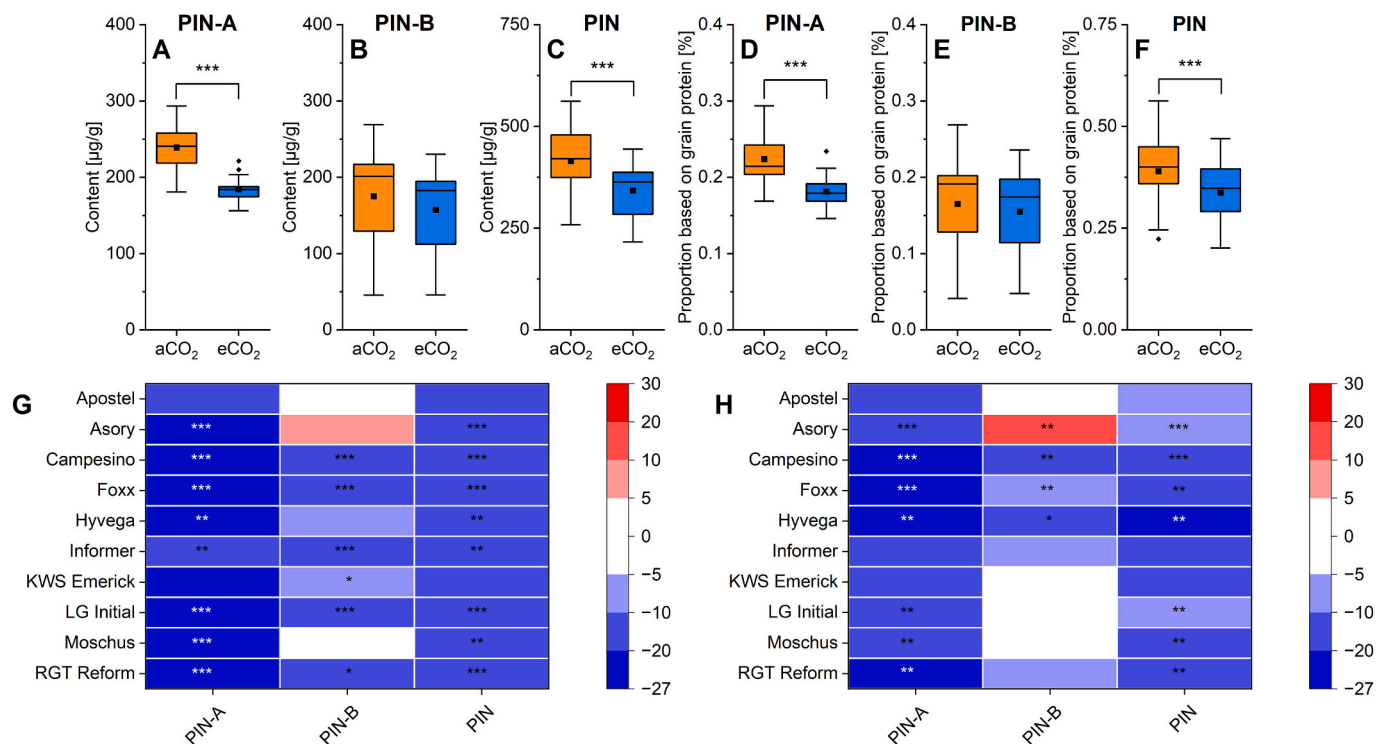


Fig. 3. Absolute content of puroindolins (PIN) and PIN proportion based on grain protein in dry mass in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration ($n = 30$): A, content of PIN-A; B, content of PIN-B; C, content of total PIN as sum of PIN-A and PIN-B; D, proportion based on grain protein of PIN-A; E, proportion based on grain protein of PIN-B; F, proportion based on grain protein of total PIN. Data are displayed as in Fig. 1. Significant differences are marked with asterisks based on Benjamini-Hochberg-adjusted p -values (two-sample t -test, *** $p < 0.001$). Percentage change per cultivar ($n = 6$): G, content; H, proportion based on grain protein. Data are displayed as in Fig. 2. Significant differences are marked with asterisks (two-sample t -test, * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Table 1

CD-active peptides with their CD-active epitope sequence according to Sollid et al. (2020) after reversion of deamidation. Cleaved forms according to Norwig et al. (2024).

Peptide	Amino acid sequence	Epitope sequence	Epitope name
ω-CD-P1	QPQQPFPQPQPQPFPQPQPFPW	QPQQPFPQ QPFPQPQPQ	DQ2.5-glia-γ4c DQ8-glia-γ1a DQ2.5-glia-γ5 DQ2.5-glia-ω1 DQ2.5-glia-ω2 DQ2.5-glia-α3
α-CD-P1	RPQQYPQPQPQY	PFPQPQPQPF PQPQPFPW	DQ2.5-glia-α1a DQ2.5-glia-α1a
α-CD-P2	LQLQFPQPQLPY	PFPQPQLPY	DQ2.5-glia-α1b
α-CD-P3	LQLQFPQPQLPYQPQLPYQPQLPYQPQPF	PYPQPQLPY PQPQLPYQ PFPQPQLPY	DQ2.5-glia-α2 DQ2.5-glia-α1a DQ2.5-glia-α2
α-CD-P4	LQLQFPQPQLPYQPQPF	PQPQLPYQ	DQ2.5-glia-γ2 DQ2.5-glia-γ1 DQ8-glia-γ2 DQ8.5-glia-γ1
γ-CD-P1	GIIQPQQAQL	IQPQQAQL	DQ2.5-glut-L1 DQ2.2-glut-L1
γ-CD-P2	LQPQQPQQSFPQQQPL	PQQSFPQQQ	DQ2.5-glut-L2 DQ8-glut-H1 DQ8.5-glut-H1
LMW-CD-P1	SQQQQPVLQPQPF	SQQQQPV [#]	
LMW-CD-P2	SQQQQSPF	SQQQQSPF ⁺	
HMW-CD-P1	QQGYTSPQQL	QQGYTSPQ	

* cleaved form of FRPQQYPQ.

[#] cleaved form of PFSQQQPV.

⁺ cleaved form of FSQQQSPF.

HMW-GS 1 and 3 was stable, whereas HMW-GS 4 and 5 decreased under eCO₂ compared to aCO₂.

Even though we did not determine the gluten content by e.g., HPLC, our findings indicate a lower gluten content under eCO₂, whereas ATI content increased. The biological mechanisms underlying these opposite

responses remain unclear. Previous studies suggest that a decline in protein content cannot be explained solely by a lack of nitrogen uptake or availability (Hay et al., 2022; Taub et al., 2008). One possible explanation may be differences in amino acid composition and metabolic regulation among protein groups. Gluten proteins are particularly

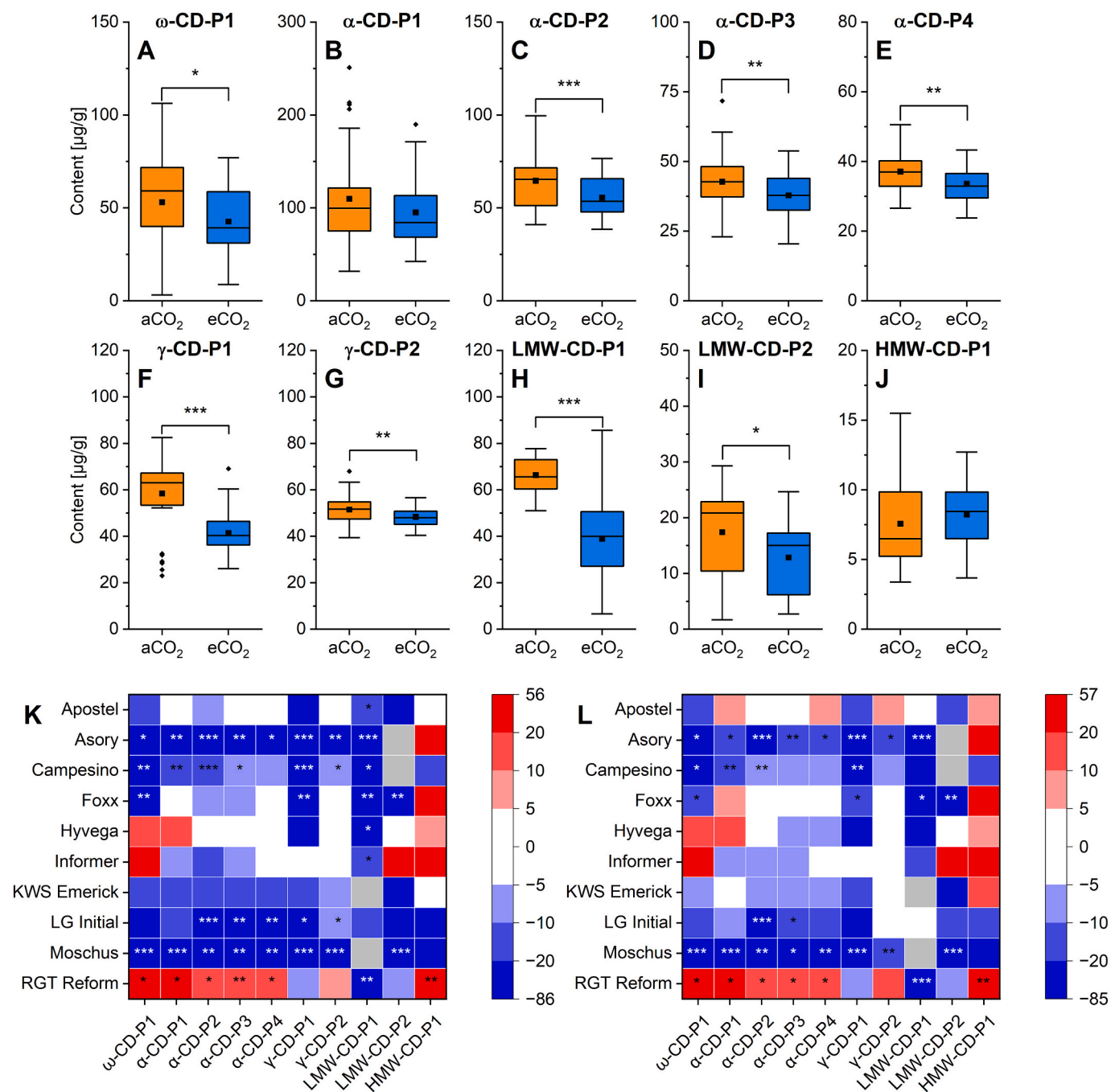


Fig. 4. Absolute content of gluten peptides with CD-active epitope in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration ($n = 30$ per treatment): A, ω -CD-P1; B, α -CD-P1; C, α -CD-P2; D, α -CD-P3 (33-mer); E, α -CD-P4; F, γ -CD-P1; G, γ -CD-P2; H, LMW-CD-P1; I, LMW-CD-P2; J, HMW-CD-P1. Data are displayed as in Fig. 1. Significant differences are marked with asterisks based on Benjamini-Hochberg-adjusted p -values (two-sample t -test, * $p \leq 0.03$; ** $p < 0.01$; *** $p < 0.001$). Percentage change per cultivar ($n = 6$): K, content; L, proportion based on grain protein. Data are displayed as in Fig. 2. Significant differences are marked with asterisks (two-sample t -test, * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$).

rich in glutamine and proline (Wieser et al., 2023), whereas ATI have a more balanced amino acid composition. Interestingly, relative to total protein, proline, glutamine and glutamic acid were reported to decrease under eCO₂ (Högy, Wieser, et al., 2009), which may contribute to the reduced abundance of gluten proteins observed in the present study. However, the biological mechanisms underlying the contrasting responses of ATI and gluten proteins remain unclear and warrant further study.

3.5. Identification of tolerant and sensitive cultivars towards eCO₂

Cultivars were classified as sensitive when at least 50% of the analytes within a given protein group showed significant changes under eCO₂ compared to aCO₂. The four cultivars Asory, Informer, LG Initial, and Moschus were sensitive to eCO₂ (Fig. 2, Table S51 and S52) and in contrast, the five cultivars Apostel, Foxx, Hyvega, KWS Emerick and RGT Reform were tolerant. The two cultivars Campesino and Foxx stood out because the absolute content of two and three individual ATI, respectively, was significantly decreased, making them promising candidates for breeding programs for a lower ATI content.

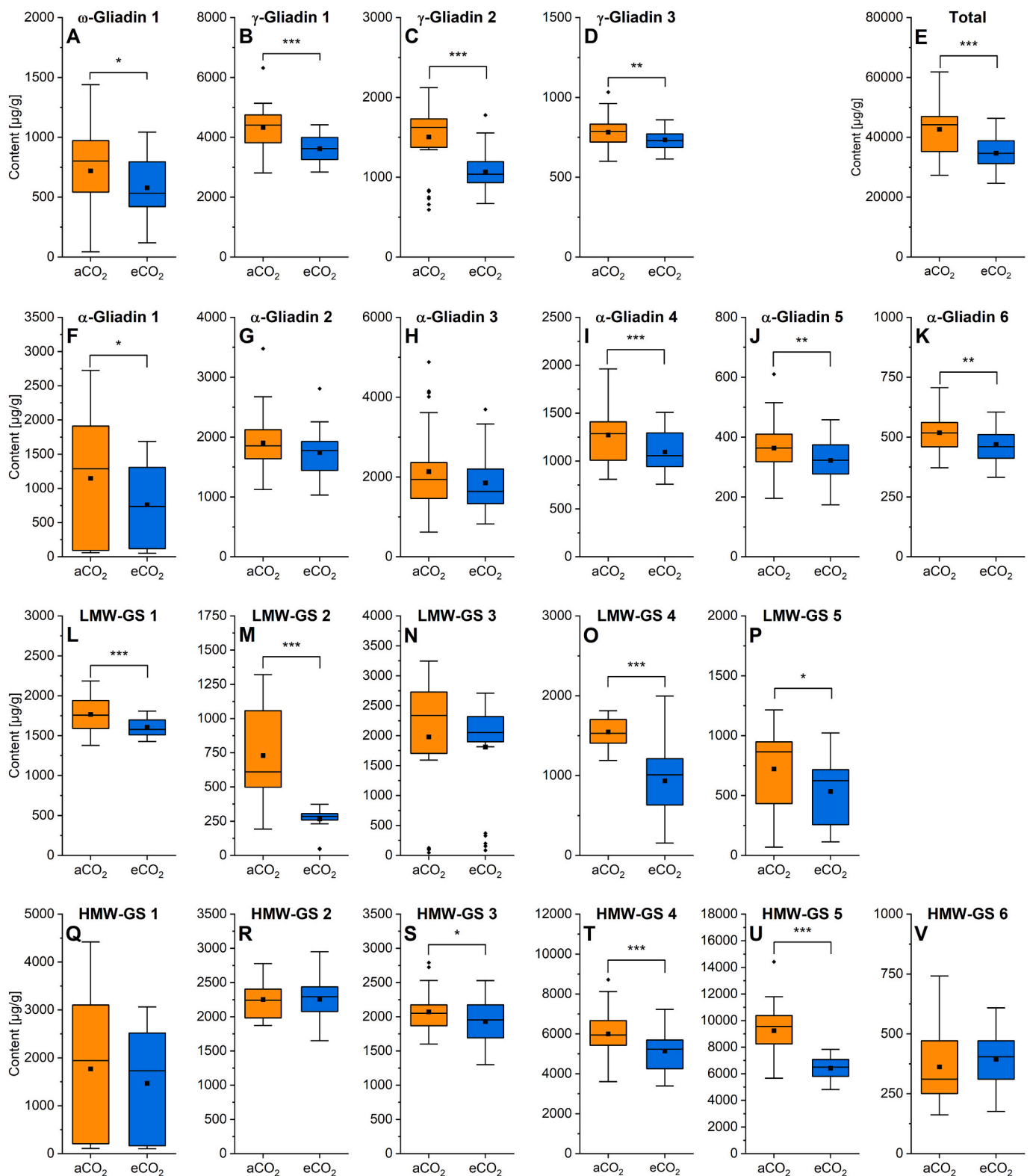


Fig. 5. Absolute content of gluten proteins related to baking quality in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration ($n = 30$ per treatment): A, ω-gliadin 1 (ω-CD-P1); B, γ-gliadin 1 (γ-BQ-P1); C, γ-gliadin 2 (γ-CD-P1); D, γ-gliadin 3 (γ-CD-P2); E, sum of all gluten proteins; F, α-gliadin 1 (α-BQ-P1); G, α-gliadin 2 (α-BQ-P2); H, α-gliadin 3 (α-CD-P1); I, α-gliadin 4 (α-CD-P2); J, α-gliadin 5 (α-CD-P3); K, α-gliadin 6 (α-BQ-P4); L, LMW-GS 1 (LMW-BQ-P1); M, LMW-GS 2 (LMW-BQ-P2); N, LMW-GS 3 (LMW-BQ-P3); O, LMW-GS 4 (LMW-CD-P1); P, LMW-GS 5 (LMW-CD-P2); Q, HMW-GS 1 (HMW-BQ-P1); R, HMW-GS 2 (HMW-BQ-P2); S, HMW-GS 3 (HMW-BQ-P3); T, HMW-GS 4 (HMW-BQ-P4); U, HMW-GS 5 (HMW-BQ-P5); V, HMW-GS 6 (HMW-CD-P1). Data are displayed as in Fig. 1. Significant differences are marked with asterisks based on Benjamini-Hochberg-adjusted p -values (two-sample t -test, * $p \leq 0.03$; ** $p < 0.01$; *** $p < 0.001$).

The eight cultivars Asory, Campesino, Foxx, Hyvega, Informer, LG Initial, Moschus and RGT Reform were sensitive to eCO₂ with regard to PIN-A, PIN-B, and total PIN (Fig. 3, Table S53 and S54). Considering the grain protein content, the three cultivars Apostel, Informer and KWS Emerick were tolerant to eCO₂. Asory was noticeable as the proportion of PIN-B was higher under eCO₂ compared to aCO₂.

The five cultivars Apostel, Foxx, Hyvega, Informer, and KWS Emerick were tolerant to eCO₂ regarding CD-active peptides (Fig. 4, Table S55 and S56) and the other five cultivars were sensitive. The cultivars Hyvega (three peptides), Informer (three peptides), and RGT Reform (six peptides) stood out due to increases in the absolute content and proportion of CD-active peptides under eCO₂. This observation may warrant further investigation when evaluating cultivars for breeding programs targeting reduced levels of disease-related marker peptides.

The five cultivars Asory, Campesino, LG Initial, Moschus and RGT

Reform were sensitive to eCO₂ regarding the content and proportion of gluten proteins related to baking quality (Fig. 6, Table S57 and S58). In contrast, the five cultivars Apostel, Foxx, Hyvega, Informer and KWS Emerick were tolerant to eCO₂.

Combining all analyses, the cultivars Asory, Campesino, LG Initial, and Moschus were sensitive to eCO₂ in all analyzed parameters, and the cultivars Apostel and KWS Emerick were tolerant. RGT Reform was sensitive in three out of four analyses (not ATI). Foxx and Hyvega were only sensitive in the PIN analysis and Informer in the both ATI and PIN analyses. Interestingly, Campesino and Foxx stood out in the ATI analysis because the content and proportion of some ATI were lower under eCO₂ than under aCO₂. At the same time these cultivars showed a higher content and/or the proportion of CD-active peptides under eCO₂ than under aCO₂.

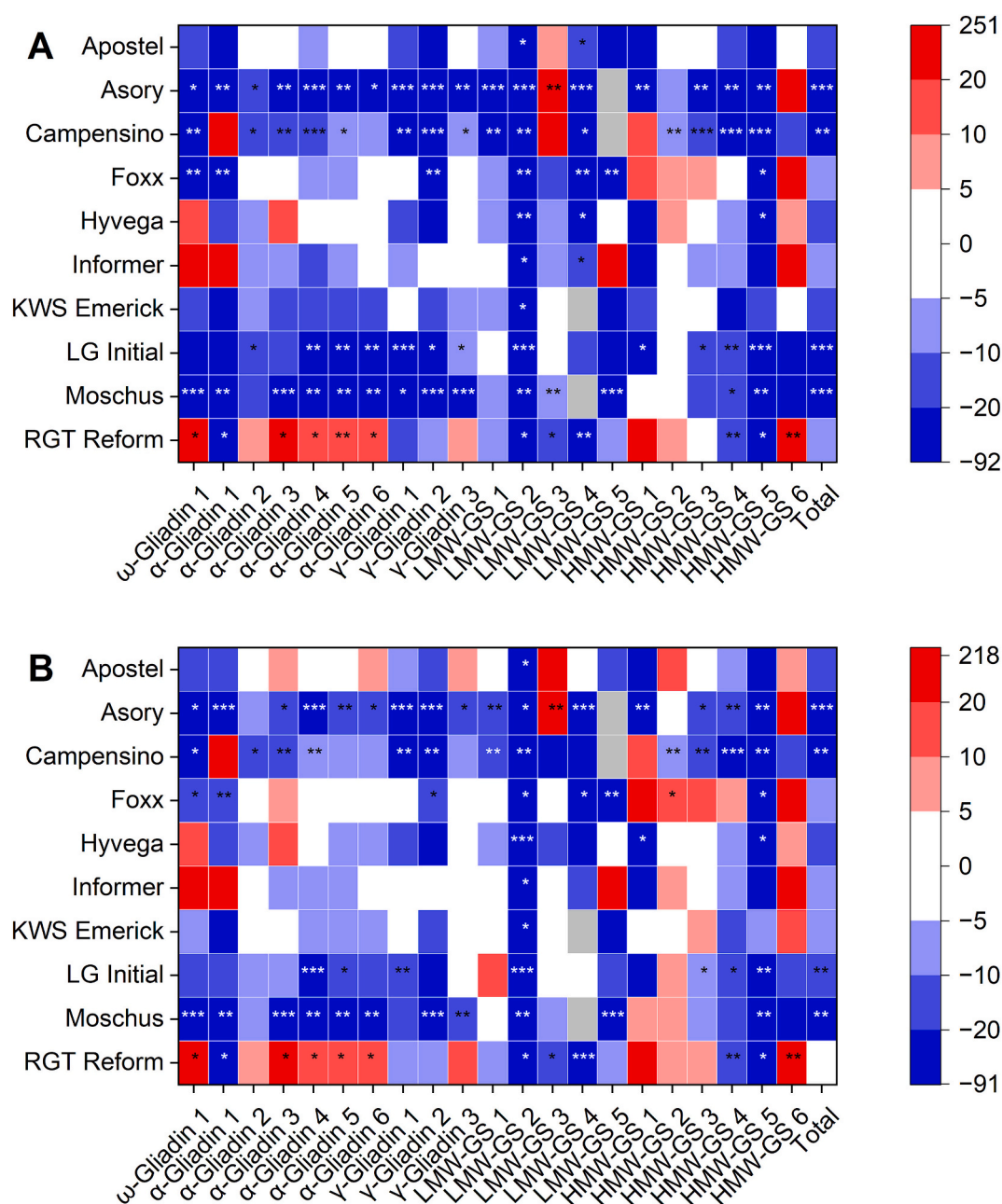


Fig. 6. Percentage change of gluten proteins per cultivar in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration (n = 6): A, content; B, proportion based on grain protein. Data are displayed as in Fig. 2. Significant differences are marked with asterisks (two-sample t-test, * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.6. Effect of eCO₂, genotype and their interaction on protein composition

Among the analyzed ATI, the treatment (i.e., aCO₂ or eCO₂) had a higher influence (higher η_p^2 ; two-way ANOVA; all η_p^2 can be found in Appendix B) only on the content of CM2 (0.20 vs. 0.65), but the genotype had a higher influence on the content of 0.19 (0.62), CM3 (0.37 vs. 0.33), CM17 (0.54 vs. 0.08), CM16 (0.50 vs. 0.16), 0.28 (0.76), CMX1/2/3 (0.72 vs. 0.06), WCI (0.54 vs. 0.13), CM1 (0.54 vs. 0.04), 0.53 (0.84 vs. 0.02), WTI (0.80), WASI (0.37 vs. 0.04), and total ATI (0.53 vs. 0.10). Significant interactions between genotype and treatment were observed for the content of CM3 (0.05), CM17 (0.07), CM16 (0.05), CM2 (0.04), WCI (0.06), CM1 (0.09), 0.53 (0.04), WTI (0.04), and total ATI (0.06). Similar observations were made for the proportion of individual ATI and total ATI. The treatment had a higher influence on the proportion only of CM3 (0.39 vs. 0.26). The effect of genotype was higher on the proportion of 0.19 (0.66 vs. 0.03), CM17 (0.42 vs. 0.16), CM16 (0.33 vs. 0.27), 0.28 (0.66 vs. 0.03), CM2 (0.45 vs. 0.15), CMX1/2/3 (0.70 vs. 0.10), WCI (0.46 vs. 0.17), CM1 (0.47 vs. 0.11), 0.53 (0.74), WASI (0.47 vs. 0.10), WTI (0.78 vs. 0.01), and total ATI (0.31 vs. 0.22). Significant interactions between genotype and treatment were observed for all ATI proportions (0.04–0.10) except for CMX1/2/3.

It was noticeable that the influence of the treatment was not significant for the content and/or proportion of the monomeric and dimeric amylase-inhibitors 0.19, 0.28, and 0.53. In contrast, the effect of the treatment was significant for the tetrameric ATI CM1, CM2, CM3, CM16, and CM17, indicating that these proteins were affected by eCO₂. The monomeric 0.28 is encoded on 6B+6D, the dimeric 0.19 and 0.53 on 3B+3D and the tetrameric CM1 on 7B, CM2 on 7A+7D, CM3 on 4B + 4D, CM16 on 2A+4B+4D and CM17 on 4B+4D (Altenbach et al., 2011; Juhász et al., 2018), indicating that the expression of different gene loci is more or less sensitive to eCO₂. However, because no change of ATI due to eCO₂ is reported in the literature until now, only speculation about the underlying genetic mechanisms is possible, and further genomic studies are required to elucidate the mechanism behind the accumulation of tetrameric ATI and the stability of mono- and dimeric ATI. This is supported by a study showing that the expression levels of some ATIs were increased, but some were also decreased in the starchy endosperm under high temperature (Juhász et al., 2018). Although the increase in ATI content under eCO₂ was statistically significant, its biological relevance remains unclear and requires confirmation by functional and immunological studies.

The treatment had a higher influence on the content of PIN-A (0.54 vs. 0.13) compared to the genotype, but the genotype had a higher influence for the content of PIN-B (0.95 vs. 0.01) and total PIN (0.71 vs. 0.17). Similar observations were made for the proportion, as the treatment had a higher influence on PIN-A (0.36 vs. 0.27), but the genotype on PIN-B (0.95 vs. 0.01) and PIN (0.77 vs. 0.09). Significant interactions between genotype and treatment were found for the PIN-B content (0.01) and the proportion of PIN-B (0.01) and PIN (0.02).

PIN-A and PIN-B are encoded on the short arm of chromosome 5D (Bhave & Morris, 2008). If PIN-B is reduced or not present, the kernel texture is harder, which is true for Asory and Moschus. Even though the treatment's effect on PIN-B was significant, there was no significant difference between aCO₂ and eCO₂ for Asory and Moschus (Fig. 3), but for most of the other cultivars. Similar to ATI, further genomic studies are required to elucidate the mechanism behind the reduction of PIN-A, PIN-B and PIN under eCO₂.

The effect of the genotype was higher on the content of ω -gliadin 1 (0.29 vs. 0.04), all α -gliadins (α -gliadin 1: 0.77 vs. 0.06; α -gliadin 2: 0.67 vs. 0.03; α -gliadin 3: 0.47 vs. 0.02; α -gliadin 4: 0.46 vs. 0.12; α -gliadin 5: 0.65 vs. 0.06; α -gliadin 6: 0.32 vs. 0.09), γ -gliadin 3 (0.29 vs. 0.07), for three out of five LMW-GS (LMW-GS 3: 0.91 vs. 0.01; LMW-GS 4: 0.17; LMW-GS 5: 0.28 vs. 0.07), for five out of six HMW-GS (HMW-GS 1: 0.71 vs. 0.01; HMW-GS 2: 0.46 vs. 0.06; HMW-GS 3: 0.43 vs. 0.06; HMW-GS 4: 0.42 vs. 0.13; HMW-GS 6: 0.20), and total gluten (0.31 vs. 0.22). The

effect of the treatment was higher only for two out of three γ -gliadins (γ -gliadin 1: 0.22 vs. 0.20; γ -gliadin 2: 0.26 vs. 0.21), for the other two LMW-GS (LMW-GS 1: 0.14 vs. 0.09; LMW-GS 2: 0.48 vs. 0.12) and HMW-GS 5 (0.42 vs. 0.13). The interaction was significant for ω -gliadin 1 (0.22), α -gliadin 1 (0.04), α -gliadin 3 (0.19), α -gliadin 4 (0.16), α -gliadin 5 (0.10), α -gliadin 6 (0.16), γ -gliadin 1 (0.09), γ -gliadin 2 (0.15), γ -gliadin 3 (0.20), LMW-GS 3 (0.01), LMW-GS 4 (0.12), LMW-GS 5 (0.15), HMW-GS 1 (0.06), and total gluten (0.08).

The effect of the genotype was higher on the proportion of ω -gliadin 1 (0.31 vs. 0.02), all α -gliadins (α -gliadin 1: 0.79 vs. 0.05; α -gliadin 2: 0.67; α -gliadin 3: 0.46; α -gliadin 4: 0.42 vs. 0.07; α -gliadin 5: 0.68 vs. 0.03; α -gliadin 6: 0.25 vs. 0.03), two out of three γ -gliadins (γ -gliadin 2: 0.24 vs. 0.20; γ -gliadin 3: 0.31), for three out of five LMW-GS (LMW-GS 3: 0.93; LMW-GS 4: 0.21; LMW-GS 5: 0.33 vs. 0.04), for five out of six HMW-GS (HMW-GS 1: 0.74; HMW-GS 2: 0.30 vs. 0.06; HMW-GS 3: 0.31; HMW-GS 4: 0.42 vs. 0.09; HMW-GS 6: 0.21 vs. 0.04), and total gluten (0.28 vs. 0.19). The effect of the treatment was higher only for γ -gliadin 1 (0.17 vs. 0.12), for LMW-GS 2 (0.50 vs. 0.11) and HMW-GS 5 (0.41). The interaction was significant for ω -gliadin 1 (0.20), α -gliadin 1 (0.04), α -gliadin 3 (0.17), α -gliadin 4 (0.15), α -gliadin 5 (0.07), α -gliadin 6 (0.12), γ -gliadin 1 (0.11), γ -gliadin 2 (0.12), γ -gliadin 3 (0.12), LMW-GS 1 (0.13), LMW-GS 3 (0.01), LMW-GS 4 (0.14), LMW-GS 5 (0.12), HMW-GS 1 (0.05), and HMW-GS 6 (0.12).

The α -gliadins are encoded by the *Gli-2* loci on the short arms of chromosomes group 6, and the γ -gliadins and ω -gliadins by the *Gli-1* loci on the short arms of chromosomes group 1. LMW-GS are encoded by the *Glu-3* loci on the short arms of chromosomes group 1 and HMW-GS by the *Glu-1* loci on the long arms of chromosomes group 1 (Juhász et al., 2018). Thus, mostly other chromosomes encode for gliadins and glutenins compared to ATI and PIN. In contrast to our findings, high temperature led to an increase of immunoreactive ω -gliadins (up to 26%) and α -gliadins (up to 33%) in three cultivars (Juhász et al., 2018). It is therefore of great interest to see how climate change, characterized by higher temperatures and elevated CO₂ levels, will actually affect the content of these proteins. In addition, no clear pattern in the expression levels of 63 immunoreactive peptides under high temperature were detected within three cultivars (Juhász et al., 2018) indicating that further genomic research is required to understand the underlying genetic mechanisms.

Overall, the effect size analysis showed that genotype generally explained a larger proportion of the variance than CO₂ treatment for most of the analyzed proteins. Although the study was conducted at a single location during one growing season, all cultivars were analyzed using three independent field plots and two technical replicates each. This design provided a robust basis for evaluating genotype-specific responses within the FACE experiment. However, the CO₂ treatment was represented by a single eCO₂ FACE ring and a single aCO₂ control ring. Consequently, the observed differences should be interpreted as responses within the specific FACE experiment conducted and should be confirmed across additional environments, growing seasons, and independent FACE units.

Although the target CO₂ concentration of 550 ppm was reached or exceeded during only 53% of the period, the mean daytime CO₂ concentration in the eCO₂ treatment was 546 ppm and therefore close to the intended target level. Studies with wheat in FACE indicate that fluctuating CO₂ concentrations can attenuate photosynthetic, growth, and yield responses relative to more constant enrichment at the same mean concentration (Allen et al., 2020; Bunce, 2012; Hendrey et al., 1997). Importantly, no major interruptions occurred during flag leaf development, while only short interruptions were observed during heading, flowering, and grain development (Appendix B). Nevertheless, fluctuations in CO₂ enrichment may have influenced treatment responses and should be considered when interpreting the results and some effects may be conservative relative to those under more sustained enrichment. Such fluctuations are difficult to avoid in FACE experiments, which are technically challenging and less controllable than greenhouse studies,

but provide a more realistic representation of future field conditions.

The eCO₂ concentration (546 ppm) is close to atmospheric CO₂ concentrations projected for the mid-21st century (Arias et al., 2021) and is therefore relevant for future climate scenarios. However, CO₂ responses are nonlinear and trait-specific (Broberg et al., 2019; Poorter et al., 2022) and no conclusions regarding nonlinear responses or saturation for ATI, PIN and gluten proteins can be drawn from the present study.

3.7. Correlation of PIN, gluten proteins and baking quality parameters

The bread volume (−4.1%) and the sedimentation volume (−11.7%) were significantly lower for cultivars grown under eCO₂ compared to aCO₂ (Fig. 6, Tables S59–S61). There was no significant change in kernel hardness, wet gluten content, falling number, and gluten index.

The decrease in bread volume is in accordance with literature, where decreases of −8.3% (Panozzo et al., 2014) and −2.3% (Fernando, Panozzo, Tausz, Norton, Neumann, et al., 2014) were reported, but the decrease is also dependent on water and nitrogen supply (Kimball et al., 2001). Our reported decrease of sedimentation volume is in contrast to Blandino et al. (2020), where no change is documented. The insignificant change of grain hardness (Blandino et al., 2020; Kimball et al., 2001; Panozzo et al., 2014) and falling number (Panozzo et al., 2014) are in accordance with literature, but Fernando, Panozzo, Tausz, Norton, Neumann, et al. (2014) reported an increase of kernel hardness (+4–9%), again depending on watering conditions. To the best

knowledge, no comparable findings for wet gluten and gluten index have been reported.

Only PIN-B was negatively correlated with bread volume and kernel hardness, but not PIN-A (Fig. 7, Table S62). As already stated, Asory and Moschus had a very low PIN-B content, and at least in Moschus this was associated with a very high kernel hardness (aCO₂: 62 vs. mean of 56; eCO₂: 61 vs. mean of 55). The kernel hardness of Asory was only slightly higher than the mean under aCO₂ (57) or even lower under eCO₂ (54).

Except for gluten index, which had negative correlations, most gluten proteins were positively correlated with baking quality parameters including bread volume, kernel hardness, wet gluten, sedimentation volume and, with limitations, falling number. Only LMW-GS 3 and HMW-GS 1 were characterized by no significant correlations and LMW-GS 4, LMW-GS 5 and HMW-GS 6 by a low number of significant correlations. This showed that especially HMW-GS 2, HMW-GS 3, HMW-GS 4, and HMW-GS 5, as well as α- and γ-gliadins and LMW-GS 1 and LMW-GS 2, are suitable predictors for baking quality. The overall lower content of gluten proteins under eCO₂ coincided with reduced baking quality. Together with the observed correlations and the established role of gluten proteins in baking performance, these findings support a link between molecular-level changes and functional properties.

Similar to the findings of Kaemper et al. (2026), the strongest correlations were observed between the content of gluten proteins with bread volume, kernel hardness, wet gluten, and sedimentation volume. In contrast to our results, however, they reported only significant correlations between two glutenin subunits with gluten index (one of them

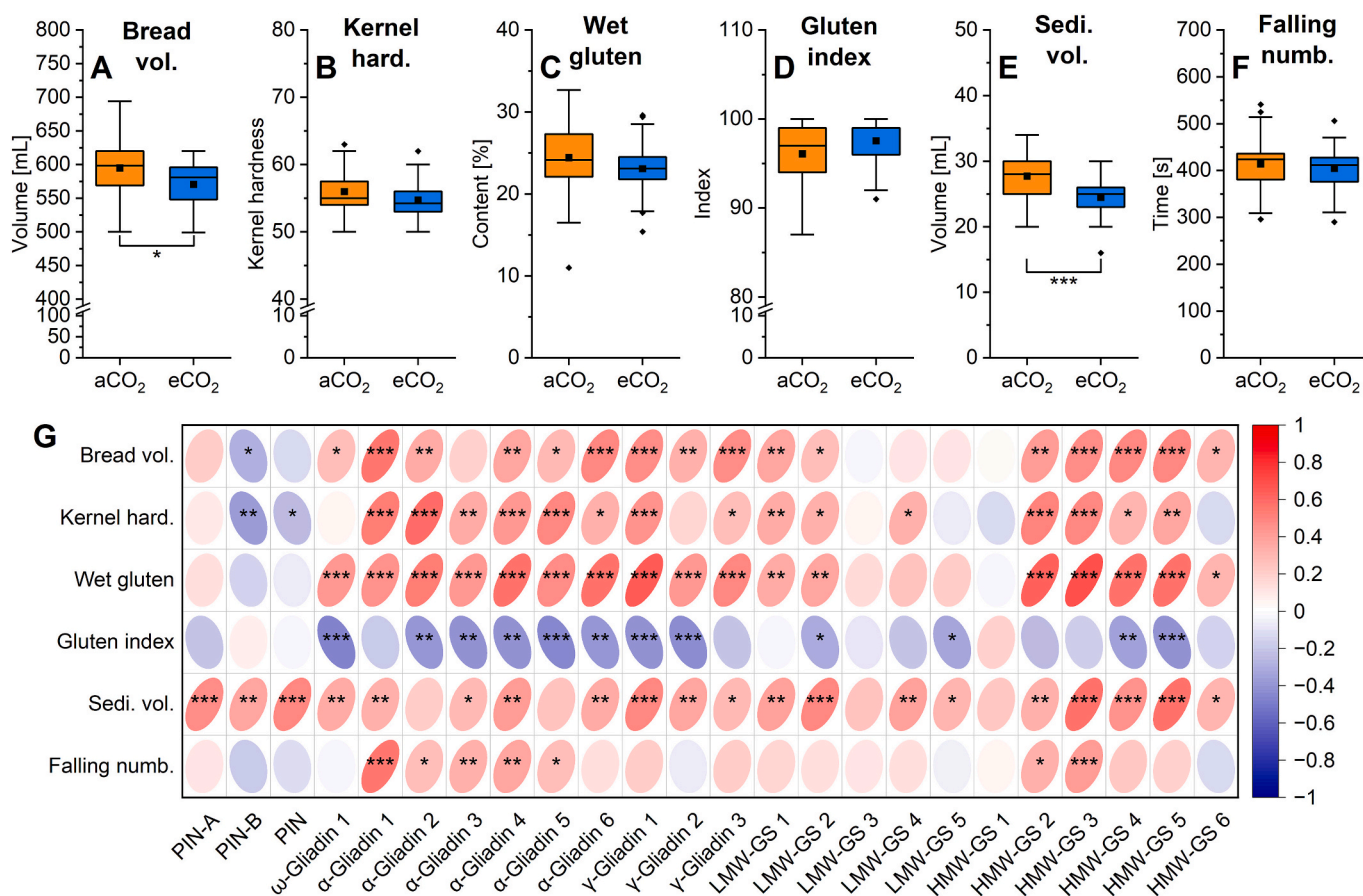


Fig. 7. Baking quality parameters in ten cultivars grown under atmospheric (aCO₂) and elevated CO₂ (eCO₂) concentration ($n = 30$ per treatment): A, bread volume (bread vol.); B, kernel hardness (kernel hard.); C, wet gluten; D, gluten index; E, sedimentation volume (sedi. vol.); F, falling number (falling numb.). Data are displayed as in Fig. 1. Significant differences are marked with asterisks based on Benjamini-Hochberg-adjusted p -values (two-sample t -test, * $p \leq 0.03$; *** $p < 0.001$). Their correlation to the content of puroindolins (PIN) and gluten proteins: G, correlation of baking quality parameters to the content of PIN-A, PIN-B and PIN, as well as gluten proteins (significant Pearson correlations are marked with asterisks, * $p \leq 0.05$; ** $p < 0.01$, *** $p < 0.001$). LMW-GS, low-molecular-weight glutenin subunits, HMW-GS, high-molecular-weight glutenin subunits.

negative) and none with falling number. At the level of individual proteins, differences were also apparent, particularly among the glutenins: LMW-GS 1 and 2 correlated with baking quality traits in this study but not in Kaemper et al. (2026), whereas the opposite was true for LMW-GS 3. This may be accounted for by the generally lower amounts of LMW-GS 1 and 2 detected in their study compared with the present one. Likewise, HMW-GS 1 showed no correlation here, whereas in their study it was reported to have the strongest individual correlation with bread volume. The remaining HMW-GS displayed similar correlations in both studies.

HMW-GS 1 corresponds to a protein encoded by the *Dy10* allele at the *Glu-D1* locus, and a possible explanation for the lack of correlations observed in this study is that only Apostel and Asory carried this allele. Nevertheless, the most likely reason for all described discrepancies might be the difference in the sample size, as Kaemper et al. (2026) analyzed 394 genotypes, making our much smaller dataset more prone to sampling bias.

4. Conclusion

The protein composition of wheat changed under eCO₂ compared to aCO₂ in ten cultivars grown in FACE experiments, but the change was dependent on the protein type and on the cultivar. The content and proportion based on grain protein of ATI generally increased, whereas those of PIN, CD-active peptides and gluten proteins decreased. Our study implies that the expression levels of some proteins are more affected by the surplus of carbon than others, but complementary genomic and transcriptomic investigations would be required to elucidate the mechanism behind our observations. For future studies, tolerant cultivars (e.g., Apostel and KWS Emerick) could be compared to sensitive ones (e.g., Asory, Campesino, LG Initial, and Moschus). Further investigations focusing on (free) amino acid composition should help to understand why gluten proteins decreased and ATI increased under eCO₂, because their amino acid composition varies significantly. As we only looked at one environment with three biological replicates, future studies should include more locations and growing seasons.

The increase in ATI under eCO₂ may be relevant from a nutritional and health perspective, but its biological significance in terms of wheat-related disorders, such as wheat allergy and NCWS remains to be established. Likewise, we found no indication that eCO₂ increased the abundance of the analyzed CD-active peptides. However, conclusions regarding overall celiac disease immunogenicity cannot be drawn, as only a subset of potentially immunoreactive peptides was analyzed and no immunological *in vivo* or *in vitro* experiments were conducted. The lower content of gluten proteins under eCO₂ coincided with reduced baking quality. Together with the observed correlations with bread volume and sedimentation volume, these findings support a link between molecular-level changes in protein composition and flour functionality, thereby contributing to a better understanding of wheat structure–function relationships.

CRediT authorship contribution statement

Sabrina Geisslitz: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Christine Kaemper:** Writing – original draft, Methodology. **Marie-Christin Norwig:** Writing – review & editing, Methodology. **Andreas Fechner:** Writing – review & editing, Investigation. **Oliver Knopf:** Writing – review & editing, Resources, Investigation. **Onno Muller:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Manuel Geyer:** Writing – review & editing, Investigation. **Lorenz Hartl:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Katharina Anne Scherf:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Compliance with ethical standards

Informed consent

Not applicable.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2026.151161>.

Data availability

Mass spectrometry data are publicly available on Panorama Public (<https://panoramaweb.org/ONh1rq.url>). All detailed values can be found within the Supplementary Material.

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