

Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid?

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Abstract Presently, the only essential therapy of celiac disease (CD) is the permanent strict withdrawal of gluten from the diet. With respect to gluten containing foods from wheat, rye, barley, and oats, CD patients have to consume surrogates that must be gluten-free according to the “Codex Alimentarius Standard for Gluten-Free Foods”. The recent “Draft Revised Standard” proposes a gluten threshold of 20 mg per kg gluten-free product. For gluten quantitation, the alcohol-soluble prolamins should be extracted and analyzed by an immunochemical method; the amount of gluten is calculated by multiplying the prolamin content by the factor of 2. To investigate, whether this calculation is valid in any case of contamination of gluten-free products by wheat, rye, barley, and oats, wholemeal or white flours from common wheat, spelt, durum wheat, kamut, emmer, einkorn, rye, barley and oats were analyzed for the ratio of prolamins to glutenins (PROL/GLUT) by a combination of extraction and reversed-phase HPLC procedures. Additionally, different industrial wheat starches were analyzed for their prolamin and total gluten content using different extraction and concentration steps followed by gel permeation HPLC. The results for the cereal flours revealed that the ratio PROL/GLUT was generally higher than 1.0 as proposed by the “Draft Revised Standard” and strongly influenced by cereal species and variety. Common wheat

showed the lowest ratio (1.5–3.1), followed by oats and spelt (1.7–3.3), barley (1.4–5.0), durum wheat and kamut (3.1–3.4), emmer (3.5–7.6), rye (6.3–8.2), and einkorn (4.0–13.9). In any case, the gluten content of gluten-free products contaminated with CD activating cereals was generally overestimated, when the prolamin content was multiplied by the factor of 2. In extreme cases, e.g., contamination with rye, the overestimation amounted to 72–79%. Completely different PROL/GLUT ratios were found in ten commercial wheat starches ranging from 0.2 to 4.9. Obviously, the quality of wheat cultivars used for starch production and/or different process parameters, e.g., washing steps, influenced the composition of gluten proteins adherent to starch granules. For wheat starch, the calculation of the gluten content by $2 \times \text{PROL}$ may either lead to underestimation (–71% at most) or overestimation (+66% at most). In conclusion, this calculation is invalid; therefore, a future task will be the development of immunoassays with antibodies against all types of storage proteins from wheat, rye, barley, and oats.

Keywords Cereal flour · Wheat starch · Prolamins · Glutelins · Gluten content

Abbreviations

CD	Celiac disease
ELISA	Enzyme-linked immunosorbent assay
GLUT	Glutelins
GP	Gel permeation
HPLC	High-performance liquid chromatography
M_r	Molecular mass
PROL	Prolamins
RP	Reversed-phase
RT	Room temperature
SDS	Sodium dodecylsulfate

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Introduction

Celiac disease (CD) is one of the most frequent permanent food intolerances worldwide. The prevalence of CD in Europe is estimated to be in a range of 1:100–300. CD is an inflammatory disease of the upper small intestine characterized by a flat mucosa with the absence of normal villi. Typical symptoms are intestinal features such as abdominal distension, chronic diarrhoea and steatorrhea as well as features resulting from malabsorption such as deficiency of minerals and vitamins (failure to thrive, anaemia, osteoporosis, infertility, and neurological abnormalities). CD is triggered in genetically susceptible individuals by the ingestion of wheat, rye, barley, and possibly oats products. The precipitating factors are the storage proteins of these cereals, which have been termed “gluten” in the field of CD. According to their solubility in aqueous alcohols they have been divided into the soluble prolamins (PROL) and the insoluble glutelins (GLUT) [1]. The current essential therapy of CD is a strict adherence to gluten-free diet, which means a permanent withdrawal of gluten from daily aliment. CD patients have to consume dietetic foods, mostly substitutes of those products that contain wheat, rye, barley and oats constituents such as bread, other baked products, breakfast cereals, pasta and beer. Common raw materials for the production of these surrogates are, for example, corn, rice, sorghum, buckwheat or amaranth. Dietetic gluten-free foods underlie the regulations of the “Codex Alimentarius Standard for Gluten-Free Foods”. This standard was established in 1981, however, without having a method to determine gluten [2]. Since that time, a revision of this standard has been underway. The most recent “Draft Revised Standard” edited in 2008 proposes a threshold level of 20 mg gluten per kg of the original product [3]. For gluten determination, PROL should be extracted with about 40–70% ethanol and quantified by means of an immunochemical method, for which the R5-ELISA developed by Valdes et al. [4] is recommended. According to the “Draft Revised Standard”, the PROL content is generally taken as 50% of gluten so that the gluten content has to be calculated by multiplying the PROL content by a factor of 2. The objective of the present study was to investigate, whether this calculation is valid in any case of contamination of gluten-free products by wheat, rye, barley as well as oats and in the case of wheat starches.

Materials and methods

Flours and starches

The origin of grains and the production of flours from common wheat, spelt, durum wheat and rye are described in

previous papers [5–10]. Common wheat samples were contained in three sets of white flours. Set I included flours from ten European winter wheat cultivars and three North American spring wheat cultivars grown in different countries and a commercial Dutch flour [5]. Set II consisted of thirtytwo flours from thirteen German winter wheat cultivars grown at two German locations and at different levels of nitrogen fertilization [6]. One of these cultivars (Herzog) was a wheat/rye hybrid (1B/1R translocation line). Set III contained eight flours from one German spring wheat cultivar (Star) grown in pots with two different soil compositions at four different levels of sulfur fertilization [7]. Spelt samples comprised 28 cultivars from Germany, Switzerland, and Belgium grown at four different locations in Southern Germany either under conventional or ecological farming [8]. The only sample available for durum wheat was flour from cultivar Biodur [9]. Rye flours were produced from the German cultivars Danko and Halo [10]. Wholemeal flours from 23 einkorn cultivars grown at different German locations were obtained from Dr. Jodlbauer Food Consulting GmbH, Hannover, Germany. Grains from emmer and barley were purchased from the Institute of Agricultural Chemistry, University of Goettingen, Germany. Emmer samples comprised six flours from three cultivars grown at two locations in Northern Germany; one further sample was previously analyzed [9]. Seven naked and one hulled barley cultivars were also grown at two locations in Northern Germany. Additionally, two hulled barley cultivars (Aura, Barke) from unknown origin were included in this study. Grains from eighteen oats cultivars representing South Tyrol landraces were provided by the Centro di Sperimentazione Agraria e Forestale, Laimburg, Italy. Kamut grains were purchased from the market. All grains were milled into wholemeal flours by means of a laboratory mill. Ten samples of wheat starches were obtained from four different European starch companies.

Analysis of flours

The PROL and GLUT content of cereal flours were analyzed by a combination of a modified Osborne fractionation with reversed-phase (RP-) HPLC described previously [11]. Briefly, flours were extracted stepwise with 0.4 mol/L NaCl plus 0.067 mol/L H₂NaPO₄, pH 7.6 (2 × 1 mL) at room temperature (RT ≈ 20 °C), with 60% (v/v) ethanol (3 × 0.5 mL) at RT (PROL), and with 50% (v/v) 1-propanol containing 2 mol/L urea, 0.05 mol/L Tris-HCl, pH 7.5 and 1% (w/v) dithioerythritol (2 × 1.0 mL) at 60 °C under nitrogen (GLUT). After centrifugation the corresponding supernatants were combined and diluted to 2.0 mL with the respective solvent. Aliquots of the PROL and GLUT extracts were quantified by RP-HPLC on C₈ silica gel at

50 °C using a solvent gradient of increasing acetonitrile concentration in the presence of 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.0 mL/min and the detection wavelength was 210 nm. Two determinations (extraction + RP-HPLC) were performed; the average coefficient of variation was $\pm 2.6\%$.

Analysis of wheat starches

About 1 g of wheat starch was suspended in 10 mL of 50% (v/v) 2-propanol containing 0.08 mol/L Tris-HCl, pH 8.0 and 0.5% (w/v) dithioerythritol under nitrogen and shaken for 15 min at RT and then magnetically stirred for 20 min at 60 °C. After standing for 15 min at RT, the sample was centrifuged for 15 min at $8.000 \times g$ and RT. 2×4.0 mL of the supernatants (total extractable proteins) were dried by means of a vacuum centrifuge at 40 °C and redissolved in 0.5 mL of the chromatographic solvent described below. For PROL extraction 1 g of starch was suspended in 10 mL of 60% (v/v) ethanol and shaken for 15 min at RT. After standing for 15 min at RT the sample was centrifuged and 2×4.0 mL of the supernatant were dried as described above and redissolved in 0.5 mL of 60% (v/v) ethanol. Gel permeation (GP-) HPLC of total extractable proteins and PROL, respectively, was performed using a Superdex 200 HR 10/30 column (Supelco 54801-U) at RT. The injection volume for both extracts was 100 μ L. The elution solvent was 1.5% (w/v) sodium dodecylsulfate (SDS) buffered with 62.5 mmol/L Tris-HCl, pH 7.0. The flow rate was 0.6 mL/min and the detection wavelength was 206 nm. Two determinations (extraction + GP-HPLC) were performed; the average coefficient of variation was $\pm 4.6\%$.

Results and discussion

In general, gluten from two different categories may be present in gluten-free products. First, naturally gluten-free foods, for example produced from corn, rice or buckwheat, could be contaminated by wheat, rye, barley or oats. Secondly, wheat starch used as an additive for gluten-free foods may contain adherent gluten. The “Draft Revised Codex Standard” recommends an immunochemical method (ELISA) for gluten quantitation [3]. However, commercially available ELISA-kits only exist for the determination of PROL of wheat, rye, and barley, but not for PROL of oats, and, not at all, for GLUT of CD toxic cereals. Therefore, HPLC in combination with UV detection was used for the quantitation of PROL and GLUT fractions or total gluten protein. Previous studies have shown that the HPLC absorbance measured at 210 nm is highly correlated with the amount of protein [11].

Studies on cereal flours

Wholemeal or white flours from all CD toxic cereals (wheat, rye, barley, oats) were investigated in this study. With respect to wheat all well-established species were analyzed: the hexaploid common wheat and spelt, the tetraploid durum wheat, kamut and emmer, and the diploid einkorn. Flour proteins were fractionated according to a modified Osborne procedure into albumins and globulins, PROL and GLUT. PROL and GLUT were then quantified by RP-HPLC on C_8 silica gel. The results for common wheat, spelt, durum wheat, and rye were adopted from previous studies [5–10]. Table 1 summarizes the number of analyzed samples, the ratio of PROL/GLUT, the real factor for the calculation of gluten content via the PROL content and the percentage of error in comparison to the calculation $2 \times \text{PROL} = \text{gluten}$. Figure 1 gives an overview of the distribution of the individual ratios within each sample group. Fifty-four cultivars of common wheat revealed a relatively broad range of PROL/GLUT ratios from 1.50 to 3.14. Cultivars with soft gluten and poor baking quality showed a tendency to have the highest ratios [5]. The intensity of fertilization, too, influenced the ratio: wheat with low N-supply had lower ratios [6], whereas S-deficiency provoked higher ratios [7]. The ratio for the wheat/rye hybrid Herzog was within the limits of the other common wheat samples [6]. Altogether, the real factors for the calculation of gluten via PROL were in a range from 1.32 to 1.66 (Table 1). The calculation of gluten by the formula $2 \times \text{PROL}$ would lead to a considerable overestimation of up to +52%. The PROL/GLUT ratio of twenty-eight spelt cultivars was between 1.73 and 3.32, on an average a little bit higher than those of common wheat. The maximum overestimation of gluten by $2 \times \text{PROL}$ would amount to +54%.

Only one sample was available for durum wheat and kamut, respectively. The PROL/GLUT ratios (3.10, 3.39) and the overestimation of gluten by $2 \times \text{PROL}$ (+50, +55%) were comparable. The seven emmer cultivars showed great differences in their PROL/GLUT ratios (3.51–7.55), and the maximum error for gluten calculation via $2 \times \text{PROL}$ would amount to as much as +77%. Twenty-four einkorn samples revealed a range of PROL/GLUT ratios from 4.04 to 13.86, and thus, the highest values on an average. Figure 1 reflects the great differences between single cultivars. In some samples, the proportion of PROL was more than 90% and that of GLUT less than 10% of total gluten proteins, and the error based on the calculation $2 \times \text{PROL}$ reached 87% of overestimation of the gluten content.

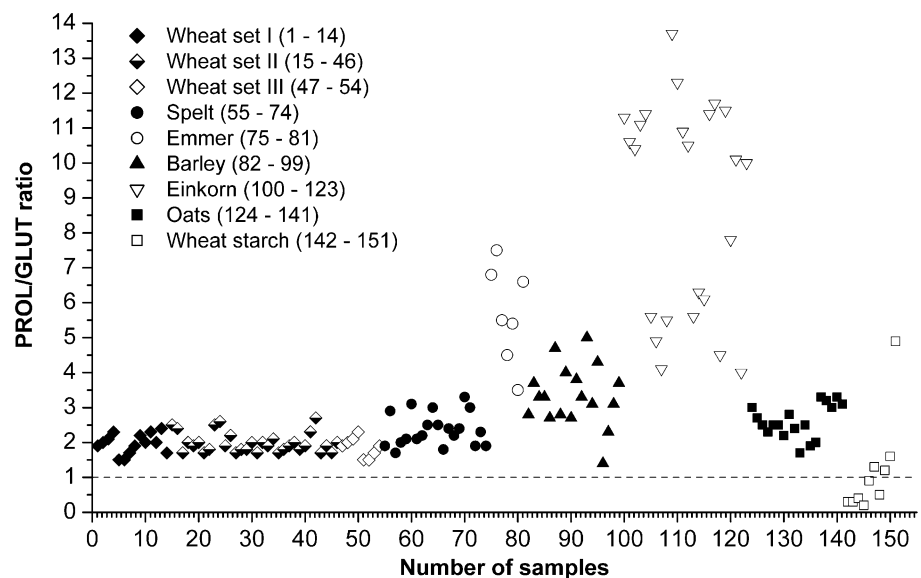
Both rye flours analyzed had also very high PROL/GLUT ratios (6.27–8.23), whereas those of the barley cultivars (1.40–4.98) were lower and strongly dependent on the genotype (Fig. 1). The maximum errors for gluten calcula-

Table 1 Ratio of amounts of prolamins and glutelins (PROL/GLUT) and comparison of factors for the calculation of gluten

Sample	Number of samples	PROL/GLUT	Real factor f^a	Error (%) ^b	References
Common wheat	54	1.50–3.14	1.32–1.66	+20 to +52	[5–7]
Spelt	28	1.73–3.32	1.30–1.58	+27 to +54	[8]
Durum wheat	1	3.10	1.33	+50	[9]
Kamut	1	3.39	1.29	+55	
Emmer	7	3.51–7.55	1.13–1.28	+56 to +77	
Einkorn	24	4.04–13.86	1.07–1.25	+60 to +87	
Rye	2	6.27–8.23	1.12–1.16	+72 to +79	[10]
Barley	18	1.40–4.98	1.20–1.71	+17 to +67	
Oats	18	1.65–3.27	1.30–1.61	+24 to +54	
Starch	10	0.17–4.86	1.21–6.81	–71 to +66	[13]

^a $f \times \text{PROL} = \text{gluten}$

^b As compared to gluten content = $2 \times \text{PROL}$

Fig. 1 Ranges of PROL/GLUT ratios of flours from different cereal species and of wheat starches. The dotted line represents a ratio of 1 used in the calculation of the gluten content by $2 \times \text{PROL}$ 

tion by $2 \times \text{PROL}$ were +79 and +67%, respectively. The data for the eighteen oats samples showing PROL/GLUT ratios from 1.65 to 3.27 were similar to those of common wheat. Summarizing, the gluten content of gluten-free products that are contaminated with CD activating cereals is generally overestimated, when the PROL content is multiplied by the factor of 2. In extreme cases, e.g., contamination with rye, the overestimation amounts to 72–79%.

Studies on wheat starch

Wheat starch contains much less protein (≈ 0.2 – 1.0%) than wheat flour. Column chromatography in combination with UV detection is not sensitive enough for the direct analysis of extract aliquots. Therefore, the alcoholic extracts were dried by means of a vacuum centrifuge and redissolved in a small volume of an appropriate solvent. Preliminary studies on a PROL extract of wheat flour demonstrated that recovery and repeatability analyzed by RP-HPLC after drying and redissolving were excellent. However, first experiments

with wheat starch extracts revealed that PROL quantitation was not possible because PROL interfered with dominant non-gluten proteins in the rear part of the chromatogram. Membrane centrifugation and subsequent RP-HPLC analysis indicated molecular masses (M_r) < 10,000 of these proteins; their identity remained unclear. Removal of non-gluten proteins by membrane centrifugation and quantitation of PROL by RP-HPLC resulted in a very poor repeatability. As a consequence, GP-HPLC on Superdex 200 with a separation range of $M_r = 10,000$ – $600,000$ was chosen for further tests. This column was effectually applied for the characterization of a wheat PROL (gliadin) reference showing that albumins and globulins, which were present in minor amounts in this sample, were well separated from gluten proteins [12]. The detection wavelength was reduced from 210 (RP-HPLC) to 206 nm, which increased the detector response by 22%. Ten industrial wheat starches, not designed to be used for the production of gluten-free food, were extracted by means of two different procedures: total gluten proteins were extracted with 50% 2-propanol under

reducing conditions and increased temperature (60 °C), and PROL were extracted with 60% ethanol at RT. Both extracts were dried, redissolved and quantified via the absorbance areas of GP-HPLC. The amount of GLUT was determined by subtracting the PROL content from the total gluten content. Examples of chromatograms and detailed data have already been published [13]. The PROL/GLUT ratios important for the present paper were in a broad range from 0.17 to 4.86 (Table 1; Fig. 1). In some samples, the proportion of GLUT was much higher than that of PROL, in other samples the opposite was the case. There was no correlation between the crude protein content of the starches [13] and the PROL/GLUT ratios. Possibly, the quality of wheat cultivars used for starch production and different process parameters, e.g., washing steps or concentration of solubles in the washing water, influenced the composition of gluten proteins adherent to starch granules. The calculation of the gluten content by $2 \times$ PROL would lead either to underestimation (–71% at most) or overestimation of the gluten content (+66% at most).

Conclusion

The results presented here demonstrate that the calculation of the gluten content by multiplying the prolamin content by factor 2 is not valid. In the case of gluten-free products contaminated with wheat, rye, barley, and oats, the gluten content is overestimated. In the case of wheat starch, the gluten content can be either over- or under-estimated.

Therefore, a future task will be the development of immunoassays with antibodies not only against PROL, but also against GLUT.

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