

Simple Determination of Gluten Protein Types in Wheat Flour by Turbidimetry

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ABSTRACT

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A simple method based on turbidimetry has been developed for the quantitative determination of total gliadins, glutenin subunits, and high and low molecular weight (HMW and LMW) subunits of glutenin. The standard procedure includes the subsequent extraction of wheat flour (100 mg) with a salt solution, with 50% 2-propanol (gliadins), and with 50% propanol under reducing conditions and increased temperature (glutenin subunits). Aliquots of the gliadin and the glutenin extracts are mixed with 2-propanol to a final concentration of 83%, and the turbidity of the precipitates is measured photometrically at 450 nm and 20°C after 40 min. Another aliquot of the glutenin extract is mixed with acetone to a final concentration of 40% acetone, and precipitated HMW subunits are determined turbidimetrically after 30 min. The sample is then filtered, and an aliquot of the filtrate is mixed with 2-propanol to a final concentration of 77% to determine the precipitated LMW subunits. Control analyses

with reversed-phase HPLC on C₈ silica gel indicate that the precipitation of the different protein types is quantitative and specific, and studies of 16 different wheat flours demonstrate the strong correlation between quantification by HPLC and turbidimetry. The turbidimetric measurements are reproducible, linear over a wide absorbance range (0.2–1.7), and sufficiently sensitive to analyze 40 µg of protein or 20 mg of flour. The absolute amounts of protein types in flour can be determined by means of calibration curves with protein standards (gliadins, HMW, and LMW subunits). Altogether, the developed method is simple, accurate, sensitive, and specific for the different protein types. The total procedure takes ≈6 hr for the analysis of six flour samples in parallel or ≈4 hr for three samples in overlapping extraction steps. The chemicals used are inexpensive, scarcely toxic, and easy to dispose.

It is commonly accepted that gluten proteins strongly determine the technological properties and quality of wheat flour. On the one hand, structural differences of single gluten proteins have been proposed to be responsible for quality variations, especially of wheat genotypes. On the other hand, numerous studies have demonstrated that the quantitative differences in the composition of gluten protein are similarly important and can explain the influence not only of growing conditions, but also of genotype on quality. Since the classical work of Osborne (1907) at the beginning of the century, the ratio of monomeric proteins (gliadins) and polymeric proteins (glutenins) is considered to contribute essentially to flour properties. More recently, the quantities and proportions of single gluten protein types have been related to different quality parameters and have been used as potential tool to predict quality. Modern analytical methods like HPLC and gel electrophoresis have been applied to the quantification of gluten proteins either in a nearly native state or in the state of reduced subunits. The equipment for such analysis, however, is expensive and simpler methods (Marion et al 1994) are time-consuming and insensitive.

The aim of the present work was, therefore, to develop an inexpensive, accurate, and sensitive method for the quantification of total gliadins, total glutenin subunits, and high and low (HMW and LMW) molecular weight subunits, which are most important in the determination of dough and gluten properties (Wieser et al 1994a,b). The principle of measurement should be turbidimetry, which is both an old and modern method. By turbidimetry, the nonabsorbed light passing through a turbid sample is measured with an ordinary photometer. A modification of turbidimetry is nephelometry, by which the scattered light is measured. Nephelometry is more sensitive than turbidimetry but requires a special instrument, a nephelometer. The application of turbidimetry (or nephelometry) is widespread in such applications as monitoring of fluid systems in industry, determining the quality of drinking water and controlling waste water, measuring the growth of bacteria, or measuring the homogenization process of milk and other emulsions. In recent years, turbidimetry has become one of the most important methods in clinical diagnostics, in particular with respect to antigen-antibody reactions (Lottspeich 1998). Based on

the precipitation with trichloroacetic acid, Choi et al (1993) introduced a simple assay for the determination of proteins in solution. In cereal analysis, Skeritt and Martinuzzi (1986) developed a simple and rapid turbidity test for sulfur deficiency in wheat grain.

MATERIALS AND METHODS

Extraction Methods

The standard extraction procedure is similar to that of a previous study (Wieser et al 1998). Nondefatted flour (100 mg) was extracted stepwise (S1–S3) with 0.4 mol/L of NaCl + 0.067 mol/L of HKNaPO₄ (pH 7.6) (S1: 2 × 1 mL, 20°C, 10 min), with 50% (v/v) 2-propanol (S2: 3 × 0.5 mL, 20°C, 10 min) and with 50% (v/v) 2-propanol containing 1% (w/v) dithioerythritol and 0.08 mol/L of Tris-HCl (pH 8.0) under nitrogen (S3: 2 × 1 mL, 60°C, 20 min). The suspensions were centrifuged (15 min, 6,000 × g, 20°C), and the corresponding supernatants were combined and diluted to 2.0 mL with the respective extraction solvent. In alternative experiments, 60% ethanol was used instead of 50% 2-propanol, extraction with S1 was omitted, and gliadins were extracted directly from flour with S2. Furthermore, extraction was performed with different amounts of flour (10–200 mg).

Precipitation

For the standard procedure, 0.10 mL of gliadin extract and 0.15 mL of total glutenin extract were mixed with 0.40 mL of S2 and 0.35 mL of S3, respectively, and 1.00 mL of 2-propanol (S4) was added for protein precipitation. Another aliquot of the total glutenin extract (0.50 mL) was mixed with 0.50 mL of S3 and 0.67 mL of acetone (S5) to precipitate HMW subunits. After the turbidimetric measurement, the sample was filtered through a 0.45-µm membrane, and a 0.50 mL-aliquot of the filtrate was mixed with 1.00 mL of S4 to precipitate LMW subunits. Different volumes of the extracts (10–180 µL of gliadins; 20–250 µL of total glutenin subunits; 100–1,000 µL of HMW and LMW subunits) were taken for the determination of the linear range of absorbance.

Turbidimetric Measurement

The absorbance of turbid samples was determined in 1 cm-microcuvettes using a spectrophotometer (554, Perkin-Elmer) against blanks containing the same combination of solvents. In preliminary experiments, the influences of the wavelength (400–800 nm), the temperature (15, 20, 25, 30°C), as well as the development and stability of turbidity (0–60 min) were studied.

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For routine analysis, 450 nm, 20°C, and 30 min (gliadins, total glutenin subunits), or 40 min (HMW, LMW subunits) were chosen. Usually, absorbances were determined in duplicates.

Reversed-Phase (RP) HPLC

Analytical RP-HPLC on C₈ silica gel was used for the qualitative and quantitative characterization of extracts, redissolved precipitates, and filtrates from turbid samples under the conditions described previously (Wieser et al 1998) with a linear solvent gradient of 0 min 24% B, 50 min 56% B.

Protein Standards

The preparation procedures of gliadin, LMW, and HMW subunit standards were described previously (Wieser et al 1998). Protein content (N × 5.7) was 84.1% (gliadin), 88.0% (HMW subunits), and 78.5% (LMW subunits) (Wieser et al 1998). The nonprotein components of the standards were probably salts and water that were not completely removed by dialysis and lyophilization. Gliadin standard (6 mg) was dissolved in 2.0 mL of S2 containing 0.1 mol/L of NaCl, magnetically stirred at room temperature for 20 min, and filtered through a 0.45-μm membrane. Aliquots of the solutions (10–100 μL) were filled up to 0.50 mL with S2 and mixed with 1.0 mL of S4. After standing for 40 min, turbidity was measured at 450 nm. LMW standard (6 mg) was dissolved in 2.0 mL of S3, stirred at 60°C for 20 min, and filtered after cooling to 20°C. Aliquots of the solutions (10–140 μL) were filled up to 0.5 mL with S3 and mixed with 1.0 mL of S4. After standing for 30 min, turbidity was measured at 450 nm. HMW standard (6 mg) was dissolved in 2.0 mL of S3, stirred at 60°C for 20 min, and filtered after cooling to 20°C. Aliquots of the solutions (10–120 μL) were filled up to 1.0 mL with S3 and mixed with 0.67 mL of S5. After standing for 30 min, turbidity was measured at 450 nm.

RESULTS AND DISCUSSION

Extraction and Precipitation Procedure

For preliminary experiments, flour of the cultivar Rektor was used. RP-HPLC on C₈ silica gel was applied as control method for the qualitative and quantitative protein analysis (Wieser et al 1998). The conditions for the extraction procedures were chosen in accordance with previous studies. The first approaches were based on

literature describing the precipitation of gliadin with a salt solution (Kasarda 1980), the precipitation of HMW subunits with acetone (Melas et al 1994), or with 60% propanol (Marchylo et al 1989), and the insolubility of gliadins at high concentrations of alcohol (Wieser et al 1994c).

Gliadins were extracted directly from flour with 60% ethanol and then precipitated with salt solutions containing different concentrations (0.1–1.0 mol/L) of NaCl. RP-HPLC of the filtered samples, however, demonstrated that the precipitation of gliadins was incomplete throughout. Moreover, repeatability was poor, and the results of turbidimetry and HPLC obtained for Rektor and flours from three other wheats (Canadian Western Red Spring, Herzog, Obelisk) did not correlate. In contrast, increased ethanol concentration from 60 to 90% led to the quantitative precipitation of all gliadins. Albumins and globulins have previously been coextracted with gliadins (Wieser et al 1998), and HPLC chromatograms of redissolved precipitates indicated that they were also precipitated with 90% ethanol and, consequently, disturbed gliadin quantification. Similar to HPLC analysis, albumins and globulins were removed by extraction with a salt solution. Gliadins were then extracted with aqueous alcohol; the use of 50% 2-propanol increased to 83% led to the same results of turbidimetric measurements as 60% ethanol increased to 90%.

Total glutenin subunits were extracted after gliadins with 50% 2-propanol under reducing conditions and increased temperature (Melas et al 1994) and then they were also precipitated by increasing the alcohol concentration to 83%. HPLC analysis of the filtered sample demonstrated that precipitation was quantitative. For a specific separation of HMW and LMW subunits, the precipitation of HMW subunits by a final propanol concentration of 60% (Marchylo et al 1989) and by the addition of acetone (Melas et al 1994) was compared. The results of turbidimetric measurements were similar, but the latter procedure appeared to be more specific and accurate, according to control HPLC analysis. After filtration of the turbid sample, LMW subunits were precipitated by a final propanol concentration of 77% and could also be determined turbidimetrically.

To make the extraction procedure as quick and simple as possible, extraction and centrifugation times were reduced to an acceptable minimum, which was controlled by HPLC. The scheme of the resulting extraction and precipitation procedure is presented in Fig. 1. As shown previously (Wieser et al 1998), at least three extraction steps (3 × 0.5 mL) with aqueous alcohol were necessary for a sufficient extraction of gliadins. For comparative investigations of wheat cultivars, a two-step extraction (2 × 1.0 mL) is considered informative enough and would shorten the procedure by ≈0.5 hr.

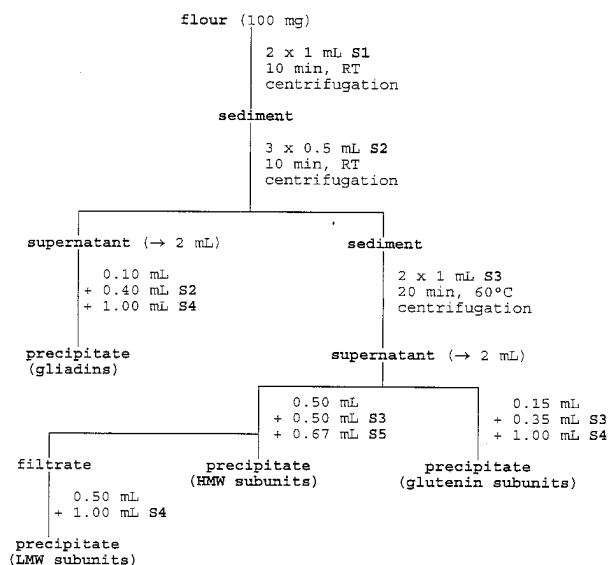


Fig. 1. Scheme of extraction and precipitation procedure. S1: 0.4 mol/L of NaCl + 0.067 mol/L of HKNaPO₄ (pH 7.6); S2: 50% (v/v) 2-propanol; S3: 50% (v/v) 2-propanol + 1% (w/v) dithioerythritol + 0.08 mol/L of Tris × HCl (pH 8.0) + N₂; S4: 2-propanol; S5: acetone.

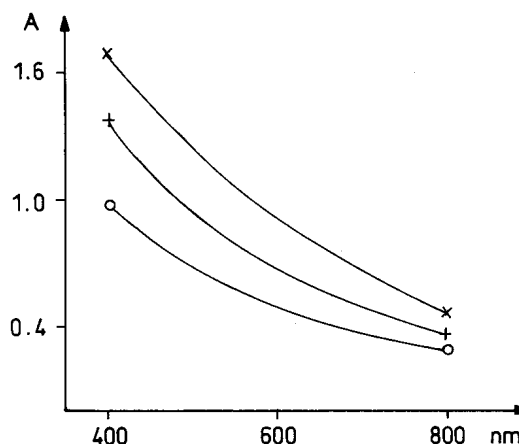


Fig. 2. Absorbance spectra of turbid samples (20°C; cv. Rektor); × = gliadins (0.10 mL), + = total glutenin subunits (0.15 mL), ○ = HMW subunits (0.50 mL).

TABLE I
Influence of Temperature on Absorbance (450 nm) of Turbid Samples (cv. Rektor)^a

| Temperature (°C) | Gliadins (0.10 mL) | HMW Subunits (0.50 mL) |
|------------------|--------------------|------------------------|
| 15 | 1.434 (0.1) | 0.865 (3.1) |
| 20 | 1.373 (0.6) | 0.850 (2.4) |
| 25 | 1.226 (1.0) | 0.785 (3.0) |
| 30 | 1.096 (3.6) | 0.711 (0.6) |

^a Mean values of two determinations (\pm % variation)

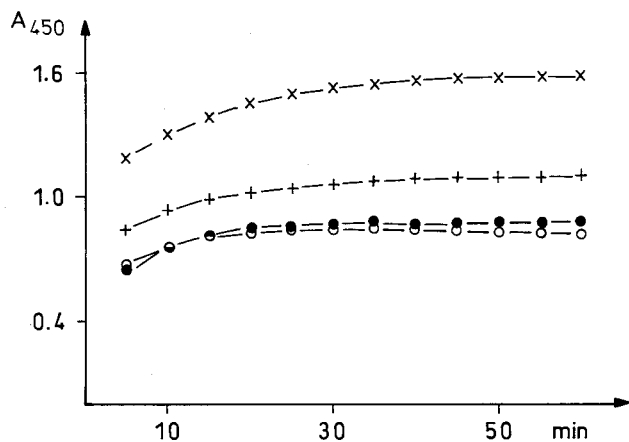


Fig. 3. Development and stability of turbidity (20°C, cv. Rektor) \times = gliadins (0.10 mL), $+$ = total glutenin subunits (0.15 mL), \circ = HMW subunits (0.50 mL) \bullet = LMW subunits (0.50 mL).

Turbidimetric Measurement

Different turbid samples of precipitated gliadins and glutenin subunits were used for the optimization of turbidimetric measurement. Because the photometric analysis should be as simple as possible, only wavelengths of visible light were considered. Spectral scans from 400 to 800 nm indicated that any visible wavelength can be used. As shown in Fig. 2, short wavelengths allowed a more sensitive detection than longer ones. Therefore, 450 nm was chosen as standard wavelength, which was in accordance with a turbidimetric determination of protein precipitated by trichloroacetic acid (Choi et al 1993). Measurements were performed in plastic cuvettes (1 cm) against the solvent combination present in the precipitated sample. Development and stability of turbidity are shown in Fig. 3. Most of turbidity developed during the first 20 min after the addition of the precipitating solvent and was stable for at least 60 min. Only the absorbance of precipitated HMW subunits decreased slightly after 35 min. For the standard procedure, samples were measured after 30 min (LMW and HMW subunits) and 40 min (gliadins and total glutenin subunits), respectively.

The influence of temperature was studied for precipitated gliadins and HMW subunits. In both cases, remarkable differences between 15, 20, 25, and 30°C could be detected (Table I). Therefore, a constant temperature of 20°C during turbidity development and measurement was ensured.

The completeness of precipitation was controlled by RP-HPLC. The analyses of the gliadin extract and the corresponding filtrate obtained from the turbid sample demonstrated that precipitation was quantitative (Fig. 4). The same was true for total glutenin subunits and LMW subunits (chromatogram not shown). The specificity and the completeness of the precipitation of HMW subunits was confirmed by HPLC of the filtered sample compared with total glutenin subunits (Fig. 5). In the elution range of HMW subunits (21–26 min), only one small peak was still visible; it probably represented an ω -gliadin coeluting with HMW subunits (Marchylo et al 1989). Based on corresponding amounts injected into the HPLC loop, the absorbance area of LMW subunits agreed with that of the total extract.

TABLE II
Repeatability of Turbidimetric Determination and Correlations with HPLC Analysis^a

| | Gliadins | Glutenin Subunits | HMW Subunits | LMW Subunits |
|------------------------------|-----------|-------------------|--------------|--------------|
| Turbidimetry ^b | | | | |
| <i>n</i> | 20 | 24 | 24 | 16 |
| cv (%) | ± 1.2 | ± 2.0 | ± 2.0 | ± 3.3 |
| Total procedure ^c | | | | |
| <i>n</i> | 16 | 16 | 16 | 6 |
| cv (%) | ± 3.9 | ± 4.1 | ± 4.0 | ± 3.9 |
| Correlations ^d | | | | |
| <i>n</i> | 20 | 15 | 16 | 16 |
| <i>r</i> | 0.972 | 0.968 | 0.940 | 0.969 |

^a *n* = number of determinations, cv = coefficient of variation, *r* = linear regression coefficient.

^b Analysis of the same extract on two consecutive days (extracts from four cultivars).

^c Analysis within four weeks (extracts from four cultivars) (Fig. 1).

^d Extracts from *n* cultivars.

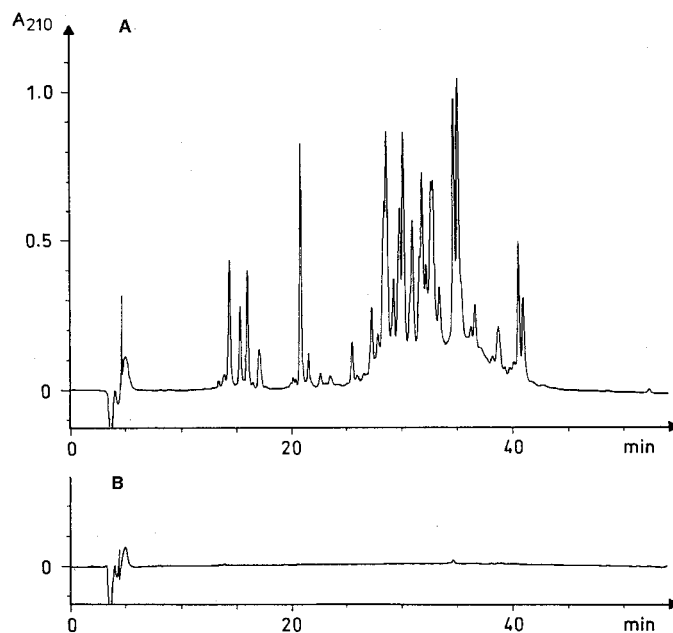


Fig. 4. Reversed-phase HPLC of gliadin extract (80 μ L) (A) and filtered precipitate (400 μ L) (B) of cv. Rektor.

Repeatability of turbidimetry was assessed by measuring the same extract on two consecutive days (Table II). Altogether, 24 samples from each of total glutenin subunits and HMW subunits, 20 gliadin, and 16 LMW subunits samples obtained from four wheat flours were analyzed. The coefficient of variation was $\pm 2\%$ or lower, only the coefficient for LMW subunits ($\pm 3.3\%$) was relatively high. The day-to-day variations of the HPLC analyses were in a somewhat lower range (1–2%). The repeatability of the total procedure (extraction, precipitation, turbidimetric measurement) determined within a period of four weeks was $\approx \pm 4\%$ for all protein types.

For the determination of the linear range of measurements, different volumes of the extracts were taken for precipitation. As can be seen in Fig. 6, the curves are linear in a range from ≈ 0.2 –1.7 absorbance units. The sample volumes that were suggested for standard analysis (0.10 mL of gliadin, 0.15 mL of total glutenin subunits, 0.50 mL of HMW and LMW subunits) are in a linear absorbance range for most common wheat flours. When different amounts of flour were analyzed, similar curves were obtained. In the case of Rektor flour, linear relations for all protein types were found in the range of 20–120 mg (curves not shown); thus, the method is sufficiently sensitive for a single kernel analysis.

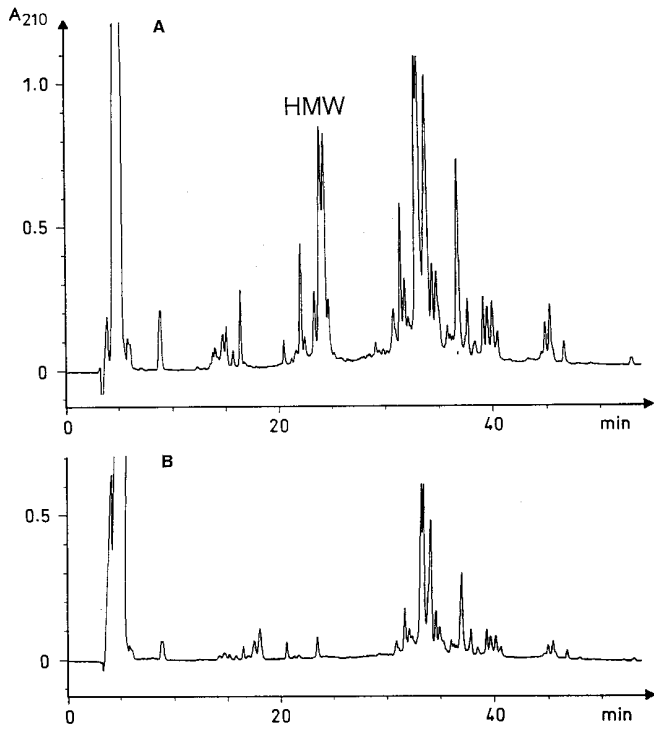


Fig. 5. Reversed-phase HPLC of glutenin subunits (120 μ L) (A) and filtrate (120 μ L) obtained after precipitation of HMW subunits (B) of cv. Rektor.

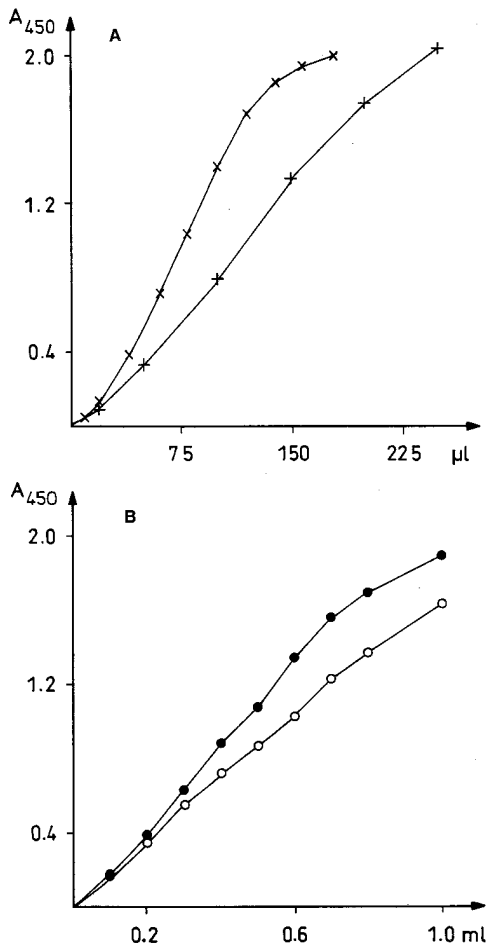


Fig. 6. Turbidimetric measurement of samples with varying volumes of extracts. A, \times = gliadins (0–180 μ L); + = glutenin subunits (0–250 μ L) B, \circ = HMW subunits (0–1,000 μ L); \bullet = LMW subunits (0–1,000 μ L) of cv. Rektor.

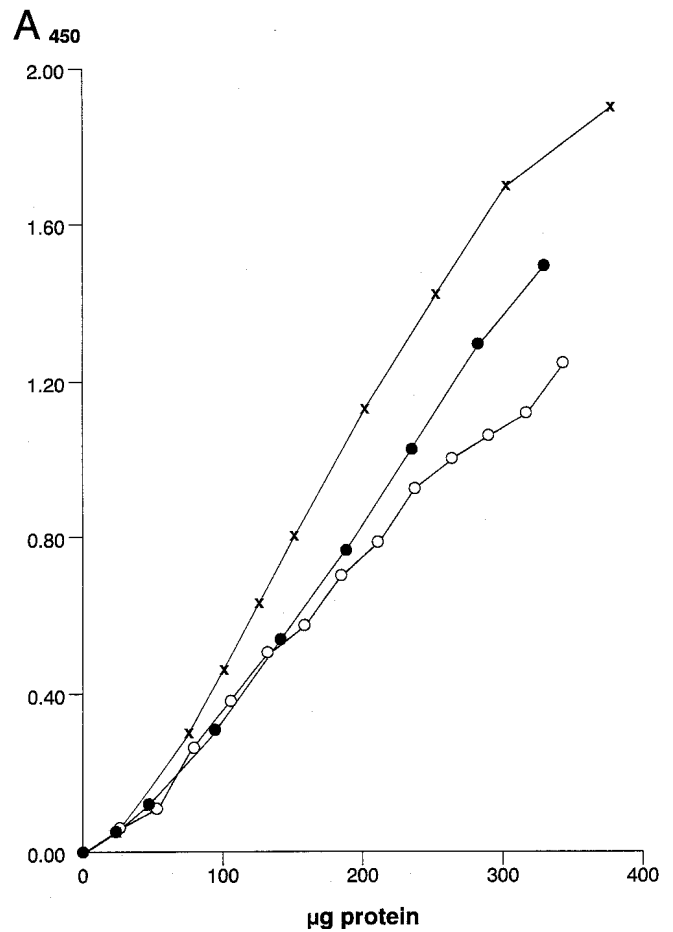


Fig. 7. Calibration curves of gliadins (\times), HMW (\circ), and LMW (\bullet) subunits.

To determine the absolute quantities of protein types in the extracts, and correspondingly in flour, calibration curves with the previously described standards for gliadins, HMW, and LMW subunits of glutenins (Wieser et al 1998) were set up. Each standard was dissolved in corresponding solvents, and in the case of gliadin standard, a small amount of NaCl (0.1 mol/L) was added to the solvent to get a quantitative precipitation. Aliquots of the solutions were precipitated according to Fig. 1 and analyzed. As shown in Fig. 7, 20 μ g of protein were clearly detected, and the linear range of the curves was 50–250 μ g of protein. The sensitivity of the method could even be increased by the use of nephelometry instead of turbidimetry.

The accuracy of the turbidimetric analyses was determined by comparison with the HPLC method, which was previously shown to be strongly correlated with the amounts of protein (Wieser et al 1998). Flours of 16 different wheat cultivars were extracted according to Fig. 1, and the relative amounts of gliadins, total glutenin subunits, HMW, and LMW subunits were determined by both turbidimetry (absorbance at 450 nm) and HPLC (absorbance units at 210 nm). Regression analysis indicated that correlations were high, ranging from $r = 0.940$ (HMW subunits) to $r = 0.972$ (gliadins) (Table II). Even when extraction and quantitative analysis were performed by two different research groups, the correlations between turbidimetry and RP-HPLC were high for all protein types (Eder 1997).

In routine work, at least six flour samples could be analyzed in parallel; the standard procedure shown in Fig. 1 took ≈ 6 hr. Alternatively, time was reduced to ≈ 4 hr when gliadins and glutenin subunits were extracted in separate, overlapping steps. For example, gliadins of three flour samples were analyzed according to Fig. 1. Simultaneously, three further samples of the same flours

were extracted with 50% propanol, and the extracts (albumins, globulins, gliadins) were discarded. Glutenin subunits were then extracted from the residues and analyzed according to Fig. 1.

CONCLUSIONS

The developed turbidimetric determination of gluten protein types in wheat flour is simple, accurate, sensitive, and specific for total gliadins and total glutenin subunits, and also for HMW and LMW subunits of glutenin. It appears also possible to determine the quantity of HMW gliadin (also called aggregated gliadin or ethanol-soluble glutenin) by a specific precipitation step described by Sapirstein and Fu (1998). The chemicals used are inexpensive, scarcely toxic, and easy to dispose. Repeatability is much more influenced by the extraction procedure than by turbidimetric measurement. Using standard proteins or a standard flour, the absolute quantities of the different protein types in flour can be determined.

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