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Investigation of the effects of sample preparation on gluten quantitation in rye and barley flours

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ABSTRACT

Proper gluten quantitation is essential for providing safe gluten-free food for patients living with celiac disease (CD). However, gluten quantitation faces several challenges: the lack of a reference method and certified reference materials, the variability of methods and the effects of genetic and environmental factors on gluten. Among all these challenges our research group focuses on gluten reference material development. Gluten content is determined by enzyme linked immunosorbent assay (ELISA) methods to obtain comparable data for the selection of cultivars used in our reference material development efforts. As ELISA methods are developed for determining low gluten concentrations, application for these special research purposes requires a 10,000-fold dilution. The formerly performed process was a post-extraction liquid dilution that proved to be sufficient for wheat samples. However, gluten contents of rye and barley samples were found to be overestimated by ELISA methods. One of the suggested reasons is the structural and solubility changes of gluten proteins during the dilution process. Therefore, our present study focuses on the comparison of the original dilution method and a revised version using solid-phase dilution in a gluten-free matrix.

KEYWORDS

ELISA, sample preparation, high gluten content

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1. INTRODUCTION

Celiac disease (CD) is an autoimmune condition triggered by dietary gluten in genetically predisposed individuals. In CD, the consumption of gluten generates an abnormal immune response that leads to inflammation of the upper small intestine, villous atrophy, and decreased absorption of essential nutrients and vitamins (Holmes, 2022). With the prevalence of around 1%, CD is one of the main food related hypersensitivities (Makharia et al., 2022). To date, the only effective treatment of CD is a strict lifelong gluten free diet (Meresse et al., 2009; Ribeiro et al., 2018; Hoilat et al., 2022).

In Europe, the Commission Implementing Regulation (EU) No. 828/2014, in line with the recommendation of Codex Alimentarius (Codex Standard 118-1979, 2015), declares that "The statement 'gluten-free' may only be made where the food as sold to the final consumer contains no more than 20 mg kg^{-1} of gluten".

To provide safe gluten-free food, proper analytical methods are needed to ensure compliance to the 20 mg kg^{-1} threshold. While there are no reference methods for gluten analysis, the Codex Alimentarius recommends the enzyme-linked immunosorbent assay (ELISA) using the R5 antibody, but any other with similar performance parameters is acceptable. However, these immunoanalytical methods have various limitations. The lack of a reference method leads to the use of various analytical solutions for gluten determination, and the results often show significant differences. These uncertainties are caused either by the variations in the methodology or by the complexity of the gluten proteins (Bugyi et al., 2013; Lexhaller et al., 2016). The most significant methodological differences are the use of antibodies with varying specificities, differences in the extraction protocols or in the calibrating materials due to the lack of a proper reference material. As gluten is a group of complex proteins including many different structures, proper quantification is challenging. Moreover, the genetic and environmental variability also has a huge impact on the number of detectable epitopes (Lester, 2008; Scherf and Poms, 2016; Juhász et al., 2020; Xhaferaj et al., 2020; Scherf et al., 2021; Makharia et al., 2022).

The lack of a certified reference material is one of the most critical points of determining the gluten content of foods. Currently the available standard materials, e.g. PWG gliadin (van Eckert et al., 2006, 2010), and a reference candidate wheat flour mixture (Hajas et al., 2018; Schall et al., 2020a, 2020b) are based on wheat proteins, which sets further difficulties for gluten determination in rye and barley as the ELISA antibodies bind their proteins with different affinities. Therefore, rye and barley should also be included in the reference material development (Lester, 2008; Tanner et al., 2013; Lexhaller et al., 2017; Huang et al., 2020; Xhaferaj et al., 2020; Makharia et al., 2022).

Commercially available gluten ELISA kits are dedicated to determining gluten levels under 200 mg kg^{-1}, however, these methods are also applicable in research to obtain information on differences in gluten content of cereal cultivars. As these methods are optimised for determining gluten around 20 mg kg^{-1}, proper measurement of gluten in samples with very high gluten content, such as wheat, rye, or barley flours, require a notable dilution. According to former studies and our pre-experiments, a 10,000-fold dilution appeared to be suitable for getting the gluten concentrations of the flour extracts into the ELISA calibration range (Schall et al., 2019). In case of the wheat samples, this dilution step was obtained after the extraction of the gluten proteins, however, in rye and barley samples increase in variances of results and overestimation of gluten content was observed (Lexhaller et al., 2017). The applied dilution method might result

in changes of the protein solubility and structures and potential formation of neoepitopes, which might be one reason of this variability and overestimation. Therefore, the former dilution method was revised, and rye and barley flours were diluted before the extraction with a gluten-free matrix in solid phase.

Our current study focuses on the optimisation of the ELISA sample preparation for these special high gluten protein content rye and barley samples to make it possible to include ELISA methods for gluten reference material development in case of these cereals as well.

2. MATERIALS AND METHODS

2.1. Rye and barley samples

Rye (Reformer (Germany), Hazlet (Canada), and Dankowskie-Diament (grown in Hungary)) and barley (H38 (Hungary), Copeland (Canada), and Daishi-Mochi (grown in Hungary)) cultivars were selected based on our former experiments to include samples with similar and different gluten content as well. Grains were milled (FQC 109, Metefém, Budapest, Hungary) and three characteristic size fractions (<150 μm (S1), 150–250 μm (S2), and >250 μm (S3)) were separated by sieving (micro sieve, Metefém, Budapest, Hungary).

Homogenisation of samples diluted in a gluten free (GF) brown rice flour matrix was obtained manually by two consecutive 100-fold dilutions in a laboratory mortar. According to our pre-experiments, the S1 fractions were more suitable for obtaining homogenous solid diluted samples than whole flours since the particle size distribution of the S1 fraction is more similar to that of the GF matrix, however, gluten content of whole flours should be considered as the relevant data. Homogeneity was tested with parallel extractions ($n = 10$ in case of one rye and one barley cultivar and $n = 5$ for the other samples). Gluten content of the three size fractions were compared for all samples to examine whether whole flours can be represented by the S1 fractions.

2.2. Experimental design

Gluten contents of the S1 size fractions were measured i) directly with post-extraction 10,000-fold liquid dilution and ii) after 10,000-fold dilution in gluten-free matrix. The S1 fraction was selected for the solid dilution to improve homogeneity. The S2 and S3 fractions were analysed with the original post-extraction liquid dilution.

Gluten contents of the size fractions were compared for checking whether the S1 fractions could represent the gluten content of the whole flour, and homogeneity of the 10,000-fold solid phase dilution was also tested. Analyses of the ELISA measurements were carried out in triplicates of each independent extraction.

2.3. Gluten quantitation with ELISA

Samples were analysed by an R5 gluten ELISA test kit (RIDASCREEN Gliadin Assay (R7001, R-Biopharm, Darmstadt, Germany)). Extraction and measurement of the gluten proteins were carried out according to the manufacturer's instructions. In case of the flour samples that were extracted in their native form, a further 10,000-fold dilution was obtained by a 2-step dilution procedure. The absorbances were determined at 450 nm using a microplate reader (iMarkTM

Microplate Absorbance Reader, Bio-Rad, Hercules, CA, USA). The gluten concentration was calculated from the gliadin concentration values determined by the Bio-Rad Microplate Manager 6 software (Bio-Rad Laboratories Inc., USA) using the cubic spline curve fit.

2.4. Data analysis

Statistical analysis was carried out by *t*-tests for comparison of dilution methods, one-way analysis of variances (ANOVAs) and nested design one-way ANOVAs considering the hierarchic structure of the data for comparison of size fractions and replicate extractions at a confidence level of 0.95 using STATISTICA v12.5 software (StatSoft Inc, Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1. Analysis of the size fractions

No significant differences were found between the fractions considering that ELISA measurement results may have a variance up to 30% (Méndez et al., 2005). This means that the S1 fractions might be used as a representative for the whole flours. The results also show that the difference between the individual extractions were significant in most cases, therefore, this factor dominates the overall variances (online supplement).

3.2. Homogeneity of the GF matrix diluted samples

Solid phase dilutions of the rye and the barley S1 fractions were tested for homogeneity (Fig. 1). According to the one-way ANOVA results, no significant differences were detected in case of the rye samples ($P = 0.1213$, $F = 1.8475$, $F_{crit} = 2.3928$), however, barley samples showed significant variance ($P = 1.7582\text{E}{-13}$, $F = 104.1359$, $F_{crit} = 2.5102$). Gluten free brown rice (blank) samples were also tested, and gluten content was around the declared LOQ value (5 mg kg^{-1}) of the kit.

Variability of the solid and the liquid phase diluted samples were analysed, and calculated RSD% values (Table 1) showed similar variances in both extraction methods in rye samples. In contrast, solid phase diluted barley samples were found to have higher RSD% values than the same samples diluted in liquid phase. This, with the low well-to-well standard deviations, suggests that the extraction step may affect the repeatability of the method more in barley than in rye samples. The same was observed after repeated homogenisation processes. According to the results, homogenous mixture was obtained from the rye samples, however, barleys were not fully suitable for the applied solid dilution sample preparation.

3.3. Comparison of the sample preparation methods

The measured gluten contents of the liquid and solid phase diluted samples (Fig. 2) were compared with *t*-tests (Table 1). The measured gluten content of Reformer and Dankowskie-Diament were found to be significantly higher after solid phase dilution, and for Hazlet no significant difference was detected. During extraction of liquid diluted rye samples an aggregation was observed in the extraction tubes, which might be the reason for the lower gluten contents, possibly due to the reduced extractability of these samples.

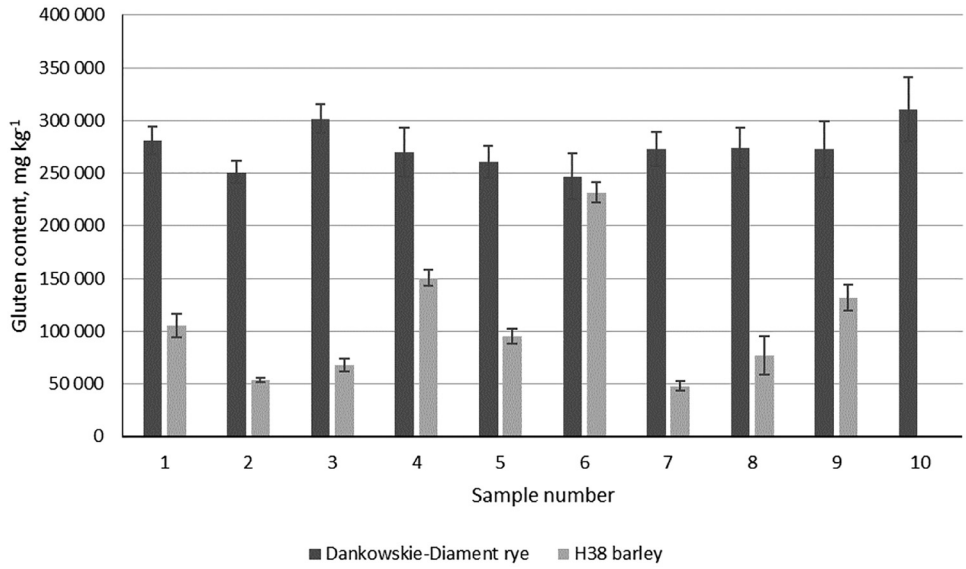

Fig. 1. Homogeneity of Dankowskie-Diament rye and H38 barley S1 fraction samples in solid phase dilution

Table 1. Results and comparison of solid and liquid phase dilution methods. Confidence level for the *t*-test was 95%. RSD% (relative standard deviation) values were compared to the ELISA overall variability of 30% (Mendéz et al., 2005)

	Gluten content, solid (mg kg^{-1})	RSD% solid phase dilutions (n = 5)	Gluten content, liquid (mg kg^{-1})	RSD% liquid phase dilutions (n = 3)	P (α = 0.05)
			Rye		
Reformer	191,023 ± 43,296	22.67	140,667 ± 29,364	20.87	7.35E–03
Hazlet	231,368 ± 30,746	13.29	250,382 ± 43,744	17.47	2.24E–01
Dankowskie-Diament	278,160 ± 32,695	11.75	168,058 ± 21,091	12.55	1.93E–11
			Barley		
H38	106,832 ± 56,368	52.76	142,394 ± 39,054	27.43	8.92E–02
Copeland	125,711 ± 36,601	29.12	251,672 ± 39,888	15.85	1.97E–06
Daishi-Mochi	195,329 ± 61,144	31.30	516,624 ± 63,267	12.25	2.45E–11

In case of barleys, the variances of the solid phase diluted samples were significantly higher than the variances observed after the liquid phase dilution method, therefore, only limited conclusions can be drawn. The gluten content of the liquid phase diluted samples in Copeland and Daishi-Mochi was found to be about 2 and 2.5-times the gluten content of the solid phase

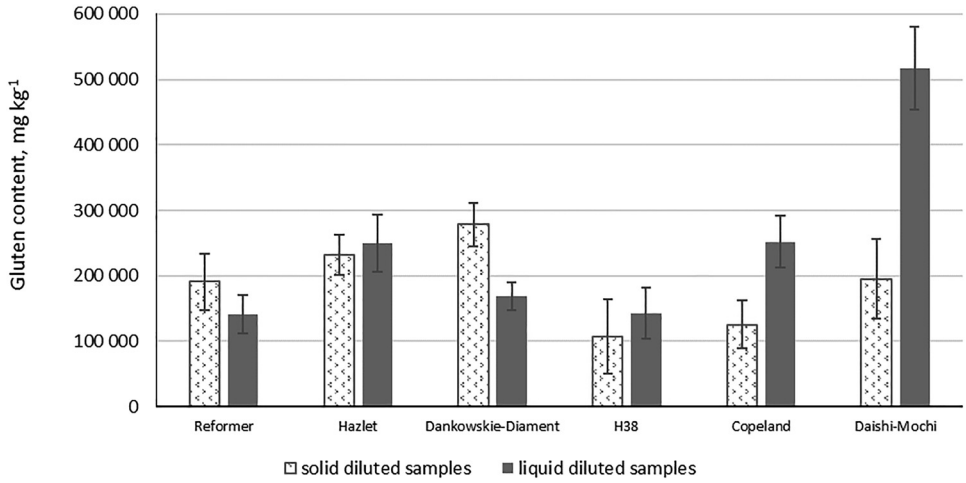

Fig. 2. Gluten content of solid and liquid diluted rye and barley samples

diluted samples, respectively. In case of H38 barley, no significant difference could be detected due to the high variance of the solid phase diluted samples. Unlike the results of ryes, this difference cannot be explained with any visually detectable event during the sample preparation; however, one possible reason could be the formation of neoepitopes during the dilution process due to some structural changes in the gluten proteins. Similar results were reported by Kanerva et al. (2006) for barley contaminated oat samples.

4. CONCLUSIONS

The R5 ELISA methods are optimised for determining gluten around the 20 mg kg^{-1} threshold, however, determination of gluten content in high gluten containing cereal flour samples, requiring a notable dilution, may also be important for research purposes like gluten reference material development. In our present study, the liquid phase dilution formerly used in wheat-based reference material developments was revised and the rye and barley flours were also diluted with a gluten-free matrix in solid phase before the extraction, and the two methods were compared.

According to our results, the measured values highly depend on the cereal matrix: in case of rye samples the solid, in case of barley samples the liquid dilution method resulted in higher measured gluten contents. However, with the use of the same method, different cultivars still compare the same way, therefore, both methods are applicable for describing the tendencies of differences between the samples, that is essential for cultivar selection in reference material development.

The proper explanation of our findings needs to be further examined; however, it might be suggested that the changes in protein structure and solubility during the sample preparation steps have a greater effect on the results than it was observed in wheat samples (Schall et al., 2019). The improvement of the sample preparation method for high gluten content samples

assists the proper determination of gluten in those samples examined for determining the genetic and environmental differences between rye and barley cultivars, selection of appropriate varieties and after all, gluten reference material development. With a proper dilution process, the ELISA measurements can be used as a rapid high-throughput method for examining and comparing high gluten containing cereal samples.

Conflict of interest: The 2nd author, S. Tömösközi is a member of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and he did not take part in the review process in any capacity.

SUPPLEMENTARY MATERIALS

Supplementary data to this article can be found online at https://doi.org/10.1556/066.2022.00177.

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REFERENCES

Bugyi, Zs., Török, K., Hajas, L., Adonyi, Z., Popping, B., and Tömösközi, S. (2013). Comparative study of commercially available gluten ELISA kits using an incurred reference material. *Quality Assurance and Safety of Crops & Foods*, 5(1): 79–87.

Codex Standard 118–1979, (2015). *Codex standard for foods for special dietary use for persons intolerant to gluten*. Codex Alimentarius Commission. Revision 1, Amendment 2.

EU (2014): Regulation No 828/2014 of the European Parliament and of the Council. *OJ*, 228: 5–8.

Hajas, L., Scherf, K.A., Török, K., Bugyi, Z., Schall, E., Poms, R.E., Koehler, P., and Tömösközi, S. (2018). Variation in protein composition among wheat (*Triticum aestivum* L.) cultivars to identify cultivars suitable as reference material for wheat gluten analysis. *Food Chemistry*, 267: 387–394.

Hoilat, G.J., Altowairqi, A.K., Ayas, M.F., Alhaddab, N.T., Alnujaidi, R.A., Alharbi, H.A., Alyahyawi, N., Kamal, A., Alhabeeb, H., Albazee, E., Almustanyir, S., and Abu-Zaid, A. (2022). Larazotide acetate for treatment of celiac disease: a systematic review and meta-analysis of randomized controlled trials. *Clinics and Research in Hepatology and Gastroenterology*, 46(1): 101782, https://doi.org/10.1016/j.clinre.2021.101782.

Holmes, G.K.T. (2022). Chapter 2 - gluten-related disorders: an evolving story. In: Rostami-Nejad, M. (Ed.), *Gluten-related disorders*. Academic Press, pp. 7–32.

Huang, X., Ma, K., Leinonen, S., and Sontag-Strohm, T. (2020). Barley C-hordein as the calibrant for wheat gluten quantification. *Foods*, 9(11): 1637, https://doi.org/10.3390/foods9111637.

Juhász, A., Colgrave, M.L., and Howitt, C.A. (2020). Developing gluten-free cereals and the role of proteomics in product safety. *Journal of Cereal Science*, 93: 102932, https://doi.org/10.1016/j.jcs.2020.102932.

Kanerva, P.M., Sontag-Strom, T.S., Ryöppy, P.H., Alho-Lehto, P., and Salovaara, H.O. (2006). Analysis of barley contamination in oats using R5 and ω-gliadin antibodies. *Journal of Cereal Science*, 44(3): 347–352.

Lester, D.R. (2008). Gluten measurement and its relationship to food toxicity for celiac disease patients. *Plant Methods,* 4: 26, https://doi.org/10.1186/1746-4811-4-26.

Lexhaller, B., Tompos, C., and Scherf, K.A. (2016). Comparative analysis of prolamin and glutelin fractions from wheat, rye, and barley with five sandwich ELISA test kits. *Analytical and Bioanalytical Chemistry*, 408(22): 6093–6104.

Lexhaller, B., Tompos, C., and Scherf, K.A. (2017). Fundamental study on reactivities of gluten protein types from wheat, rye and barley with five sandwich ELISA test kits. *Food Chemistry,* 237: 320–330.

Makharia, G.K., Singh, P., Catassi, C., Sanders, D.S., Leffler, D., Ali, R.A.R., and Bai, J.C. (2022). The global burden of coeliac disease: opportunities and challenges. *Nature Reviews Gastroenterology and Hepatology,* 19: 313–327.

Méndez, E., Vela, C., Immer, U., Janssen, F.W. (2005). Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. *European Journal of Gastroenterology & Hepatology*, 17(10): 1053–1063.

Meresse, B., Ripoche, J., Heyman, M., and Cerf-Bensussan, N. (2009). Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. *Mucosal Immunology*, 2(1): 8–23.

Ribeiro, M., Nunes, F.M., Rodriguez-Quijano, M., Carrillo, J.M., Branlard, G., and Igrejas, G. (2018). Next-generation therapies for celiac disease: the gluten-targeted approaches. *Trends in Food Science & Technology*, 75: 56–71.

Schall, E., Bugyi, Z., Hajas, L., Török, K., and Tömösközi, S. (2019). Applicability of ELISA methods for high gluten-containing samples. *Acta Alimentaria*, 48: 365–374.

Schall, E., Scherf, K.A., Bugyi, Z., Hajas, L., Török, K., Koehler, P., Poms, R.E., D'Amico, S., Schoenlechner, R., and Tömösközi, S. (2020a). Characterisation and comparison of selected wheat (*Triticum aestivum* L.) cultivars and their blends to develop a gluten reference material. *Food Chemistry*, 313: 126049, https://doi.org/10.1016/j.foodchem.2019.126049.

Schall, E., Scherf, K.A., Bugyi, Z., Török, K., Koehler, P., Schoenlechner, R., and Tömösközi, S. (2020b). Further steps toward the development of gluten reference materials – wheat flours or protein isolates? *Frontiers in Plant Science*, 11: 906, https://doi.org/10.3389/fpls.2020.00906.

Scherf, K.A., Catassi, C., Chirdo, F.G., Ciclitira, P.J., Feighery, C.F., Gianfrani, C., Koning, F., Lundin, K.E.A., Masci, S., Schuppan, D., Smulders, M.J.M., Tranquet, O., Troncone, R., and Koehler, P. (2021). Statement of the Prolamin Working Group on the determination of gluten in fermented foods containing partially hydrolyzed gluten. *Frontiers in Nutrition*, 7: 626712, https://doi.org/10.3389/fnut.2020.626712.

Scherf, K.A. and Poms, R.E. (2016). Recent developments in analytical methods for tracing gluten. *Journal of Cereal Science,* 67: 112–122.

Tanner, G.J., Blundell, M.J., Colgrave, M.L., and Howitt, C.A. (2013). Quantification of hordeins by ELISA: the correct standard makes a magnitude of difference. *Plos One* 8(2): e56456, https://doi.org/10.1371/journal.pone.0056456.

van Eckert, R., Berghofer, E., Ciclitira, P.J., Chirdo, F., Denery-Papini, S., Ellis, H.J., Ferranti, P., Goodwin, P., Immer, U., Mamone, G., Méndez, E., Mothes, T., Novalin, S., Osman, A., Rumbo, M., Stern, M., Thorell, L., Whim, A., and Wieser, H. (2006). Towards a new gliadin reference material–isolation and characterisation. *Journal of Cereal Science*, 43(3): 331–341.

van Eckert, R., Bond, J., Rawson, P., Klein, Ch.L., Stern, M., and Jordan, T.W. (2010). Reactivity of gluten detecting monoclonal antibodies to a gliadin reference material. *Journal of Cereal Science*, 51(2): 198–204.

Xhaferaj, M., Alves, T.O., Ferreira, M.S.L., and Scherf, K.A., (2020). Recent progress in analytical method development to ensure the safety of gluten-free foods for celiac disease patients. *Journal of Cereal Science*, 96: 103114, https://doi.org/10.1016/j.jcs.2020.103114.