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Old and modern wheat (*Triticum aestivum* L.) cultivars and their potential to elicit celiac disease

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ABSTRACT

One potential explanation for the increasing prevalence of celiac disease (CD) over the past decades is that breeding may have inadvertently changed the immunoreactive potential of wheat. To test this hypothesis, we quantitated four CD-active peptides, namely the 33-mer and peptides containing the DQ2.5-glia-α1a/DQ2.5-glia-α2 (P1), DQ2.5-glia-α3 (P2) and DQ2.5-glia-γ1 (P3) epitopes, in a set of 60 German hexaploid winter wheat cultivars from 1891 to 2010 and grown in three consecutive years. The contents of CD-active peptides were affected more by the harvest year than by the cultivar. The 33-mer and P1 peptides showed no tendency regarding their absolute contents in the flour, but they tended to increase slightly over time when calculated relative to the α-gliadins. No trends in relative or absolute values were observed for the P2 and P3 peptides derived from α- and γ-gliadins. Therefore, the immunoreactive potential of old and modern wheat cultivars appears to be similar.

1. Introduction

Wheat is a staple food of mankind, but its consumption is also linked to wheat-related disorders, such as celiac disease (CD), wheat allergy or non-celiac gluten sensitivity. CD affects about 1% of the population worldwide and is characterized by chronic inflammation of the small intestine, which results in destruction of the epithelial cells and consequently in nutrient malabsorption (Caio et al., 2019). In genetically predisposed individuals, CD can be initiated by ingestion of the storage proteins of wheat (gliadins, glutenins), barley (hordeins) and rye (secalins) (Abadie, Sollid, Barreiro & Jabri, 2011). Wheat storage proteins comprise gliadins and glutenins and are usually classified into gliadin types (ω5-, ω1,2-, α-, and γ-gliadins) and high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) (Scherf, Koehler & Wieser, 2016). As their name suggests, prolamins are rich in the amino acids proline and glutamine (Arentz-Hansen et al., 2002; Shan et al., 2005). The disability of human gastrointestinal enzymes to cleave proteins before or after proline and glutamine results in the persistence of large CD-active peptides with nine or more amino acid residues, which are absorbed into the lamina propria and initiate the inflammatory reaction in CD patients (Plugis & Khosla, 2015).

All gluten proteins contain CD-active epitopes with distinct levels of stimulatory potential (Tye-Din et al., 2010), but gliadin peptides are usually considered to be most toxic, *inter alia*, because the incomplete digestion of gliadins results in a 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) (Qiao et al., 2004). The 33-mer is derived from α2-gliadin of hexaploid wheats, but not present in tetraploid emmer and durum wheat and diploid einkorn, because the D-genome, which encodes α2-gliadin, is absent (Ozuna, Lehisa, Gimenez, Alvarez, Sousa & Barro, 2015). The 33-mer is considered to be the most immunodominant peptide (Qiao, Bergseng, Molberg, Jung, Fleckenstein & Sollid, 2005; Shan et al., 2005), because it contains six partly overlapping epitopes, in particular PFPQPQLPY (DQ2.5-glia-α1a, one repetitive unit), PYPQPQLPY (DQ2.5-glia-α1b, two repetitive units) and PQPQLPYPQ (DQ2.5-glia-α2, three repetitive units) (Arentz-Hansen et al., 2000; Sollid, Tye-Din, Qiao, Anderson, Gianfrani & Koning, 2020). Two monoclonal antibodies (mAbs, A1 and G12) have been developed against the 33-mer for use in enzyme-linked immunosorbent assays (ELISA) for gluten detection (Morón, Bethune et al., 2008; Morón, Cebolla et al., 2008). The two most commonly used sandwich ELISA test kits are based on the R5 (Valdés, García, Llorente & Méndez, 2003) and G12 mAbs (Morón, Bethune et al., 2008; Morón, Cebolla

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Table 1
LC-MS/MS parameters for the quantitative analysis of CD-active peptides. Multiple reaction monitoring (MRM) parameters for the 33-mer, P1 and P2 derived from α-gliadins, P3 derived from γ-gliadins and the stable isotope labelled peptide standards.

Peptide	Amino acid sequence	Precursor ion [*m/z*] (charge state)	Product ion[1] [*m/z*]	Collision energy [V]
33-mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	979.0 (4+)[2]	263.3 (y2)[2]	14
		1305.2 (3+)[3]	973.5 (y8)[3]	12
*33-mer	LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F	987.0 (4+)[2]	279.0 (y2)[2]	14
		1316.0 (3+)[3]	996.0 (y8)[3]	12
P1	LQLQPFPQPQLPYPQPQPF	755.20 (3+)	262.96(y2)[2]	18
			973.64 (y8)[3]	20
*P1	LQLQPFPQPQLPYPQPQ*P*F	760.50 (3+)	278.96 (y2)[2]	14
			989.64 (y8)[3]	10
P2	RPQQPYPQPQPQY	814.24 (2+)	407.12 (y3)[2]	14
			770.48 (b6)[3]	10
P3	LQPQQPQQSFPQQQQPL	1011.42 (2+)	839.02 (y7)[2]	20
			228.96 (y2)[3]	18

*Labelled with *P: L-[$^{13}C_5$][^{15}N]-proline and *F: L-[$^{13}C_9$][^{15}N]-phenylalanine, [1]charge state: 1+, [2]precursor to product ion transition was used as quantifier, [3]precursor to product ion transition was used as qualifier.

et al., 2008).

One hypothesis to explain the rising prevalence of CD in the population (Gatti et al., 2020; Singh et al., 2018) is that wheat breeding may have contributed to increasing the immunostimulatory potential of modern wheats (cultivars developed after 1950) compared to old wheats and landraces (van den Broeck et al., 2010). Several studies have already set out to verify or falsify this hypothesis, but with partly contradictory results. A recently developed method based on a stable isotope dilution assay (SIDA) paired with liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to quantitate the 33-mer in 15 old and 23 modern common wheat cultivars, but no trends towards increased contents of the 33-mer in modern cultivars were identified (Schalk, Lang, Wieser, Koehler & Scherf, 2017; Schalk, Koehler & Scherf, 2018). Malalgoda, Meinhardt & Simsek (2018) used SIDA LC-MS/MS to quantitate two CD-active epitopes, namely DQ2.5-glia-α1 (PFPQPQLPY) and DQ2.5-glia-α3 (FRPQQPYPQ), present in α-gliadins of 30 different spring wheat cultivars from North Dakota of the past 100 years. This study suggested a random variation of the epitope contents over all cultivars and no relation to the cultivar release year. Boukid et al. (2017) determined the antigenicity of 100 Tunisian durum wheat cultivars of the 20th century and the results indicated a high variability in the contents of immunogenic peptides resulting from different environmental conditions, but again not related to the release year of the cultivars. A comparable study found that the DQ2.5-glia-α1a peptide was present in all durum wheat cultivars studied and that the amount was affected more by the cultivar than by the environmental conditions (Prandi, Mantovani, Galaverna & Sforza, 2014). The same group determined ten immunogenic peptides in old and modern Italian common wheat cultivars and showed that the old cultivars had higher contents of immunogenic peptides than modern ones (Prandi, Tedeschi, Folloni, Galaverna & Sforza, 2017). Ribeiro, Rodriguez-Quijano, Nunes, Carrillo, Branlard & Igrejas (2016) analyzed 53 modern wheat varieties and 19 old landraces using the R5 competitve ELISA and found that the reactivity did not increase over time, but that landraces even had higher reactivity. Another study by Escarnot, Gofflot, Sinnaeve, Dubois, Bertin, & Mingeot (2018) used A1 and G12 mAbs to analyze 195 wheat accessions and determined no significant differences in reactivity of landraces, old, mid and modern wheat varieties. In contrast, van den Broeck, Cordewener, Nessen, America & van der Meer (2015) performed a label-free determination of the immunogenic epitopes DQ2.5-glia-α1a, DQ2.5-glia-α2 and DQ2.5-glia-α3 in three wheat cultivars, demonstrating that one modern wheat cultivar contained a higher amount of the epitopes. In a previous study, van den Broeck et al. (2010) analyzed 36 modern European wheat cultivars and 50 landraces using ELISA. The results indicated that higher amounts of DQ2.5-glia-α1a were present in modern wheat cultivars than in landraces, with the exception of a few modern cultivars.

Taken together, the evidence whether old or modern wheat cultivars have higher or lower contents of CD-active peptides is inconclusive so far, because only small sample sets were studied or samples that were not grown under the same conditions to ensure comparable environmental conditions. Therefore, the aim of our study was to investigate the influence of breeding on the contents of four major CD-active peptides by LC-MS/MS in a very well-characterized sample set of 60 German hexaploid wheat cultivars from 1891 to 2010 grown in three consecutive harvest years. All cultivars and selected gliadin fractions and types were also investigated regarding their influence on the ELISA responses, using the R5 and G12 mAbs to get further insights into their immunoreactive potential.

2. Materials and methods

2.1. Materials

Analytical grade quality or higher was used for all chemicals and reagents, unless stated otherwise. The reagents were purchased from VWR Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), LECO (Kirchheim, Germany) and Sigma-Aldrich (Steinheim, Germany). α-Chymotrypsin (from bovine pancreas, TLCK-treated, 40 U/mg protein) was used for gluten digestion. Water for reversed-phase high-performance liquid chromatography (RP-HPLC) was deionized by an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Two commercially available sandwich ELISA test kits for gluten detection in foods were used: RIDASCREEN® Gliadin (R-Biopharm, Darmstadt, Germany) (R5) and AgraQuant® ELISA Gluten G12 (Romer Labs, Tulln, Austria) (G12). The peptides LQLQPFPQPQLPYPQPQLPYPQPQPLPYPQPQPF (33-mer), LQLQPFPQPQLPYPQPQPF (P1), RPQQPYPQPQPQY (P2), LQPQQPQQSFPQQQQPL (P3) and the stable isotope labelled peptides LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F (*33-mer) and LQLQPFPQPQLPYPQPQ*P*F (*P1) with *P: L-[$^{13}C_5$][^{15}N]-proline and *F: L-[$^{13}C_9$] [^{15}N]-phenylalanine, were purchased from Genscript (Hongkong, PR China) with a purity of > 90% (Table 1).

2.2. Grain samples

In this study 60 different hexaploid wheat cultivars (*Triticum aestivum* L.), common between 1891 and 2010 in Germany, were analyzed. The five most widely grown cultivars for each of the twelve decades were selected (Supplementary Table S1) and provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany as 20 g of cleaned grains. All cultivars were harvested in three different years, namely 2015, 2016 and 2017, and cultivated in a randomized field order without fertilization (Pronin, Börner, Weber & Scherf, 2020). The grains were milled into wholemeal flour using a

laboratory grinder (Bosch, Stuttgart, Germany) and sieved to a particle size of 0.2 mm.

On the basis of clear differences between RP-HPLC chromatograms as described in Pronin, Geisslitz, Börner & Scherf (2020), 16 particular cultivars were selected (samples 5, 10, 14, 19, 24, 25, 29, 35, 38, 40, 44, 45, 48, 51, 58 and 59) and propagated in the years 2016 and 2017 for further analysis. The grains were milled into flour using a Quadrumat Junior mill (Brabender, Duisburg, Germany), sieved to a particle size of 0.2 mm and left to rest for two weeks.

2.3. Quantitation of CD-active peptides by LC-MS/MS

2.3.1. Sample preparation

The quantitative analysis of CD-active peptides was performed according to Schalk, et al. (2017). Wheat flours (150–200 mg) were defatted twice with pentane/ethanol (95/5, v/v, 2.0 mL). Then, the albumin/globulin fraction was extracted from the flour with 2 × 1.0 mL of buffered salt solution (0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6). In the next step, the residues were extracted three times with 0.5 mL 60% (v/v) ethanol to obtain the gliadin fraction. For each extraction step the suspensions were vortex mixed for 2 min at 22 °C followed by magnetic stirring for 10 min and centrifugation (4600*g*, 25 min, 22 °C). The supernatants of the albumin/globulin fraction were discarded, while the supernatants of the gliadin fraction were combined and dried by centrifuging under reduced pressure (40 °C, 6 h, 800 Pa). After re-suspension in a TRIS-HCl-buffer (2.0 mL, 0.1 mol/L TRIS-HCl, pH 7.8, urea 120 mg/mL) the labeled *P1 and *33-mer peptides were added (300 µL; 10 µg/mL). The mixture was hydrolyzed with α-chymotrypsin (enzyme-to-protein (E:P) ratio of 1:200) for 24 h at 37 °C. The digestion was stopped with trifluoracetic acid (TFA) (5 µL) and dried by centrifuging under reduced pressure (40 °C, 6 h, 800 Pa). The residue was re-dissolved in formic acid (FA) (0.1%, v/v, 500 µL), filtered (0.45 µm) and analyzed by targeted LC-MS/MS (Table 1). α-Chymotrypsin was used for enzymatic hydrolysis instead of trypsin, because α-chymotrypsin has been shown to release the 33-mer from gluten proteins in its intact form (Schalk et al., 2017).

The determination of 33-mer and P1 was performed for each harvest year (2015, 2016, 2017), whereas P2 and P3 were only determined as an average over the three harvest years in order to focus on the genetic variability rather than the environmental effect. Therefore, 300 mg of flour from each wheat cultivar per harvest year were combined to a pooled sample and subsequently prepared as described above. All determinations were performed in triplicate.

2.3.2. Calibration

First the 33-mer, P1, P2 and P3 and the labelled *33-mer and *P1 standards were each dissolved in FA (0.1%, v/v, 10 µg/mL). For calibration, the stock solutions of P1 and 33-mer were mixed with *P1 and *33-mer in molar ratios *n*(peptide)/*n*(*standard peptide) between 1.0 and 9.1 (1 + 1, 1 + 4, 1 + 9, 4 + 1, 9 + 1), respectively. The calibration for P2 and P3 was performed based on *P1 as reported previously by Schalk et al. (2018), since no labelled standards were available. Therefore, different amounts of P2 and P3 stock solution (0 µL, 50 µL, 100 µL, 250 µL, 500 µL, 1000 µL) were each added to a mixture of wheat samples 24, 38 and 51, which were pooled from three different harvest years. The obtained mixture was prepared with addition of *P1 as described above to create a response line based on standard addition. The quantitation was performed as matrix-matched calibration relative to the labeled *P1 standard peptide.

2.3.3. Targeted LC-MS/MS

The following system was used: UltiMate 3000 HPLC system (Dionex, Idstein, Germany) coupled to a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany). A XBridge Peptide BEH-C_{18} column (1.0 × 150 mm, 3.5 µm, 13 nm; Waters, Eschborn, Germany) was used for peptide separation with the following conditions: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in acetonitrile; gradient 0–5 min isocratic 5% B, 5–22 min linear 5–55% B, 25–30 min isocratic 90% B; 30–35 min linear 90–5% B, 35–45 min isocratic 5% B; flow rate, 0.1 mL/min; injection volume, 10 µL; column temperature, 22 °C. The ion source was operated in the ESI positive mode and the following source parameters were set: spray voltage, 4500 V; vaporizer temperature, 50 °C; sheath gas pressure, 40 arbitrary units (au); aux gas pressure, 5 au; capillary temperature, 300 °C. A declustering voltage of −10 V was set for all transitions.

2.4. Quantitation of the gliadin/gluten content with ELISA

All ELISA measurements were carried out in a separate, closed room where the surfaces and equipment had been cleaned with 60% (v/v) ethanol to prevent gluten contamination. The gliadin/gluten contents of the 60 wheat cultivars were measured by two commercially available ELISA test kits: R5 RIDASCREEN® Gliadin (R-Biopharm, Darmstadt, Germany) and G12 AgraQuant® Gluten (Romer Labs, Tulln, Austria). Wheat flours (0.025 g) were incubated with 2.5 mL Cocktail solution (R5) or 2.5 mL extraction solution (G12) for 40 min at 50 °C, followed by shaking with 7.5 mL ethanol (80%, v/v in water) for 1 h at 20 °C. The samples were diluted 1:500 (R5) and 1:1000 (G12) with ethanol (60%, v/v in water) and the last dilution step was performed with the sample dilution buffer provided by the manufacturers (1:12.5 for R5, 1:10 for G12). All extractions were carried out in triplicate. The following ELISA procedures were performed according to the manufacturers' protocols. The absorbance was measured at 450 nm with a microplate reader (Expert 96 microplate reader, Asys Hitech, Eugendorf, Austria). Calibration curves with the respective standard provided in each test kit were created using the cubic spline function of the Rida® Soft Win Software (R-Biopharm).

2.5. Characterization of the protein fractions and types with ELISA

2.5.1. Sample preparation

To obtain protein fractions, the 16 propagated wheat samples were used. Each wheat flour (120 g) was defatted twice with 250 mL pentane/ethanol (95/5, v/v) for 30 min, followed by stirring once with 250 mL pentane. The suspensions were centrifuged (4600*g*, 20 min, 22 °C) and the solvent was discarded. After vacuum-drying overnight, the defatted flours (100 g) were extracted four times each with 200 mL buffered salt solution (0.067 mol/L K_2HPO_4/KH_2PO_4-buffer, 0.4 mol/L NaCl, pH = 7.6) by homogenizing with an Ultra Turrax blender (16 000 rpm, IKA-Werke, Staufen, Germany) for 5 min at 22 °C and centrifuged (4600*g*, 20 min, 22 °C). The supernatants of the obtained albumin/globulin fraction were discarded. The flour residues were extracted three times each with 200 mL 60% (v/v) ethanol as described for the albumin/globulin fraction to obtain the gliadin fraction. Then, the residues were extracted three times each with 200 mL of the glutenin extraction solution (50% (v/v) 1-propanol, 0.1 mol/L TRIS-HCl, pH 7.5, 0.06 mol/l (w/v) dithiothreitol) by homogenizing with an Ultra Turrax blender for 5 min under nitrogen and stirring for 30 min at 60 °C, cooling and centrifugation (4600*g*, 20 min, 22 °C). The supernatants of the gliadin and glutenin fractions were each concentrated under reduced pressure, dialyzed (molecular weight cut-off: 12,000–14,000, Medicell Membranes, London, UK) and lyophilized.

To obtain gliadin and glutenin types, preparative RP-HPLC was used according to Schalk, Lexhaller, Koehler & Scherf (2017). The lyophilized protein fractions and types were dissolved in the glutenin extraction solution (1 mg/mL), filtered (0.45 µm) and used for the ELISA experiments. They were also simultaneously analyzed by analytical RP-HPLC to determine the exact protein concentration, as reported by Lexhaller, Tompos & Scherf (2017). The stock solutions were diluted with 60% (v/v) ethanol to yield five serial dilutions each, so that the absorbance lay within the respective calibration ranges of the two

ELISA test kits (5–80 ng/ml for R5, 10–500 ng/ml for G12). The last dilution step was carried out with the sample dilution buffer provided in each test kit (1:12.5 for R5, 1:10 for G12). Each dilution was applied to two cavities of the 96-well plate (n = 4). The following ELISA procedures were performed as described above.

2.5.2. Analytical RP-HPLC

The following system was used: Jasco XLC system (Jasco, Gross-Umstadt, Germany) with Chrom Pass software; column, Acclaim™ 300 C_{18} (particle size 3 µm, pore size 30 nm, 2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany); elution solvents, TFA (0.1%, v/v) in water (A) and TFA (0.1%, v/v) in acetonitrile (B); linear gradient: 0 min 0% B, 0.5 min 24% B, 20 min 56% B, 20.1–24.1 min 90% B, 24.2–30 min 0% B; flow rate 0.2 mL/min; temperature, 60 °C; injection volume, 40 µL for gliadins and 40 µL for glutenins, 40 µL for ω5- and ω1,2-gliadins, 10 µL for α- and γ-gliadins; detection, UV absorbance at 210 nm. Prolamin Working Group (PWG)-gliadin (2.5 mg/mL in 60% ethanol) was used as a calibration reference for the calculation of the protein contents (van Eckert et al., 2006).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's test at a significance level of $p < 0.05$ using SigmaPlot 12.0 (Systat Software, San José, CA, USA) was used to determine statistically significant differences between the contents of 33-mer, P1, P2 and P3 of the five wheat cultivars per decade. The significant differences between the contents of P1 and 33-mer in all cultivars and the harvest years (2015, 2016, 2017) were analyzed by two-way ANOVA with harvest year and cultivar as factors. Principle component analysis (PCA) was performed with XLSTAT 2016 (Addinsoft, New York, NY, USA) to analyze whether the contents of the CD-active peptides, α-gliadins, γ-gliadins and gliadins can be used to differentiate between old and modern wheat cultivars. Pearson's product moment correlations were calculated between RP-HPLC contents of gliadins and the corresponding contents analyzed by R5 and G12 ELISA. Since the interpretation of correlation coefficients (r) varies significantly among research areas and there are no absolute rules to interpret their strength (Akoglu, 2018), we defined them according to Thanhaeuser, Wieser & Koehler (2014) ($r > 0.78$, strong correlation; 0.67–0.78, medium correlation; 0.54–0.66, weak correlation; $r < 0.54$, no correlation). The mean concentrations determined by RP-HPLC (n = 3) plotted against the corresponding mean ELISA absorbances (n = 4), while taking the five serial dilutions into account, were applied to create quadratic fits ($f = y0 + ax + bx^2$).

Next, the 16 curves of the gliadin fraction and also gliadin types were taken together for dynamic fitting to obtain the overall best fit and also 95% confidence bands and 95% prediction bands, as in Schopf & Scherf (2018).

3. Results and discussion

The contents of the CD-active 33-mer, P1, P2 and P3 peptides in the 60 wheat cultivars from 1891 to 2010 from three different harvest years (2015–2017) are presented in Supplementary Tables S2 and S3. The contents of crude protein and gluten proteins (ω5-, ω1,2-, α- and γ-gliadins, HMW-GS and LMW-GS) were already reported for the whole sample set (Pronin et al., 2020).

3.1. Determination of the 33-mer peptide in the 60 wheat cultivars

The proportions of 33-mer were calculated based on the contents of α-gliadins for each particular cultivar (Fig. 1), but also as absolute values and proportions based on the contents of crude protein (Supplementary Figs. S1 and S2). The 33-mer was present in all cultivars and showed a range from 2.7% (sample 50) to 0.6% (sample 10) considering the average over all three harvest years (Fig. 1D). Concerning each harvest year, the values ranged between 2.7% (sample 50) and 0.6% (sample 31) in 2015 (Fig. 1A), between 3.4% (sample 50) and 0.5% (sample 10) in 2016 (Fig. 1B) and between 2.3% (sample 60) and 0.5% (sample 10) in 2017 (Fig. 1C). The five cultivars per decade showed comparatively low variability in some decades, e.g., the 1920s and 1960s, but also higher variability, e.g., in the 1940s and 1950s. Considering the absolute values based on flour weight and the proportions relative to crude protein contents for the 33-mer averaged over three years, we observed no clear change. When looking at the 33-mer proportions based on α-gliadin contents, there was no clear trend in 2015, but a slight increasing trend in the year 2016, because there was a significant increase between the old (1901–1930) and modern cultivars (1981–1990, 2001–2010), but also already to samples from the 1930s. In 2017, there was a significant difference between old (1891–1930) and the most modern cultivars (2001–2010). The average values of all three years showed significantly lower 33-mer proportions in the old cultivars (1901–1930) compared to the most modern ones (2001–2010). However, due to large variability within the five samples per decade, there was no significant difference between the oldest (1891–1900) and the most modern cultivars (2001–2010). This result can be explained by the large influence of environmental factors, e.g., the harvest year, on 33-mer contents, as has been reported earlier (Schalk et al., 2017). We also observed no correlation between the contents of 33-mer and α-gliadins ($r = 0.41$, $p < 0.001$).

3.2. Determination of CD-active peptides P1, P2 and P3 in the 60 wheat cultivars

The results for P1 based on α-gliadin contents for each harvest year, the absolute contents and the proportions based on crude protein contents are presented in Supplementary Figs. S3–S5. The absolute values and the proportions based on the crude protein contents for P2 and P3 are available in Supplementary Figs. S6 and S7. P1 was present in 59 out of 60 cultivars, with the exception of sample 12 (Fig. 2A). Apart from this sample, the proportions of P1 based on α-gliadins averaged over all three harvest years were between 0.6% (sample 52) and 0.3% (sample 31), with a comparatively narrow range. The proportions of P1 based on α-gliadins showed an increasing trend for the average over the three harvest years with significant differences between the samples from 1891 to 1940 and also from 1951 to 1970 to those from 1971 to 2010. Concerning the absolute contents of P1, no apparent trend was identified, because the overall protein contents decreased from 1891 to 2010. As already observed for the 33-mer, the contents of P1 were not correlated to the α-gliadin contents ($r = 0.45$, $p < 0.001$).

No significant trends were detected for the CD-active peptides P2 and P3 over the past 120 years (Fig. 2B, C). Compared to P1, the variability of the proportions of P2 and P3 was higher within the five cultivars per decade, even if all cultivars contained both P2 and P3. In contrast to the 33-mer and P1, correlations were observed between the contents of P3 and γ-gliadins ($r = 0.79$, $p < 0.001$) and between the contents of P2 and α-gliadins ($r = 0.75$, $p < 0.001$).

All investigated common wheat cultivars contained all four CD-active peptides investigated, except sample 12, that did not contain P1. The contents of the peptides varied across the 120 years of breeding and also within the five cultivars per decade, regardless of the harvest year. Further, we showed that two CD-active peptides (33-mer and P1) did not change over the past 120 years in terms of their absolute contents in the flours, but their proportions in α-gliadins increased. The increasing trend of the proportions in α-gliadins is in agreement with van den Broeck et al. (2010) and van den Broeck et al. (2015), who reported that modern cultivars tend to contain more CD-active peptides compared to old cultivars. However, these studies were limited either to the use of ELISA or to only three wheat cultivars, respectively. Malalgoda et al. (2018) reported a high variability of CD-active peptides in wheat cultivars without a clear trend from old to modern cultivars, which is in

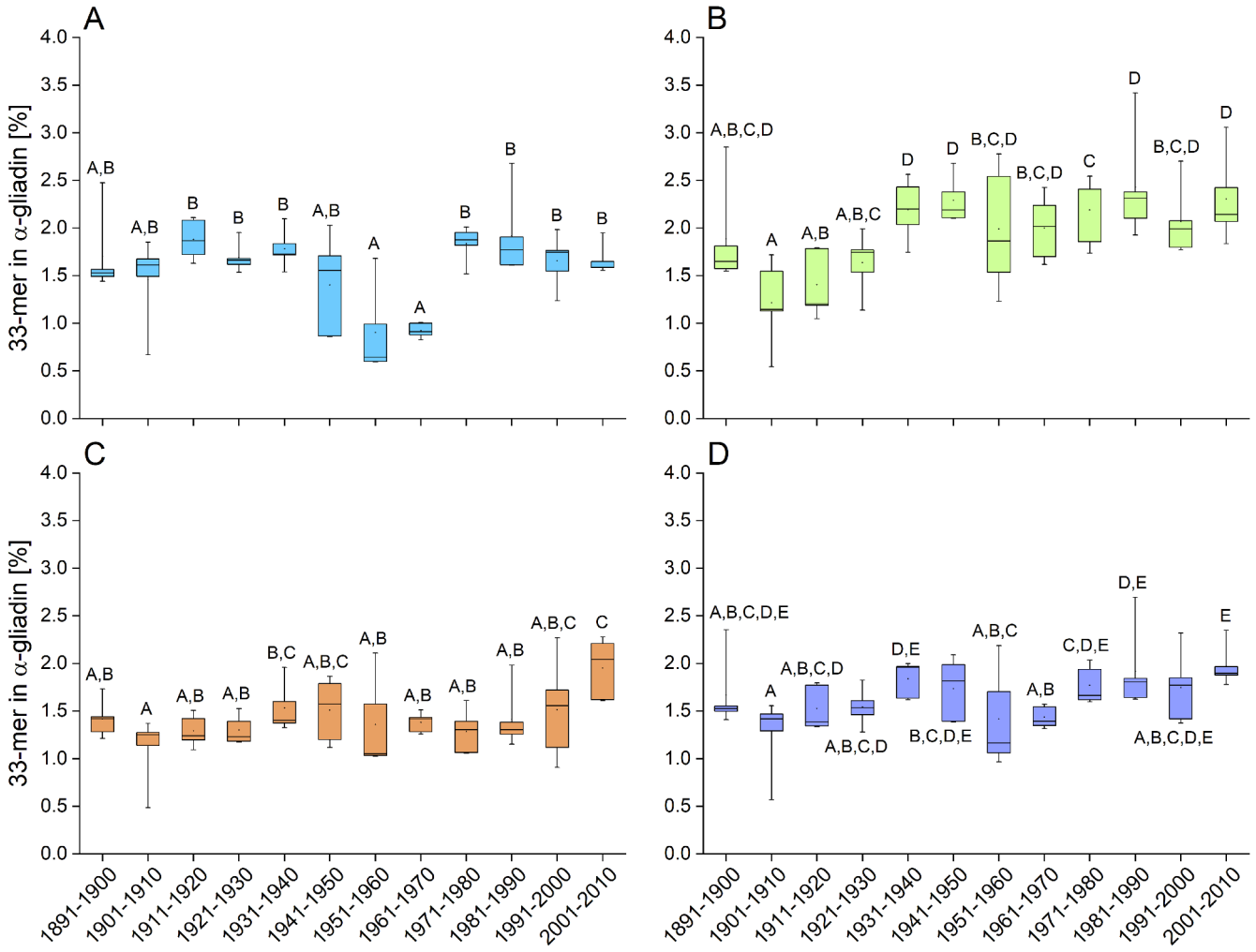

Fig. 1. Proportions [%] of 33-mer based on α-gliadin contents in five cultivars per decade of the harvest years 2015 (A), 2016 (B), 2017 (C), and averaged over three harvest years 2015–2017 (D). Boxes represent the interquartile range with the median (line in the box) and mean (point in the box) and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA, Tukey's test, $p < 0.05$).

agreement with our findings for P2 and P3. Given the fact that α-gliadin is considered the most immunoreactive fraction, the results of our study suggest that α-gliadin may not be associated with the amount of CD-active peptides. Another study by Boukid et al. (2017) on 100 Tunisian durum wheat cultivars of the 20th century found significant differences between the amounts of immunogenic peptides between abandoned and modern cultivars but did not find increased amounts in modern durum lines. In contrast to our findings, Prandi et al. (2017) reported that old Italian common cultivars had higher contents of CD-active peptides compared to modern ones.

3.3. Influence of cultivar and harvest year on the contents of 33-mer and P1

Two-way ANOVA to determine the effect of different cultivars (G variability) and different harvest years (E variability) on the contents of the 33-mer and P1 revealed a statistically significant interaction between the effects of cultivar and harvest year (G × E, (F [118, 360] = 108.2 and 7.6, respectively, $p < 0.001$)). Additionally, the effect of the harvest year for P1 was greater for old cultivars (1–30) (F [2, 180] = 3678.3, $p < 0.001$), which implies that modern cultivars (31–60) (F [2, 180] = 829.8, $p < 0.001$) are less sensitive to environmental changes. No substantial difference was observed for the 33-mer, thus old and modern cultivars were affected equally (F [2, 180] = 1258.9 and 1688.8, for old and modern cultivars respectively, $p < 0.001$).

Due to the significantly higher variance between the harvest years (F [2, 360] = 1959 and 3661.5, for 33-mer and P1 respectively, $p < 0.001$) compared to the variance within one particular harvest year, the harvest year had the greatest influence on the peptide contents. The effect of the cultivar on the peptide contents was smaller compared to that of the harvest year (F [59, 360] = 190 and 154.5, for 33-mer and P1 respectively, $p < 0.001$).

These findings are further corroborated by the fact that different cultivars had the highest/lowest contents for each harvest year. Regarding the 33-mer, the minima (86.0 μg/g, 117.6 μg/g, 79.6 μg/g) were observed for samples 34, 10 and 10 for the harvest years 2015, 2016 and 2017, respectively, whereas the maxima were found for samples 8 (541.9 μg/g), 50 (634.6 μg/g) and 54 (445.3 μg/g). Interestingly, the maxima of P1 were detected for the same samples as for the 33-mer. We observed maximal values of 235.4 μg/g (sample 8), 197.9 μg/g (sample 50) and 188.7 μg/g (sample 54) and minimal values for sample 34 (84.8 μg/g), sample 57 (71.1 μg/g) and sample 51 (55.4 μg/g) for the harvest years 2015, 2016 and 2017, respectively.

Our results are in agreement with Schalk et al. (2017), who demonstrated a significant influence of the harvest year on the contents of the 33-mer, based on the analysis of four wheat cultivars from three different harvest years. Prandi et al. (2014) on the other hand, found that the genotype affected the contents of the DQ2.5-glia-α1a peptide in durum wheat species more than environmental factors. Boukid et al. (2017) showed that modern genotypes appear to be less affected by

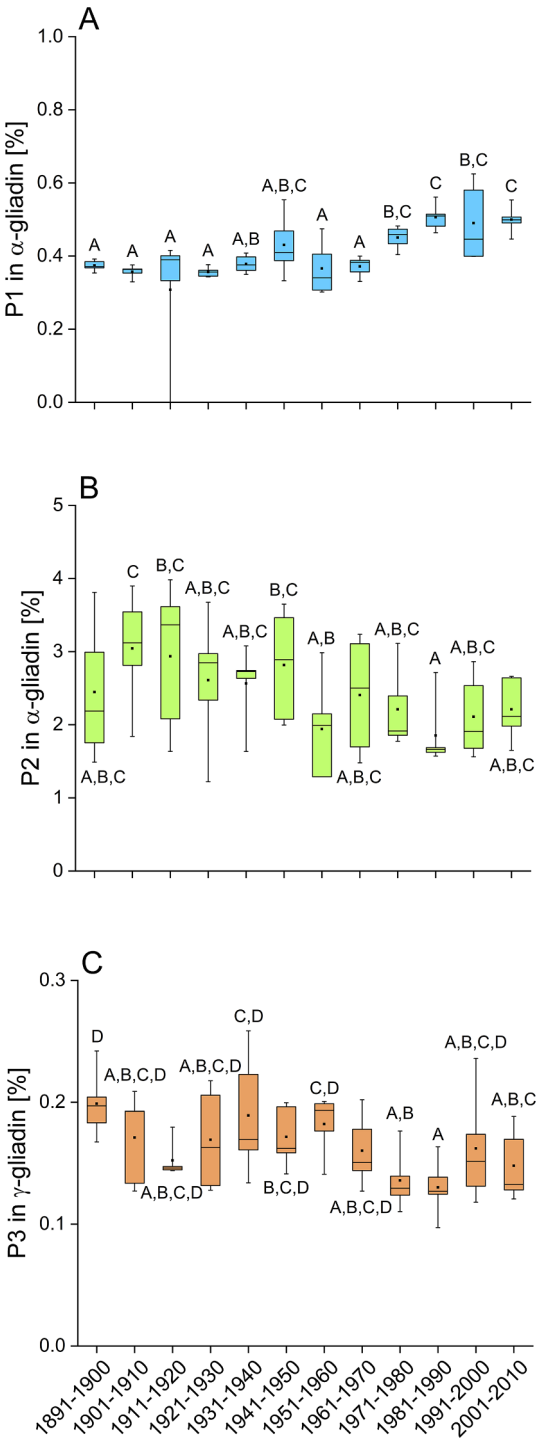

Fig. 2. Proportions [%] of P1 based on α-gliadins (A), of *P*2 based on α-gliadins (B) and of *P*3 based on γ-gliadins in five cultivars per decade (C) averaged over three harvest years 2015–2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box) and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA, Tukey's test, $p < 0.05$).

environmental changes and this is in line with our results for P1 in the present study. The same research group further identified a greater impact of the cultivar on the content of CD-active peptides in Tunisian durum wheat cultivars, which is in contrast to our results. However, these discrepancies in the findings may be caused by different environmental conditions and the fact that we studied common wheat, but no durum wheat cultivars.

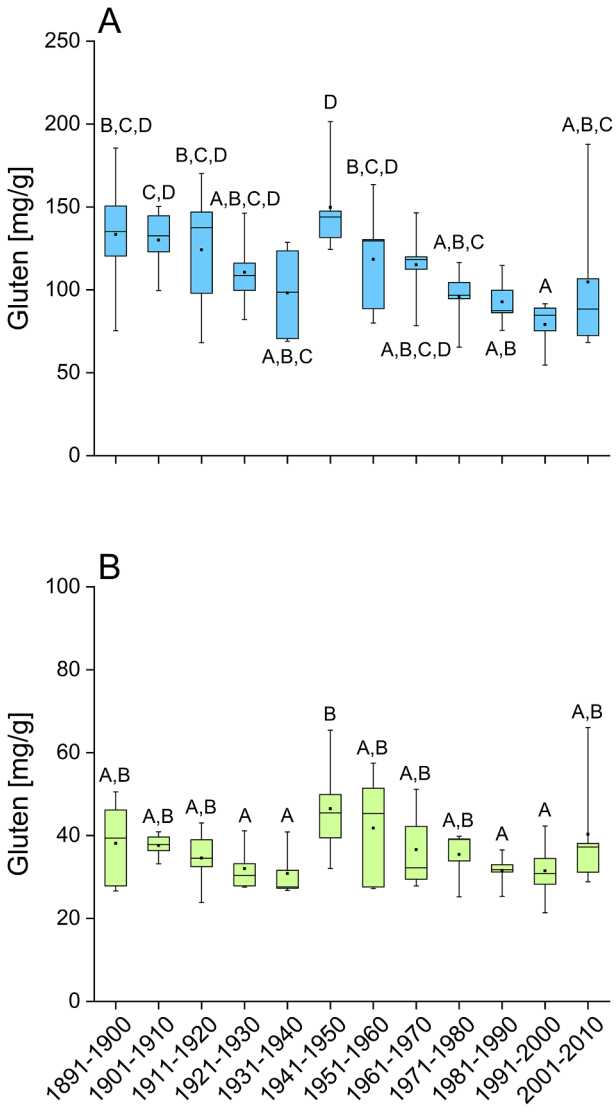

Fig. 3. Gluten contents determined by R5 (A) and G12 (B) ELISA test kits in five cultivars per decade as an average over three harvest years 2015–2017. Boxes represent the interquartile range with the median (line in the box) and mean (point in the box) and whiskers represent the minima and maxima. Different capital letters designate significant differences between the decades (one-way ANOVA, Tukey's test, $p < 0.05$).

3.4. Reactivity of common wheat cultivars with ELISA test kits

The gliadin and gluten contents of the 60 common wheat flours from three different harvest years were analyzed with two different sandwich ELISA test kits, namely R5 and G12 (Supplementary Table S4). The antibodies used in these test kits are specific for certain CD-active peptide motifs. The R5 mAb recognizes the pentapeptides QQPFP and QLPFP present in ω1,2-, γ- and α-gliadins, whereas the G12 test kit recognizes QPQLPY and QPQLPF sequences, that occur in α-gliadins (Morón, Bethune et al., 2008; Morón, Cebolla et al., 2008). Usually, the R5 test kit reports the results as gliadin concentrations and the G12 test kit as gluten concentrations. In order to allow a comparison between both test kits, a conversion factor of 2 (2 × gliadins = gluten) is used, even though this procedure has been questioned (Scherf, 2016; Wieser & Koehler, 2009). This is why we compared results by multiplying the R5 gliadin concentrations with the factor 2 and also with the actual gliadin-to-glutenin ratios (Pronin et al., 2020) and correlation of either value with the gluten contents determined by RP-HPLC. We observed medium correlations for both scenarios ($r = 0.745$ and $r = 0.685$,

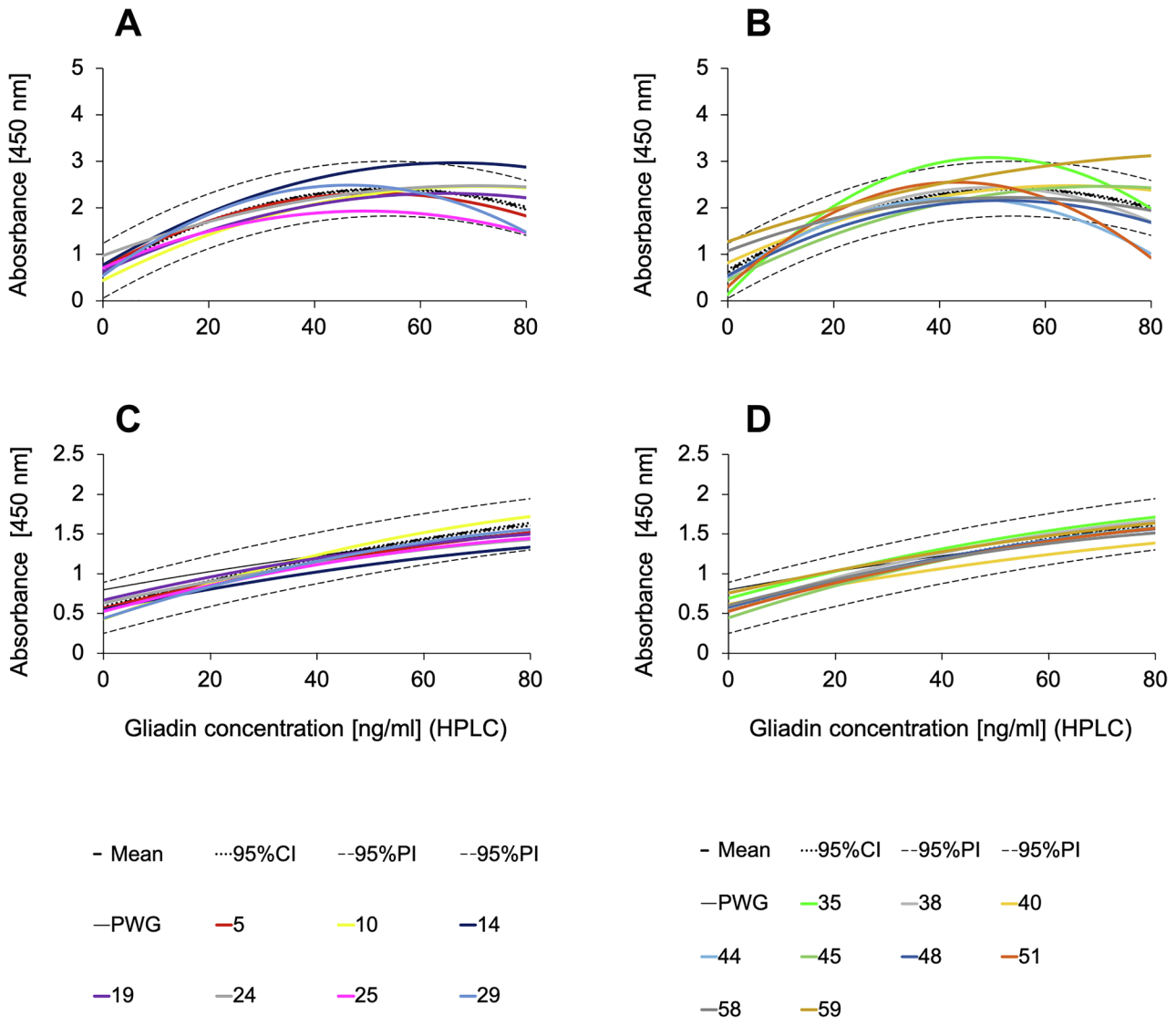

Fig. 4. Array of quadratic fits for ELISA absorbances (λ = 450 nm) as a function of the concentrations quantitated by RP-HPLC including overall mean, 95% confidence bands (CI) and 95% prediction bands (PI) as well as the PWG-gliadin reference material. Gliadin fractions from old wheat cultivars 5, 10, 14, 19, 24, 25 and 29 by R5 ELISA (A), from modern wheat cultivars 35, 38, 40, 44, 45, 48, 51, 58 and 59 by R5 ELISA (B), from the same old wheat cultivars by G12 ELISA (C), and from the same modern wheat cultivars by G12 ELISA (D).

respectively, $p < 0.001$), but the conversion factor 2 had a higher correlation coefficient (Supplementary Fig. S8).

Correlation analyses revealed no correlation between gluten contents determined by R5 ELISA and ω5-gliadin contents ($r = 0.47$, $p < 0.001$), but a strong correlation with ω1,2-gliadins ($r = 0.824$, $p < 0.001$) and medium correlations with α- and γ-gliadins ($r = 0.667$ and $r = 0.685$, respectively, $p < 0.001$). Concerning the G12 ELISA, the gluten contents were strongly correlated with the ω1,2-gliadins ($r = 0.80$, $p < 0.001$), weakly correlated with α-gliadins ($r = 0.660$ and $r = 0.548$, respectively, $p < 0.001$), but not with the ω5- and γ-gliadins ($r = 0.48$ and $r = 0.44$, respectively, $p < 0.001$). Interestingly, no correlation was observed between the gluten contents determined by G12 ELISA and the contents of 33-mer, although this mAb was raised against the 33-mer peptide ($r = 0.47$, $p < 0.001$)

In contrast to the R5 test kit, the G12 test kit showed significantly lower gluten contents, because of different gluten protein extraction procedures and characteristics towards detectable peptide motifs. As expected, both test kits showed an overestimation of the gluten contents (Hajas et al., 2017). The absolute values for both test kits are presented in Fig. 3 as an average over three harvest years and additionally for each harvest year in Supplementary Figs. S9 and S10. For both test kits, the median values were highly variable over the 120 years and also within the decades. Considering the average of the three harvest years, we found no clear trend in gluten contents irrespective of the test kit used and this is in agreement with our previous findings using RP-HPLC (Pronin et al., 2020).

3.5. Reactivity of gliadin protein fractions and protein types between ELISA test kits

To investigate the differences between gliadin protein fractions of 16 selected wheat cultivars, the total gliadin concentrations determined by RP-HPLC were plotted against the ELISA absorbances of the five serial dilutions (Fig. 4). In the specified range, the R5 test kit showed higher absorption values overall compared to the G12 test kit. One modern cultivar (sample 59) showed the highest response to the R5 mAb due to a linear increase after a strong response at low concentrations. The old (sample 14) and the modern (sample 35) cultivars showed weak R5 ELISA responses for small concentrations, but had significantly higher absorptions at their maxima, especially compared

to the old cultivar (sample 25). However, the differences in the overall absorption values between old and modern cultivars were not significant and subsequently no clear distinction could be made.

The G12 test kit did not allow any distinctions between the old and modern gliadin fractions isolated from 16 common wheat cultivars, because the absorption values for all cultivars were nearly equal. These results are in accordance with previous studies performed by Schopf et al. (2018), who did not find any tendencies for R5 or G12. We performed the same experiments for isolated ω5-, ω1,2-, α- and γ-gliadins from one old (sample 10) and one modern cultivar (sample 51), but essentially found no differences between the old and modern cultivars when looking at the same gliadin type (data not shown). Further work could focus on the relations between the presence of CD epitope sequences in the genetic information, their expression levels based on quantitative PCR analysis of cDNA and the final protein/peptide levels (Dubois, Bertin, Hautier, Muhovski, Escarnot & Mingeot, 2018).

4. Conclusions

To test the hypothesis that wheat breeding may have contributed to increasing the immunostimulatory potential of modern wheats compared to old wheats and landraces, we quantitated four CD-active peptides by LC-MS/MS and gluten contents by ELISA in a well-characterized set of 60 German common winter wheat cultivars from 1891 to 2010. We found no clear trend for gluten contents over the 120-year period studied. Regarding the CD-active peptides, we demonstrated a high variability of the contents of immunogenic peptides, both over 60 different cultivars and within the five cultivars per decade, primarily affected by the harvest year and less by the cultivar. The 33-mer and P1 peptides showed a tendency to increase over breeding, but only when looking at the proportions of peptides relative to the respective protein type, the α-gliadins, not when looking at the absolute contents. No trends in relative or absolute values were observed for the P2 and P3 peptides present in α- and γ-gliadins. Taken together, our results suggest that the contents of some CD-active peptides may have changed over the course of breeding, but that the effect of different harvest years is much more important compared to the genetic effect, because we identified both old and modern cultivars containing high and low contents of CD-active peptides, respectively.

CRediT authorship contribution statement

Darina Pronin: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. **Andreas Börner:** Conceptualization, Investigation, Project administration, Resources, Writing - review & editing. **Katharina Anne Scherf:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127952.

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