

# Quantitative Determination of Gluten Protein Types in Wheat Flour by Reversed-Phase High-Performance Liquid Chromatography

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## ABSTRACT

Cereal Chem. 75(5):644-650

A combined extraction-HPLC procedure was developed on a microscale to determine the amounts of the different gluten protein types ( $\omega$ 5-,  $\omega$ 1,2-,  $\alpha$ - and  $\gamma$ -gliadins; high molecular weight [HMW] and low molecular weight [LMW] glutenin subunits) in wheat flour. After preextraction of albumins and globulins from flour (100 mg) with a salt solution ( $2 \times 1.0$  mL), extraction of gliadins was achieved with 60% aqueous ethanol ( $3 \times 0.5$  mL). Subsequently, the glutenin subunits were extracted under nitrogen and at 60°C with 50% aqueous 1-propanol containing Tris-HCl (0.05 mol/L, pH 7.5), urea (2 mol/L) and dithioerythritol (1%). The separation and quantitative determination of gliadins and glutenin subunits was then performed by reversed-phase HPLC on C<sub>8</sub> silica gel at 50°C using a gradient of increasing acetonitrile concentration in the presence of 0.1% trifluoro-

acetic acid. The flow rate was 1.0 mL/min, and the detection wavelength was 210 nm. Temperature and flow rate were modified for the quantitation of single underivatized HMW subunits. To determine the absolute amounts of protein types, different protein standards (gliadin, LMW and HMW subunits, bovine serum albumin) with known protein contents were compared to HPLC absorbance areas. The calibration curves were almost identical and linear over a broad range (20–220  $\mu$ g). This extraction-HPLC procedure allows an accurate, reproducible, sensitive, and relatively fast quantitative determination of all gluten protein types in wheat flour, and can be applied to quality evaluation of cereals as raw materials or in processed products.

The technological properties of wheat flour are strongly dependent on wheat genotype and growing conditions, and are mainly determined by both structure and quantity of gluten proteins. Numerous amino acid sequences of gluten proteins, mostly deduced from nucleotide sequences, have been determined and, except for the  $\omega$ -gliadins, all gluten protein types are now known in complete primary structures. Accordingly, gluten proteins can be classified into three main groups, each consisting of two or three protein types (Shewry et al 1986, Wieser 1995): a high molecular weight (HMW) group including the  $x$ - and  $y$ -type of HMW subunits of glutenin; a medium molecular weight (MMW) group (S-poor group) containing the  $\omega$ 5- and  $\omega$ 1,2-type gliadins; and a low molecular weight (LMW) group (S-rich group), consisting of LMW glutenin subunits, and  $\alpha$ - and  $\gamma$ -type gliadins. Certainly, wheat cultivars differ in the primary structures of single gluten protein components, but differences are considered to be small and restricted to only a few substitutions, deletions, or insertions of single amino acid residues or oligopeptides in the peptide chain. To judge wheat flour properties on a chemical basis, it is therefore not sufficient to characterize gluten proteins in purely structural terms; quantitative aspects also have to be regarded. For these reasons, the determination of amounts and proportions of the different gluten proteins was an object of increasing interest during recent years.

One basic approach for quantitation involves the analysis of gluten proteins in a nearly native state comprising both polymeric glutenins and monomeric gliadins. Size-exclusion (SE) HPLC has frequently been used to relate the quantity of gluten protein fractions to flour properties (see Cornec et al 1994, Gupta and Mac Ritchie 1994, El Haddad et al 1995, Gupta et al 1995, Ciaffi et al 1996, Fu and Sapirstein 1996, Gupta et al 1996, Jia et al 1996). Another approach is the quantitative determination of glutenins after reduction of disulfide bonds in the state of monomeric subunits just as gliadins. For that, reversed-phase (RP) HPLC gel electrophoresis combined with densitometry and capillary electrophoresis have been the most common methods. Because of the high separation efficiency, these methods can relate the quantity and proportions of all gluten protein types, even of single protein com-

ponents, to flour properties (see Kolster et al 1993, Andrews et al 1994, Gupta and Mac Ritchie 1994, Kolster and Vereijken 1994, Mosleth et al 1994, Peltonen and Virtanen 1994, Wieser et al 1994a-c, Hou and Ng 1995, Weegels et al 1995, Dupuis et al 1996, Hou et al 1996a,b). Most of these studies, however, present quantitative data only for specific (not all) gluten protein types and only in relative (not absolute) terms.

The aim of the work presented here was, therefore, to develop a relatively simple, fast, and sensitive extraction-analysis procedure for the routine quantitation of all protein types in wheat flour in relative and absolute quantities, including the optimization of protein extraction and of quantitative analysis by RP-HPLC.

## MATERIALS AND METHODS

### Materials

**Chemicals and reagents.** Solvents and reagents used for the present studies were analytical quality or better, unless described differently. For the preparation of HPLC solvents, trifluoroacetic acid (TFA) of sequencing grade (Fluka 91699) and acetonitrile (ACN) of gradient grade (Merck 100030) or of preparative grade (Merck 113358) were used. Bovine serum albumin (BSA standard) was purchased from Serva (11920), and propyl-4-hydroxybenzoate from Fluka (54790).

**Flours.** The kernels of the German wheat cultivars Astron and Rektor, and of the Canadian Western Red Spring (CWRS) class were milled to flours with an ash content of 0.55% using a laboratory mill (Brabender Quadrumat Jr.). Portions of the flours were defatted twice with light petroleum (boiling range 40–60°C) and air-dried.

### Extraction Methods

**Solvents.** A1: 0.4 mol/L of NaCl + 0.067 mol/L of HKNaPO<sub>4</sub> (pH 7.6); A2: 0.4 mol/L of NaCl. B1: 60% (v/v) ethanol (EtOH); B2: 50% (v/v) 1-propanol (1-PrOH); B3: 50% (v/v) 2-propanol (2-PrOH). C1: 50% (v/v) 1-PrOH + 2 mol/L of urea + 0.05 mol/L of Tris-HCl (pH 7.5) + 1% (w/v) dithioerythritol (DTE) under nitrogen; C2: 50% (v/v) 2-PrOH + 0.08 mol/L of Tris-HCl (pH 8.0) + 1% (w/v) DTE under nitrogen.

**Standard extraction procedure.** Nondefatted flour (100 mg) was weighed into a centrifuge tube (10 mL) and extracted stepwise twice with 1.0 mL of solvent A1 at room temperature (RT  $\approx$  20°C), three times with 0.5 mL of solvent B1 at RT, and twice with 1.0 mL of solvent C1 at 60°C under nitrogen. Each extraction step was initiated with vortexing for 2 min at RT and continued with mag-

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netic stirring for 10 min (A1, B1) and 20 min (C1), respectively. The suspensions were centrifuged for 15 min (solvent A) and 20 min (other solvents), respectively, at 7,000 rpm ( $6,000 \times g$ , RT) using a Jouan C412 centrifuge. The corresponding supernatants were combined and diluted to 2.0 mL with the respective extraction solvents. Aliquots (300  $\mu$ L) of the extracts were filtered through a 0.45- $\mu$ m membrane before HPLC analysis.

**Preliminary extraction studies.** In preliminary experiments, nondefatted and defatted flour and the extractants A1, A2, B1, B2, and B3, respectively, were compared. Furthermore, four subsequent extraction steps with 0.5 mL or 1.0 mL of A1, B1, and C1 were performed, and the single supernatants were diluted to 1.0 mL. In another series, A1 was replaced by water in the third extraction step. For selected extractions steps, centrifugation times of 10, 15, 20, and 30 min were compared.

**Preparation of protein standards.** Flour of Rektor was defatted twice with light petroleum (boiling range 40–60°C). Defatted flour (50 g) was extracted stepwise three times with 200 mL of solvent A1 (albumins, globulins) and three times with 200 mL of solvent B1 (gliadins) using an Ultra Turrax homogenizer for 5 min at RT. The suspensions were centrifuged for 15 min at RT and  $40,000 \times g$  (10,000 rpm). The supernatants of the gliadin extraction were combined, concentrated to  $\approx 200$  mL using a vacuum evaporator, dialyzed against 0.01 mol/L of acetic acid until free of chloride and freeze-dried (gliadin standard). According to Melas et al (1994), the extraction residue was extracted twice with 250 mL of solvent C2 under nitrogen using magnetic stirring for 30 min at 60°C. The suspensions were centrifuged (15 min,  $40,000 \times g$ , RT), and the supernatants were combined and diluted to 500 mL with solvent C2. Acetone (333 mL) was added, shaken, and after a 10-min rest at RT and centrifugation (15 min,  $40,000 \times g$ , RT), residue (HMW standard) and supernatant (LMW standard) were dialyzed (see above) and freeze-dried. For HPLC analysis, standards (4 mg) were dissolved in 2 mL of water (BSA) and in 2 mL of solvent B1 (gliadin) by stirring for 10 min at RT or in 2 mL of C2 (LMW, HMW) by stirring for 20 min at 60°C under nitrogen.

**RP-HPLC.** Routine RP-HPLC of the filtered protein fractions extracted from flour and the different protein standards was performed using a Beckman instrument (solvent module 126, System Gold software); Nucleosil 300-5 C<sub>8</sub> column material (Machery-Nagel 71265) packed into a 4.6-  $\times$  240-mm column by an HPLC packing instrument (Shandon) under a pressure of 450 bar (which was much cheaper than a commercially available column); column temperature was 50°C; sample loop was 2 mL; injection was 200  $\mu$ L of albumins and globulins, 50 or 80  $\mu$ L of gliadins, and 100 or 150  $\mu$ L of glutenin subunits; 0.1% (v/v) TFA (500  $\mu$ L) was injected before and after sample injection. Elution system I: A) TFA (0.1%, v/v); B) ACN (preparative grade) + TFA (99.9/0.1%, v/v). Linear gradients: 0 min 20% B, 20 min 60% B (albumins-globulins); 0 min 28% B, 30 min 56% B (gliadins, glutenin subunits, gliadin standard, LMW standard, HMW standard); 0 min 39.5% B, 20 min 41.0% B (BSA standard). Flow rate was 1.0 mL/min; detection was by UV absorbance at 210 nm. After each run, the column was cleaned with 90% B (5 min) and equilibrated with the starting B concentration (10 min).

For the analysis of HMW subunits, changes included column temperature of 53°C and injection of 250  $\mu$ L of the glutenin extract. Elution system II: A) ACN (15%, v/v) + 2-PrOH (10%, v/v) + TFA (0.1%, v/v); B) ACN (30%, v/v) + 2-PrOH (8%, v/v) + TFA (0.1%, v/v). Linear gradients: 0 min 46% B, 60 min 71% B, 61–65 min 100% B. Flow rate was 0.8 mL.

Other conditions tested included: Nucleosil 300-5 C<sub>18</sub> column material (Machery-Nagel 71252); gradient grade ACN; column temperature 70°C. Gradients: 0 min 24% B, 50 min 56% B, or 0 min 27% B, 20 min 55% B (gliadins, glutenin subunits). UV absorbance was at 220 nm.

The integration procedure was automatically handled by the software. The baseline was adjusted to zero when injector was moved

to inject position. Integration was suppressed for the first part of the chromatograms (negative and positive solvent peaks). The elution borders of the protein groups were set to absorbance minima between specific peaks.

The absorbance spectra of eluted proteins were determined by a diode-array detector (Beckman module 168). Column quality was tested with propyl-4-hydroxybenzoate (1.5  $\mu$ g dissolved in 30  $\mu$ L of methanol) eluted with 50% B of elution system I or II; peak width at half peak height was taken as quality criterion.

### Further Analytical Methods

The nitrogen content of flours and protein standards was determined according to the Dumas method using a protein-nitrogen analyzer (Leco FP-328). SDS-PAGE was performed according to the procedure described by Krause et al (1988).

## RESULTS AND DISCUSSION

### Extraction Procedure

Due to the extreme complexity of the protein mixture present in wheat flour, prefractionation into different solubility groups is unavoidable. For that, the classical Osborne procedure (fractionation into albumins, globulins, gliadins, and glutenins) is still widely used, even when the results are strongly dependent on different experimental parameters, for example, on composition and sequence of extraction solvents, temperature, and type of mixing (Shewry and Mifflin 1985). For the present study, a modified Osborne fractionation on a microscale was used, and flour proteins were separated into three fractions: albumins plus globulins, gliadins, and glutenin subunits (Wieser et al 1994b).

After every extraction step, centrifugation of the sample was necessary; therefore, extraction was most conveniently performed in centrifuge tubes. Because some flours tended to conglomerate immediately after addition of solvent, the samples were intensively homogenized for 2 min at the beginning of each extraction by vortex mixing. The period following magnetic stirring was reduced in comparison with previous studies (Wieser et al 1994b) from 15 to 10 min (albumins-globulins, gliadins) and from 30 to 20 min (glutenin subunits), respectively, without significant decrease of protein yield. To get clear supernatants from the suspensions, a centrifugation time of at least 15 min (albumins, globulins) and 20 min (gliadins, glutenin subunits) at  $6,000 \times g$  at RT was necessary using a lab centrifuge. The efficiency of centrifugation was ensured, when the supernatants were clear and could easily be filtered through a 0.45- $\mu$ m membrane.

For extraction on a microscale, flour amounts in the range of 50–200 mg and solvents volumes of 0.5–1.0 mL are most common (Lookhart and Bietz 1994). The amount of flour used for the present study was 100 mg because this amount was sufficient for repeated analysis of extracts and high enough to avoid serious errors derived from flour heterogeneity. Because of the high sensitivity of HPLC detection, proteins extracted from a single kernel may also be analyzed (Bietz 1986). In agreement with results of Bietz et al (1984), defatting of flour before extraction was not necessary. Preextraction of albumins and globulins with a salt solution, however, was essential, because they were coextracted with gliadins by aqueous alcohol and disturbed gliadin analysis (Wieser et al 1994a). Common extraction solvents for albumins and globulins are NaCl solutions in concentrations of 0.1–0.5 mol/L, either with or without buffer salts. When 0.4 mol/L of NaCl solution alone (actual pH of flour suspension  $\approx 6.2$ ) and with 0.067 mol/L of HKNaPO<sub>4</sub>, pH 7.6 (actual pH  $\approx 7.0$ ) (Wieser et al 1994a,b; Weegels et al 1995) were compared for preextraction, no significant differences in the quantities of gliadins or glutenin subunits could be detected (results not shown). For further experiments, the buffered salt solution was used. Gliadins are most appropriately extracted with aqueous ethanol at RT (Lookhart and Bietz 1994). Previous studies demonstrated that an EtOH concentration of 60% (v/v) lead to the highest yield of

gliadins (Wieser et al 1994a). In the present study, 50% (v/v) aqueous 1-PrOH and 2-PrOH were compared with 60% EtOH and did not reveal any significant differences (results not shown). SDS-PAGE of the different gliadin extracts indicated that none of them contained HMW subunits of glutenin (results not shown). Finally, glutenin subunits were extracted according to Marchylo et al (1989) with 50% aqueous 1-PrOH under desaggregating (urea) and reducing (DTE, N<sub>2</sub>) conditions and increased temperature (60°C).

The extraction of protein groups should be as complete as possible without protein overlapping, but also as fast as possible. For the optimization of the extraction procedure, nondefatted Rektor flour with a crude protein content (N × 5.7) of 13.1% was used. Four extraction steps with each of the solvent types were performed, and the efficiency of 0.5- or 1.0-mL volume was tested. The extracted proteins were then quantified by RP-HPLC. The absorbance areas (AU) of HPLC for each step and each protein group, and the percentage of yield are summarized in Table I. Generally, 1 mL lead to a faster extraction of proteins than 0.5 mL, and two steps with 1.0 mL appeared to be sufficient for the nearly complete extraction of albumins-globulins and glutenin subunits. A washing step with water after the extraction with the salt solution, as done by Fu and Sapirstein (1996), cannot be recommended because a lot of additional protein was coextracted (Table I). HPLC analysis demonstrated that most of the proteins extracted by water were ω- and α-type gliadins (results not shown). In the case of gliadins, at least three steps were necessary to obtain a yield of ≈95%. In this case, differences between 0.5 and 1.0 mL were small and, thus, 3 × 0.5 mL was used for routine analysis. SDS-PAGE of the residue after all extraction steps revealed only a double band with M<sub>r</sub> ≈ 65,000 (designated as bands AB 9 and 10 by Krause et al 1988). Possibly, these bands corresponded to nongluten proteins. The crude protein content of the freeze-dried residue determined by N analysis was 0.5%. For routine analysis, albumins and globulins were extracted with 2 × 1.0 mL of A1, gliadins with 3 × 0.5 mL of B1, and glutenin subunits with 2 × 1.0 mL of C1. The time required for the total extraction procedure was ≈5.5 hr, in the course of which, six samples could be extracted in parallel.

### RP-HPLC

Since its introduction by Bietz (1983), RP-HPLC has been widely applied to cereal proteins and has proven to be a highly efficient tool for the investigation of gliadins and glutenin subunits. RP-HPLC is very efficient and sensitive in protein resolution; fast, reproducible, and relatively easy to handle.

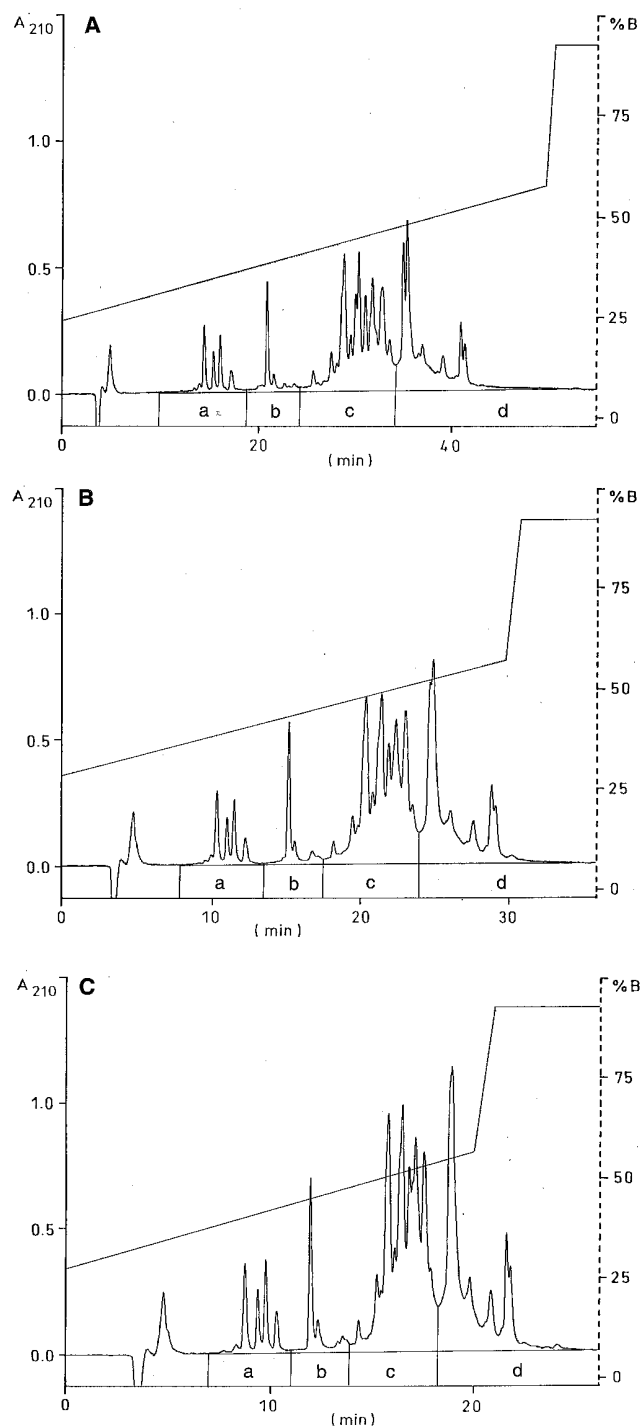
**TABLE I**  
Yields and Proportions of Protein Obtained by Stepwise Extraction of Wheat Flour (German Wheat Cultivar Rektor) with 0.5 or 1.0 mL of Solvent

Solvent (step)	0.5 mL		1.0 mL	
	AU <sup>a</sup>	%	AU	%
A1(1)	218 ± 13	71.1	262 ± 4	76.4
A1(2)	56 ± 2	18.4	54 ± 3	15.7
A1(3)	21 ± 1	6.9	21 ± 1 <sup>b</sup>	6.1
A1(4)	11 ± 2	3.6	6 ± 1	1.8
Σ A1	306 ± 12	100.0	343 ± 13	100.0
B1(1)	571 ± 25	49.3	604 ± 31	53.0
B1(2)	335 ± 5	28.9	332 ± 1	29.1
B1(3)	194 ± 7	16.7	158 ± 14	13.9
B1(4)	59 ± 4	5.1	46 ± 3	4.0
Σ B1	1,159 ± 37	100.0	1,140 ± 19	100.0
C1(1)	347 ± 25	48.1	607 ± 18	86.1
C1(2)	257 ± 3	35.6	80 ± 7	11.4
C1(3)	87 ± 6	12.1	13 ± 2	1.8
C1(4)	30 ± 0	4.2	5 ± 1	0.7
Σ C1	721 ± 28	100.0	705 ± 10	100.0
Σ A1-C1	2,186 ± 15	...	2,188 ± 56	...

<sup>a</sup> Absorbance areas of RP-HPLC per milligram of flour ± standard deviation of two determinations.

<sup>b</sup> 57 AU for extraction with water instead of salt solution.

The quantitation of proteins appears to be superior to most of the other methods (Bietz 1986). A further advantage is that the proteins are eluted according to different surface hydrophobicity in the series ω5-, ω1,2-, α-, and γ-type gliadins (gliadin extract) and glutenin-bound ω(ωb)-gliadins, HMW and LMW subunits (glutenin extract) (Bietz and Burnouf 1985, Wieser et al 1994b). Thus, each protein type is separated by RP-HPLC largely as a unique subgroup and can be quantitated without major overlap. It should be mentioned, however, that a considerable proportion of proteins extractable with aqueous alcohol are not monomeric but aggregated gliadins (also called HMW gliadin or ethanol-soluble



**Fig. 1.** Reversed-phase HPLC results for gliadin extract (50 μL) from German wheat cultivar Rektor on C<sub>8</sub> silica gel (50°C, elution system I); a-d = elution ranges for ω5-, ω1,2-, α-, and γ-gliadins. Linear gradients: 0 min 24% B, 50 min 56% B (A); 0 min 28% B, 30 min 56% B (B); 0 min 27% B, 20 min 55% B (C).

glutenin). These proteins are eluted during RP-HPLC mainly in the region of  $\alpha$ - and  $\gamma$ -type gliadins (Huebner and Bietz 1993, Wieser et al 1994b). On the other hand, gliadin-type proteins are present in the glutenin fraction when they have an odd number of cysteine residues and, therefore, are bound to glutenins by disulfide bonds (Keck et al 1995).

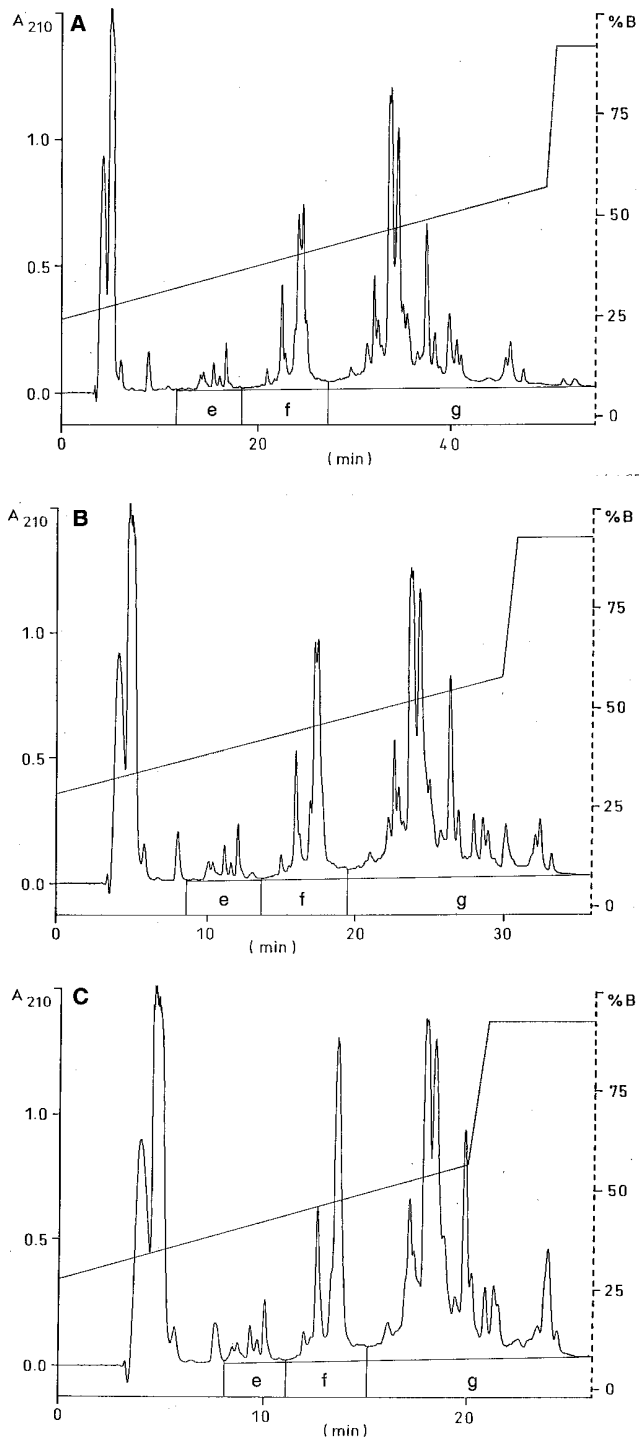
The aim of the present study was to use the same HPLC conditions for gliadins and glutenin subunits as well as for albumins, globulins, and protein standards. Among reversed-phase columns,

$C_8$  and  $C_{18}$  silica gels with average particle diameters of 5  $\mu\text{m}$  and pore diameters of 30 nm are most common. The comparison of corresponding materials (Nucleosil  $C_8$  and  $C_{18}$ , 50°C) demonstrated that separations of glutenin subunits were comparably satisfactory, whereas the elution ranges of  $\omega$ - and  $\alpha$ -type gliadins overlapped somewhat on  $C_{18}$  (results not shown). Therefore, a  $C_8$  column was used for further studies and for both gliadins (Fig. 1) and glutenin subunits (Fig. 2). Before injection, the samples were filtered through a 0.45- $\mu\text{m}$  membrane. Consequently, a precolumn was not necessary, and more than 500 runs could be performed before the column support had to be replaced. Occasionally, the quality of the column was tested by injection of propyl-4-hydroxybenzoate as control compound (Fig. 3). Both elution systems I and II could be used.

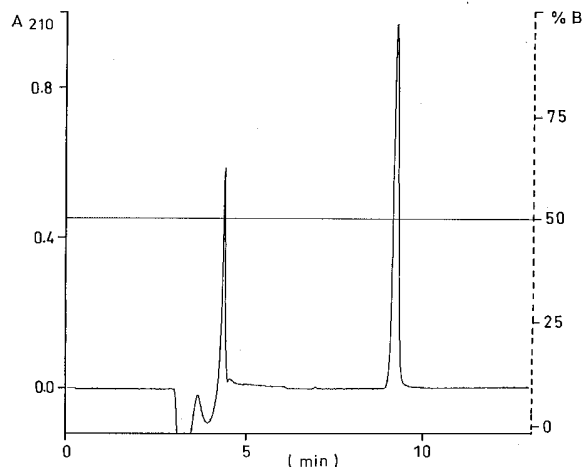
The elution system used was a simple but very common system: A) water-TFA (0.1%) and B) ACN-TFA (0.1%) (Bietz 1986). Even when the separation of single components was not optimal, the separation and quantitation of all gluten protein types was achieved. During the chromatography of glutenin subunits, the systems were held under slowly bubbling nitrogen to prevent reoxidation of glutenin subunits. For the optimal integration of absorbance areas, the baseline (determined by a blank run) should not have a remarkable increase or decrease. If this was not the case, absorbance differences between elution solvents A and B were equalized by addition of TFA. Insofar as possible, electronic baseline correction could be used instead. After the replacement of solvents by fresh ones, a blank run was performed and its absorbance area, which was usually very small, was subtracted from those of an analytical run. According to most of the literature, the quality of ACN should be HPLC-grade purity. This product is expensive, so cheaper products were tested. Thus, preparative-grade ACN was also suitable. No peaks were present in the blank run, and the absorbance of the elution system measured against distilled water was  $\approx 0.4$ –0.5 units.

Column temperature has a big effect on separation efficiency. Usually resolution of cereal proteins markedly improves at higher temperatures like 70°C (Bietz 1986). For the quantitation of gliadin subgroups, however, a temperature choice of 50°C was better because elution ranges of  $\alpha$ - and  $\gamma$ -type gliadins overlapped strongly at 70°C (results not shown).

The elution gradient was adjusted such that analysis time was as short as possible, and the first and the last few minutes of elution were free of absorbing compounds, so that an optimal area integration was achieved. In preliminary experiments, gradient running times of 20, 30, and 50 min were tested for both gliadins and glutenin subunits. As shown in Figs. 1 and 2, the time was reduced from 50 min (Wieser et al 1994b) to 30 min without an im-



**Fig. 2.** Reversed-phase HPLC results for glutenin extract (100  $\mu\text{L}$ ) from German wheat cultivar Rektor on  $C_8$  silica gel (50°C, elution system I); e–g = elution ranges for  $\omega$ -gliadins, high molecular weight subunits, and low molecular weight subunits. Linear gradients: 0 min 24% B, 50 min 56% B (A); 0 min 28% B, 30 min 56% B (B); 0 min 27% B, 20 min 55% B (C).



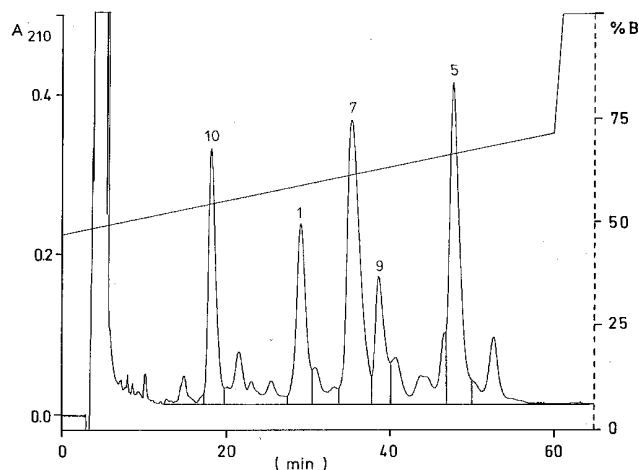
**Fig. 3.** Reversed-phase HPLC of propyl-4-hydroxybenzoate (1.5  $\mu\text{g}$  dissolved in 30  $\mu\text{L}$  of methanol) on  $C_8$  silica gel (50°C, elution system II, isocratic gradient of 50% B).

portant decrease in separation quality. When 20 min were applied, however, the separation of  $\alpha$ - from  $\gamma$ -gliadins became unsatisfactory (Fig. 1C). Thus, for standard chromatography ( $C_8$  silica gel, 50°C, system I), a linear gradient starting with 28% B and finishing with 56% B within 30 min was chosen. Column was then washed with 90% B for 5 min and equilibrated with 28% B for 10 min.

The direct injection of aliquots from the alcoholic extracts leads to remarkable losses of considerable portions of the hydrophilic proteins, which were eluted with the front peak because of the high elution power of the aqueous alcohols used for extraction (Marchylo and Kruger 1988). To avoid this problem, a 2 mL-injection loop was used, and before and after sample injection, 500  $\mu$ L of TFA (0.1%) was additionally injected according to Seilmeier et al (1991). The examination of the collected front peak eluate by RP-HPLC (Marchylo and Kruger 1988) demonstrated that it was free of protein.

The wavelength of 210 nm, most common for detection and quantitation, was also used for the present study. Thus, areas beneath peaks in the appropriate chromatogram regions are closely related to the amounts of protein (Bietz 1986). Detection at 210 nm resulted in a twofold higher sensitivity than at 220 nm or 223 nm as used previously (Seilmeier et al 1991, Wieser et al 1994a). Also, the absorbance spectrum of HMW subunits measured by an diode-array detector showed a shoulder between 222 and 234 nm when compared with those of LMW subunits or gliadins. Consequently, measurement between these wavelengths would overestimate the relative amounts of HMW subunits compared with LMW subunits. This was obviously the case with data presented previously when measured at 223 nm (Seilmeier et al 1991).

Most wheat cultivars show similarities of the general overall patterns of gliadin and glutenin subunits with characteristic elution regions for each type. In the case of wheat-rye hybrids or some spelt wheats, however, the elution border between  $\omega$ - and  $\alpha$ -type gliadins is unclear due to additional peaks in this region. The comparison of unreduced and reduced gliadins by RP-HPLC can



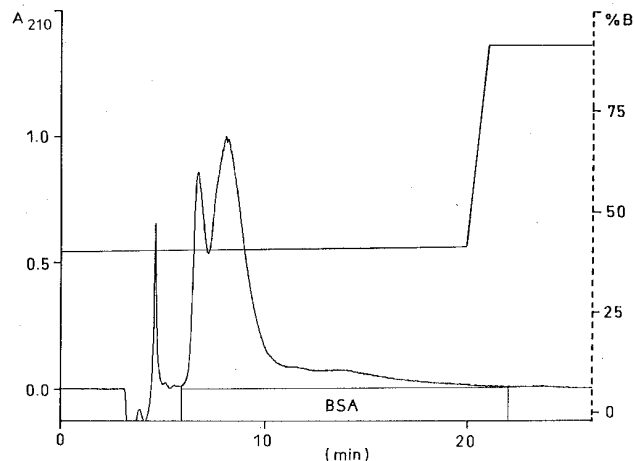
**Fig. 4.** Reversed-phase HPLC of high molecular weight subunits (250  $\mu$ L of glutenin extract) from German wheat cultivar Astron on  $C_8$  silica gel (53°C, elution system II, gradient 0 min 46% B, 60 min 71% B).

solve this problem. The  $\omega$ -type gliadins have the same retention times because of the lack of cysteine. The  $\alpha$ -type gliadins, however, show significantly increased retention times after the reduction of disulfide bonds (Bietz 1986, Wieser et al 1990) and can easily be identified.

In agreement with studies of other groups (Burnouf and Bietz 1984, Marchylo et al 1989, Lafiandra et al 1994), HMW subunits were poorly separated using the simply composed elution system I (ACN-TFA) and were quantitated only as a sum (Fig. 2). Burnouf and Bietz (1984) were the first to demonstrate that HMW subunits separated very well after alkylation with 4-vinylpyridine. The procedure described by Marchylo et al (1989) offered good possibilities for the quantitation of single pyridylethylated subunits. In previous work, a urea-containing elution system was developed that allowed the separation and quantitation of underivatized HMW subunits (Seilmeier et al 1991). The elution times, however, were rather long ( $\approx$ 140 min), and the urea-containing elution system was difficult to handle in the HPLC instrument. Therefore, further systems in combination with modified column temperature and flow rate were tested. The best results were obtained with an elution system consisting of ACN, 2-PrOH, and TFA (elution system II) at 53°C and 0.8 mL/min. HMW subunits could be determined directly from the extract. The separation of subunits 1, 5, 7, 9, and 10 from Astron is shown in Fig. 4.

### Repeatability

The repeatability of the extraction procedure and HPLC analysis was studied with five samples of CWRS flour, which were analyzed within two weeks using elution system I and the 50-min gradient for HPLC. Subsequent injections of the same extract indicated that variations were lower than  $\pm$ 2% for each protein type. Variations for the total procedure (extraction plus HPLC, Table II) were  $\pm$ 3.2% or lower (average of five determinations) and  $\pm$ 5.6% or lower (maximum of the two most different values) for all protein types except  $\omega$ b-gliadins ( $\pm$ 18.2% and  $\pm$ 32.6%, respectively), which was a very low amount. Thus, repeatability appeared satisfactory and two determinations were usually sufficient.



**Fig. 5.** Reversed-phase HPLC of bovine serum albumin (146  $\mu$ g) on  $C_8$  silica gel (50°C, elution system I, gradient 0 min 39.5% B, 20 min 41% B).

**TABLE II**  
Repeatability<sup>a</sup> of Extraction and Quantitative HPLC Determination of Gluten Protein Types<sup>b</sup>

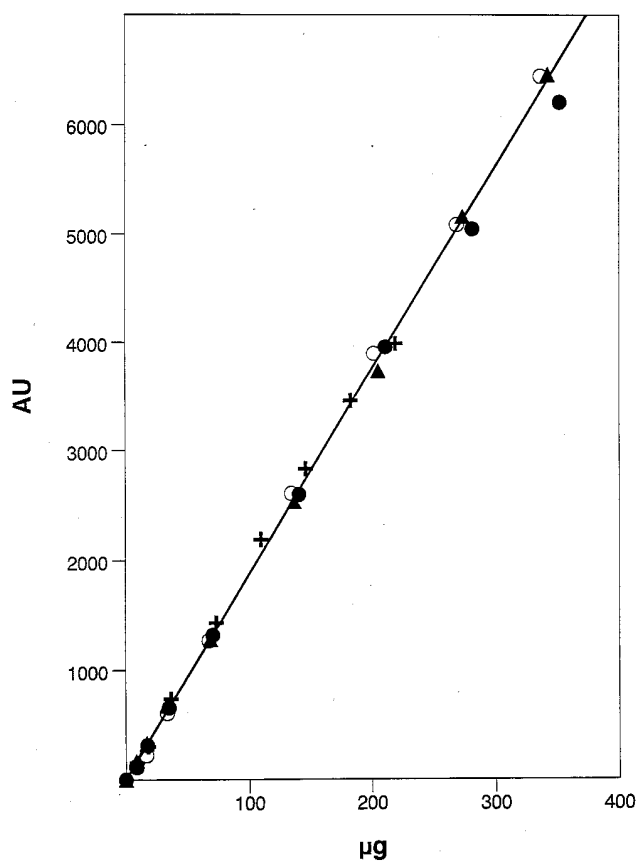
Coefficient of Variation	Gliadins					Glutenin Subunits			
	Total	$\omega$ 5	$\omega$ 1,2	$\alpha$	$\gamma$	Total	$\omega$ b	HMW	LMW
Average	1.7	2.3	1.0	3.2	1.9	2.3	18.2	2.0	3.0
Maximum	2.9	4.0	1.6	5.5	3.0	4.1	32.6	4.4	5.6

<sup>a</sup> Five determinations within two weeks.

<sup>b</sup> Gliadin extract:  $\omega$ 5-,  $\omega$ 1,2-,  $\alpha$ -, and  $\gamma$ -type gliadins. Glutenin extract: glutenin-bound  $\omega$ ( $\omega$ b)-gliadins, high molecular weight (HMW) subunits and low molecular weight (LMW) subunits.

## Protein Standards

In many cases, the comparison of the wheat samples is most important and the relative amounts of different gluten proteins determined by HPLC absorbance areas give sufficient information. When the absolute amounts in flour should be determined, calibration of absorbance areas has to be done using adequate protein standards. For this, gliadins and glutenin subunits were extracted from Rektor flour on a preparative scale, and HMW subunits were then separated from LMW subunits by precipitation with acetone (Melas et al 1994). The protein fractions were dialyzed and freeze-dried. The protein contents ( $N \times 5.7$ ) were 84.1% (gliadin standard), 88.0% (HMW standard), and 78.5% (LMW standard). Each standard was dissolved in corresponding solvents and stirred according to the extraction procedure for flour. Aliquots of the solutions (8–352  $\mu\text{g}$  of protein) were then analyzed by HPLC under standard conditions (elution system I). HPLC patterns indicated that the three standards were obtained in high purity. Regression analysis showed that the relation between absorbance areas and micrograms of protein was linear over a broad range ( $\approx 20$ –300  $\mu\text{g}$ ), and the curves for the three standards were almost identical ( $r$  for all values = 0.997). Consequently, the gliadin standard, which can be produced most easily, was suitable for the calibration of all three types. Additionally, commercially available BSA with a protein content of 91.4% ( $N \times 6.28$ ) was included in the study. Under usual conditions of HPLC, the limit of the linear range of the detector ( $\approx 2.0$  units) was reached with  $\approx 40$   $\mu\text{g}$  of protein. For the determination of higher amounts, the elution gradient had to be flattened, and BSA could then be determined up to 220  $\mu\text{g}$  (Fig. 5). The regression line (Fig. 6) demonstrated a very high correlation ( $r = 0.999$ ;  $y = 18.57x + 10.7$ ) of BSA (8–202  $\mu\text{g}$ ) and the three gluten protein standards (8–352  $\mu\text{g}$ ). Therefore, BSA can be used as a protein standard for the calibration of HPLC absorbance areas. A



**Fig. 6.** Relationship between absorbance area (AU) of HPLC and amount of standard protein ( $\mu\text{g}$ ).  $\circ$  = Gliadins;  $\bullet$  = high molecular weight subunits;  $\blacktriangle$  = low molecular weight subunits + bovine serum albumin.  $r = 0.999$ ;  $y = 18.57x + 10.7$ .

two-point calibration between 20–220  $\mu\text{g}$  appears to be sufficient. This result is in accordance with some earlier studies (Buck et al 1989) that demonstrated good protein quantitation by UV absorbance at low wavelength. Using the calibration curve of Fig. 6 for the absorbance areas of Table I (1.0 mL), Rektor flour shows 1.84% albumins and globulins, 6.13% gliadins (including HMW-gliadins), and 3.79% glutenins. A total 11.8% of the flour was extractable proteins and  $\approx 0.5\%$  was unextractable proteins in the residue. The comparison with the crude protein content of the flour determined by N analysis (13.1%) indicated a high recovery of protein by the extraction and HPLC procedure.

## CONCLUSIONS

The combined extraction-HPLC procedure described in this article allows an accurate and fast quantitative determination of the relative amounts of the different gluten protein types in wheat flour. When a protein standard such as gliadin or BSA is used for calibration, the absolute amounts in flour can be determined. The method is sensitive enough for a single kernel analysis. The procedure can be applied for the quantitative characterization of gluten proteins in flours and processed products (e.g., vital gluten, baked products) and is, in principle, transferable to the other cereals. The results obtained provide highly useful information for breeding programs and genetic improvement of cereals and for quality control of raw materials and end products.

## ACKNOWLEDGMENTS

This work was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), the AIF, and the Ministry of Economics. Project No. 10228N). We thank M. Redler and U. Schützler for excellent assistance.

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[Received January 21, 1998. Accepted May 27, 1998.]