Effects of Temperature, Sonication Time, and Power Settings on Size Distribution and Extractability of Total Wheat Flour Proteins as Determined by Size-Exclusion High-Performance Liquid Chromatography

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ABSTRACT

The size-exclusion (SE) HPLC profile of total protein extract obtained by sonication of flour samples at ambient temperature showed marked instability on reinjection. Instability was related to the presence of flour proteases that were inactivated by thermal treatment of flour samples at 60°C. Extraction of flour protein by sonication was a function of ultrasonic energy (sonication time × power product) delivered to flour sample. As protein solubility increased, the proportions of the earliest eluted SE-HPLC fractions (F1 and F2) increased. Oversonication of proteins evidenced by a decrease in F1 amount at the benefit of F2 occurred below the sonication energy level needed for total protein extraction. Ultrasonic energy level was adjusted to allow total protein extraction while limiting oversonication. The sonication procedure was applied on 27 flour samples of contrasting dough strength to extract total proteins. Absolute amount of protein extractable by sonication, determined from SE-HPLC area, was strongly correlated with flour protein content. Very significant and equivalent relationships were found between alveographic *W* index and absolute amount of either unextractable protein extract or F1 of SE-HPLC profile from total protein extract.

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Singh et al (1990a) showed that total protein from wheat flour was successfully extracted by sonication without chemical reduction by breakdown of large SDS-insoluble glutenin polymers into smaller SDS-soluble polymers. Size-exclusion (SE) HPLC was used to quantify the absolute and relative amounts of glutenin polymeric protein from total protein extract obtained by sonication of flour sample (Singh et al 1990b, Gupta et al 1992). Absolute quantity of glutenin polymer (M_r > 158,000) was strongly correlated with dough quality attributes. Subsequent studies on diverse flour sample sets refined this finding and established that the proportion of SDS-insoluble polymeric protein in total protein or in total polymeric protein (both strongly correlated) provided the most reliable biochemical criterion for predicting flour dough strength (Gupta et al 1993). Therefore, it was suggested that the size distribution of glutenin polymeric protein of wheat flour controls the rheological properties of dough (Gupta et al 1993). Rather than estimating the relative size distribution of total polymeric glutenin from SE-HPLC measurements of both total and SDS-insoluble protein fractions, Gupta et al (1993) proposed to estimate the percent of SDS-insoluble polymeric protein in total protein by dividing the SE-HPLC area of SDS-insoluble protein extracted by sonication, by the flour protein content. Other alternative methods have been developed by Ciaffi et al (1996), Bean et al (1998), and Fu and Sapirstein (1996) based on direct measurement of the amount of SDS-insoluble or 50% 1-propanol insoluble proteins.

In theory, reliable estimate of total protein content, gliadin content, total polymeric content, and an estimate of unextractable polymeric protein should be obtained from SE-HPLC analysis of total protein. To obtain this information, several requisites are needed: 1) extraction of total protein must be successfully achieved; 2) polymeric protein must be resolved from monomeric protein on SE-HPLC column; 3) unextractable polymeric protein extracted by sonication must have a size-distribution range distinct from that of SDS-soluble polymeric protein.

Nevertheless, after sonication, unextractable polymers are eluted within the size-distribution range of the original SDS-soluble polymeric proteins (see figure 1 in Gupta et al 1993). An improvement could, however, be considered by limiting the extent of the degradation of unextractable polymeric protein and by using a column with packing of larger pore size which should improve the column effectiveness for separating large polymeric protein.

In this study, we investigated the experimental variables that could affect total protein extraction by sonication and modulate the size-distribution profile of total protein as assayed by SE-HPLC. As in a previous work by Dachkevitch and Autran (1989), the size distribution of protein was analyzed on TSK G4000 SW column (TosoHaas) which, according to the manufacturer, allows the separation of globular proteins of M_r 20,000 to 500,000. This column has a wider separation range than the 10,000 to 300,000 separation range of Protein-Pack 300 (Waters) and Superose 12 HR (Pharmacia) used by Gupta et al (1993) and Ciaffi et al (1996), respectively. The TSK G4000 SW column has larger pore size (450 Å) than that of Phenomenex SEC-BIOSEP 4000 column (400 Å), which, according to Larroque et al (1996), give better resolution of wheat polymeric proteins than columns of pore size of ≈300 Å, like Protein-Pack 300.

Details of the extraction procedure and SE-HPLC analysis of total wheat flour protein are presented in this article. The procedure was applied on a set of 27 flour samples. How flour dough strength, evaluated by the alveographic index *W*, and flour protein content correlated with the absolute and relative amounts of SE-HPLC fractions from total, SDS-soluble, and SDS-insoluble protein extracts is discussed.

MATERIALS AND METHODS

Flour Samples

Flour samples were supplied by the Institut Technique des Céréales et des Fourrages (ITCF, Paris, France). ITCF prepared flours from common commercial cultivars grown in 1995 and 1996. Grain was milled on a Brabender Quadrumat Sr. laboratory mill after 24 hr of tempering to a moisture basis of 16% (dm). Flour extraction rate was 68–76%. Flour protein content (N × 5.7) was determined by the Kjeldahl method. Alveographic tests were performed according to NF ISO 5530-4 procedure (AFNOR 1992). The *W* index corresponds to the total area of the alveograph curve and is related to the strength of the dough. Protein contents of the Soissons and Thésée flour samples used for the optimization of extraction and sonication procedures were 11.6 and 10.3% (db), respectively. Dough strength of Soissons flour was higher, with a *W* index of 339 ($\times 10^{-4}$ J) instead of 197 ($\times 10^{-4}$ J) for Thésée flour. The set of 27 flour samples (from 17 cultivars) used for correlation analysis showed a range of variation in protein content and *W* values of 8.9–12.6% (mean 10.62% ±1.22, db) and 115–311 ($\times 10^{-4}$ J) (mean 195.4 ± 53.7 $\times 10^{-4}$ J), respectively.

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Sonication Procedure

A sonifier (Vibra Cell 72434, Bioblock Scientific, Illkirch, France) delivering ultrasonic vibrations at 20 kHz and equipped with a 3-mm diameter tip probe was used. The sonication power settings were 10–80% (scale 25); i.e., 1–15W power output. Sonication was applied without interruption (no overheating was noticed) probably because of the large solvent volume used.

Standard Extraction Procedures of Flour Samples

Flour sample (160 mg in a 50-mL centrifuge tube) was dispersed at 60°C for 80 min with 20 mL of 1% SDS, 0.1*M* sodium phosphate buffer (pH 6.9), using a rotary shaker set at 60 rpm. SDS-soluble protein extract was obtained by gathering the supernatant after 30 min of centrifugation at 37,000 × *g* at 20°C in a centrifuge (Beckman model JA-221). Alternately, the dispersed flour sample was sonicated (3 min at 30% power setting) to obtain total protein extract after centrifugation. The residue of SDS-soluble protein extraction was suspended by vortexing with 10 mL of SDS-phosphate buffer, and sonicated as above to extract SDS-insoluble protein.

Measuring Size Distribution of Protein by SE-HPLC

The SE-HPLC apparatus (Waters model LC Module1 plus) controlled by software (Waters Millenium). Size-exclusion analytical column (TSK G4000-SW, TosoHaas) (7.5 × 300 mm) was used with a guard column (TSK G3000-SW, TosoHaas) (7.5 × 75 mm). The columns were eluted at ambient temperature with 0.1*M* sodium phosphate buffer (pH 6.9) containing 0.1% SDS. The flow rate was 0.7 mL/min and absorbance was recorded at 214 nm. SDS-soluble, SDS-insoluble, and total protein extracts were applied on column as 20 µL of sample without being filtered. Protein extraction yields (%) of sonicated flour samples were derived from the total SE-HPLC area of the corresponding SDS-soluble protein extract, for which the protein content was determined from triplicate measurements of protein content (15-mL samples) by Kjeldhal procedure. Apparent molecular weights of the major fractions, referred to as F1 to F5, were estimated by calibrating the column with protein standards according to Dachkevitch and Autran (1989). The first fraction (F1) of SE-HPLC profile from total protein extract corresponds to polymeric protein eluted at the void volume of the column (blue dextran, M_r 2,000,000). The upper limit of the column separation range was higher than thyroglobuline bovine (M_r 669,000), a protein standard resolved on the column. According to the manufacturer, the exclusion limit for globular proteins with TSK G4000-SW column is comparable to the exclusion limit for linear polymers (M_r 500,000) when using denaturing eluent. Our calibration procedure revealed a rather larger exclusion limit. Fraction F2 corresponds to protein ranges of M_r 630,000 to 116,000. Fractions F3 and F4 correspond to proteins ranges of M_r 116,000 to 65,000 (F3) and to 21,000 (F4), and could therefore be assimilated to gliadin. Fraction F5 corresponds to the smallest monomeric proteins (M_r < 21,000) and is likely to consist of water-soluble protein.

SE-HPLC profiles from wheat flour extracts were not corrected from the SDS-phosphate buffer contribution. Blank run of SDS-phosphate buffer showed a small peak overlapping with fraction F5 from flour protein. This peak represented <1% of total SE-HPLC area and <5% of F5 from total flour protein extract.

Flour Sample Stability

To test for the presence of some proteolytically active material, effects of temperature and added protease inhibitors were investigated. Flour (160 mg, 11.6%, db) from cultivar Soissons was extracted at ambient temperature and at 60°C by rotation (80 min at 60 rpm) with 20 mL of extracting buffer (1% SDS, pH 6.9). Extractions at ambient temperature were also performed using extracting buffer supplemented with either antipaïn (1 mg/mL) or phenylmethanesulfonyl fluoride (PMSF) (1 m*M*) inhibitors of cysteine and serine proteases, respectively. Total protein extract was recovered after sonication (3 min at 30% power setting) and centrifugation. Stability of all protein extracts was investigated from SE-HPLC measurements. The four extracts placed in the sample injector were sequentially and continuously injected on the column over 19 hr.

TABLE I
Levels of Extraction Factors and Trials Worksheet

	Levels		Trials (arbitrary numbers)															
Variables	**–1**	**1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Dispersion time	40 min	120 min	–1	–1	–1	–1	–1	–1	–1	–1	1	1	1	1	1	1	1	1
SDS	1%	2%	–1	–1	–1	–1	1	1	1	1	–1	–1	–1	–1	1	1	1	1
Power setting	20%	80%	–1	–1	1	1	–1	–1	1	1	–1	–1	1	1	–1	–1	1	1
Sonication time	30 sec	90 sec	–1	1	–1	1	–1	1	–1	1	–1	1	–1	1	–1	1	–1	1

TABLE II
Effects of Experimental Variables of Sonication on Protein Extractability: Estimated Effects and Significance of Regression Models[a]

	Estimated Effects for Extraction Yield	
Experimental Variables	**Design I**	**Design II**
Average	96.84 ± 0.22	96.45 ± 0.13
Homogenization time	1.82 ± 0.48*[b]	–1.11 ± 0.28*
SDS	–0.73 ± 0.48ns	1.25 ± 0.28*
Power setting	3.39 ± 0.48**	6.85 ± 0.28****
Sonication time	5.56 ± 0.48***	5.83 ± 0.28****
Power × homogenization time	0.53 ± 0.48ns	0.02 ± 0.28ns
Power × SDS	–1.00 ± 0.48ns	0.62 ± 0.28ns
Power × sonication time	–2.60 ± 0.48**	–3.21 ± 0.28****
Sonication time × homogenization	–1.19 ± 0.48ns	0.03 ± 0.28ns
Sonication time × SDS	0.03 ± 0.48ns	0.07 ± 0.28ns
Homogenization time × SDS	1.52 ± 0.48 *	0.02 ± 0.28ns
Lack-of-fit	*	**
R^2	0.77	0.85

[a] Sonication was performed before (Design I) or after (Design II) Soissons flour sample (11.68% protein, db) dispersion at 60°C. Effect of variables determined from variance analysis of 2^4 factorial design (16 runs): SDS (1–2%), power settings (20–80%), dispersion time (40–120min), sonication time (30–90 sec). Standard error was estimated from three replicates of centerpoints; R^2 values correspond to linear regression models including all linear and interacting terms for each variables.

[b] *, **, ***, **** significant at $P < 0.1$, 0.05, 0.01, and 0.005, respectively; ns = nonsignificant.

Blank runs for PMSF and antipaïn showed that these compounds are eluted after wheat flour protein and did not contribute to the SE-HPLC profile.

Statistical Analysis

The effects of various factors on the extractability of protein from Soissons flour (11.60% protein, db) were studied according to a 2^4 screening design methodology. Factors tested at two levels (–1, 1) were dispersion time at 60°C, SDS concentration of the extracting SDS phosphate buffer, sonication time, and sonication power setting. The factor levels and the trial worksheet are given in Table I. Two independent designs (Designs I and II) were used, for which dispersion of flour samples (160 mg/20 mL) was applied before (Design I) or after (Design II) sonication. For each design, three centerpoints (SDS 1.5%, homogenization time 80 min, sonication time 60 sec, sonication power 50%) were performed to test pure error model.

Reproducibility of the standard extraction procedure of flour samples was determined using standard procedures NF V 03 110 (AFNOR 1998) and NF ISO 5725 (AFNOR 1994). Data were subjected to linear regression and analysis of variance using Statgraphics software.

RESULTS AND DISCUSSION

Sample Stability

Effects of extraction temperature and protease inhibitors on the stability of total protein extract were investigated from SE-HPLC measurements performed at regular intervals over 19 hr. For all extracts, variations in total SE-HPLC area between reinjection were low (±0.4%). This indicates that the protein extracts were stable with respect to precipitation. Nevertheless, changes in the size distribution of protein were noticed between reinjection according to the extraction procedure used. Figure 1 shows that F1 area dropped with time when protein extraction was performed at room temperature. Fraction F1 was continuously decreasing, mainly at the benefit of fractions F2 and F5, whereas fractions F3 and F4 remained almost unchanged (not shown). Instability SDS-soluble protein extracts was first noticed by Huebner and Bietz (1985), who speculated the presence of some proteolytically active material. Effect of antipaïn, a thiol protease inhibitor that was effective in preventing F1 decay from protein sample extracted at ambient temperature, confirmed the involvement of proteases. Antipaïn effect was greater than that of phenylmethylsulfonyl fluoride (PMSF) a serine protease inhibitor. Together, these two types of inhibitor should prevent most of the F1 decay. Identification of papaïnase-type proteases accounting for about half of the proteolytic activity of wheat flour was first mentioned by McDonald and Chen (1964). These authors, along with Wang and Grant (1969), demonstrated the instability of wheat flour proteases at >50°C. Later, Dachkevitch and Autran (1989) showed that stable SE-HPLC extracts could be obtained by using an extraction temperature of 60°C. Accordingly, we confirmed that extraction at 60°C greatly improved sample stability (Fig. 1).

In contrast with our findings, Batey et al (1991) showed that flour extracts prepared using the sonication procedure of Singh et al (1990a) were stable over 72 hr. The reason for these inconsistent results remains unknown, particularly because the sonication method of Singh et al (1990a) comprises a 15–30 sec sonication step during which, according to the authors, sample temperature would not exceed 60°C.

Critical Variables Governing Sonication Efficiency

Protein extractability from Soissons flour was investigated according to a 2^4 factorial screening design. The design variables were dispersion time at 60°C (40 and 120 min), SDS concentration (1 and 2%), sonication time (30 and 90 sec), and sonication power setting (20 and 80%; output 1.5 and 15W).

Flour proteases were inactivated at 60°C by sample dispersion, which was performed before (Design I) or after (Design II) sonication. In accordance with findings of Singh et al (1990a), the increase in protein solubility was related to increased solubility of polymeric protein (sum of F1 and F2 areas), while the amount of monomeric proteins (sum F3 to F5 areas) remained unchanged (Fig. 2).

Effect of SDS content. Effect of SDS was significant when using Design II, but estimated effect and significance were weak compared with those of other significant variables (Table II). With Design I, the interaction factor dispersion time by SDS exerted a positive

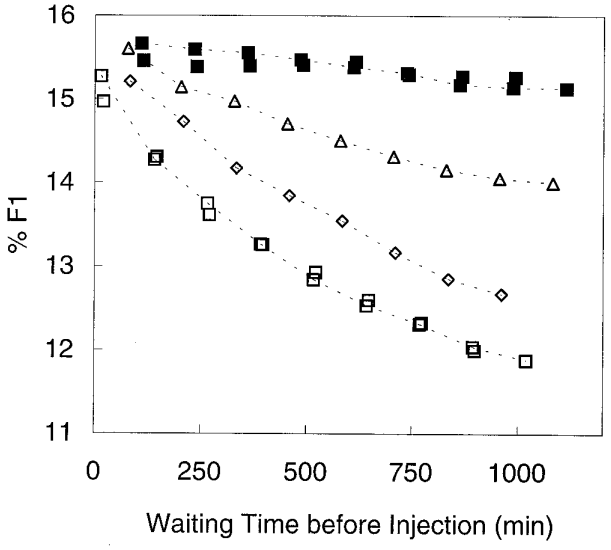

Fig. 1. Effect of protease inhibitors and dispersion temperature on stability of relative amount of F_1 area (%F1) from total protein extract analyzed by size-exclusion HPLC. Total protein extract obtained by sonication (3 min at 30% power setting) of flour sample (160 mg/20 mL) after dispersion for 80 min at ambient temperature (open symbol) or at 60°C (solid symbol). Extracting buffer (1% SDS, phosphate buffer) contained no inhibitor (■), 1 m*M* phenylmethanesulfonyl fluoride (◇) or 1 µg/mL of antipaïn (▲).

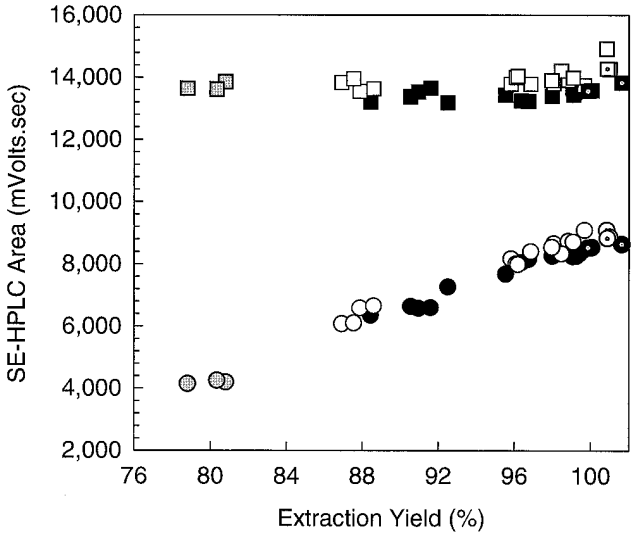

Fig. 2. Relationship between protein extraction yield of flour samples and absolute amounts of polymeric and monomeric protein measured by size-exclusion (SE) HPLC: polymeric proteins (sum of F1 and F2 areas) (○), monomeric proteins (sum of F3 to F5 areas) (□). Samples from Soissons flour (160 mg/20 mL) extracted according to a 2^4 factorial screening design (16 runs and triplicate centerpoints). Variables and levels: dispersion time at 60°C (40 and 120 min), SDS concentration (1 and 2%), sonication time (30 and 90 sec) and power settings (20 and 80%). Dispersion applied before (open symbols), after (solid symbols) or without (shaded symbols) sonication (standard SDS-soluble protein extract). Dotted symbols are center points.

effect. This means that increase in SDS concentration promoted protein extractability but only when combined with the shortest dispersion time (40 min). Batey et al (1991) showed that 0.3% SDS in phosphate buffer was sufficient to extract with sonication the total protein from a flour sample at 10 g/L. SDS can disrupt all noncovalent bonds and is an efficient dissociating detergent that is most effective at concentrations >5 m*M* (0.14%) and ≥100 m*M* (2.8%) (Jirgensons 1980). In subsequent investigations, we used 1% SDS to extract protein. This was likely to provide a broad safety margin to ensure SDS-denaturation of flour protein within a reasonable time.

Effects of sonication time and sonifier power settings. Both sonication time and power setting significantly increased protein extraction yield but there was a significant negative effect of the interaction between power and sonication time. This last result means that the gain in solubility resulting from the increase in sonication time of 30–90 sec was no longer so effective at high power setting. For example, with Design I at 20% power output, and as sonication time increased from 30 to 90 sec, mean protein extraction yield increased by 8% (from 90.4% ± 1.4% to 98.5 ± 1.2%) instead of 3% at 80% power output (from 96.4 ± 3.0% to 99.4 ± 2.4%). The nonlinearity of the increase in protein extractability during sonication was assessed by pure error model test that revealed significant lack-of-fit with both designs. In fact, the models underestimated the extraction yields obtained for centerpoints. The nonlinearity of the effects of sonication on protein extractability was previously reported by Singh et al (1990a).

Ultrasonic energy output (related to both sonication time and power) delivered to the sample during sonication, higher than the amplitude of ultrasonic vibrations (related only to sonication power setting), would account for SDS-insoluble protein breakdown.

Effect of Extraction Design

Both procedures (Designs I and II) showed similar efficiency in promoting protein extractability because almost equal average extraction yields were obtained (Table II). Nevertheless, different mechanisms could contribute to protein extractability because dispersion time showed contrasted effects according to the design. During dispersion, and in consequence of SDS binding, some of the SDS-insoluble protein complexes would be disrupted and brought into solution (Rao 1993, Couthon 1996). Conversely, it is well known that heat treatment of wetted wheat protein at ≥60°C led to a sharp decrease in gluten protein solubility (Weegels and Hamer 1998). Undoubtedly, with Design I, SDS prevented the aggregation of protein usually observed at 60°C. However, when sonication preceded dispersion (Design II), part of the SDS-insoluble proteins brought into solution by sonication precipitated during dispersion. The contrasted effect of dispersion time between both designs suggests that sonication could ensure an immediate and total saturation of the protein chains with SDS, whereas temperature could induce a slow aggregation of even SDS-saturated proteins.

Estimated effect of sonication power was lower with Design I. Because dispersion was applied before sonication, samples have higher SDS-soluble protein contents. In consequence, lower effects of sonication power would have been observed because of the nonlinear increase in protein extractability.

Sensitivity of gluten macropolymers to breakdown by sonication. Figure 3 shows that irrespective of designs, F1 area increased gradually up to 2,400 (sec × %) of ultrasonic energy. Above this level, fraction F1, which consists of very large polymers excluded from the column, tended to decrease at the benefit of fraction F2 (not shown), indicating oversonication effect. Nevertheless, decay in F1 area is not truly disclosed on Fig. 3 for the highest ultrasonic energy (7,500 sec × %) corresponding to samples sonicated at 80% sonication power setting. At this power setting, we noticed that the SDS extracting buffer tended to foam, causing a drastic drop of output energy. The phenomenon, which was random, is likely to impair the ultrasonic energy delivered to the sample. On average, the amount of excluded polymers (fraction F1 from SE-HPLC profile) was higher when using Design I (solid symbols). With Design II, and as flour sample dispersion at 60°C (performed after sonication) was prolonged from 40 min (○ and +) to 120 min (○), F1 area dropped, whereas F2 area remained rather unchanged (results not shown). These results are consistent with the negative effect of dispersion observed with Design II (Table II). Loss in protein solubility when samples were dispersed after sonication was related the precipitation of the some of the largest glutenin polymers. This suggests that the largest polymers originated from SDS-insoluble protein fraction could be especially sensitive to heat treatment.

Optimization of Wheat Flour Proteins Extraction

We attempted to optimize the sonication procedure to extract total protein, while preventing excessive breakdown of SDS-insoluble polymers. Taking into account the earlier results, we investigated power settings <50% (10–50% by 10% increments) in association with a larger range of sonication time (30, 105, and 180 sec). Pro-

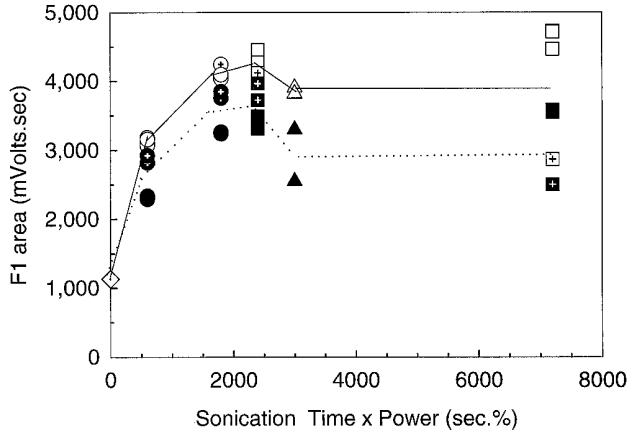

Fig. 3. Change in absolute amount of F1 area as a function of sonication time per power setting delivered during sample sonication. Samples from Soissons flour (160 mg /20 mL) were extracted according to a 2^4 factorial screening design (16 runs and triplicate centerpoints).Variables and levels: dispersion time at 60°C (40 and 120 min), SDS concentration (1 and 2%), sonication time (30 and 90 sec), and power settings (20 and 80%). Sonication power setting at 20% (○), 50% (Δ), and 80% (□). Dispersion applied before (open symbols) or after (solid symbols) sonication. Dispersion time was 40 min (+) or 120 min (unmarked).

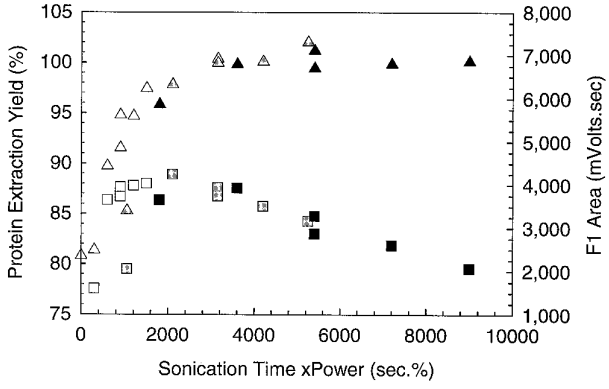

Fig. 4. Changes in protein extraction yield and absolute amount of fraction F_1 measured from size-exclusion (SE) HPLC analysis of flour samples as a function of ultrasonic energy (% × sec) delivered during sample sonication. Samples of Soissons flour (160 mg/20 mL) extracted after dispersion for 80 min at 60°C by sonication at power settings of 10–50% (increment 10%) for 30 sec (open symbols), 105 sec (shaded symbols) or 180 sec (solid symbols). Protein extraction yield (Δ), absolute amount of F_1 area (□).

cedure Design 1 was selected because it lead to larger F1 areas. Flour dispersion was set arbitrarily to 80 min and 1% SDS, phosphate buffer was used to extract protein from Soissons flour. Figure 4 presents the protein extraction yields and the F1 areas as a function of sonication time by power values (sec × %). This variable was very relevant because protein extraction yield data were distributed along an single hyperbolic curve, irrespective of sonication power. Total protein extraction was obtained as ultrasonic energy reaching 3,000 (sec × %). F1 area started to decrease below this threshold, and as ultrasonic energy output increased, showed a monotonic decay. As routine extraction procedure, we selected dispersion of flour sample (160 mg/20 mL, 1% SDS, phosphate buffer) for 80 min at 60°C, sonication at 30% of power setting for 180 sec. Thus, a 5,400 (sec × %) ultrasonic energy output was delivered to the flour sample. This level was likely to allow total protein extraction from samples with a high content in SDS-insoluble polymers, while preventing excessive oversonication of protein polymers.

Other Factors Influencing Sonication

Flour samples from Soissons and Thésée were extracted according to the standard procedure using various buffer volumes (10 or 20 mL) and flour weights (80 or 160 mg). Injection volume was adjusted to the sample solid-to-solvent ratio to compare SE-HPLC areas on the same flour content basis. Total SE-HPLC areas were rather similar irrespective of the solid-to-solvent ratio used, indicating that the extraction of total proteins was achieved in any case (Table III). Balance between F1 and F2 areas varied according to the solvent volume. With a solvent volume of 10 mL, F1 area tended to decrease at the benefit of F2 area, indicating a harder oversonication effect. The solid-to-solvent ratio or the weight of flour were not related to this shift of F1 to F2. The mechanical effects arising from acoustic cavitation would apply to a volume unit and not to a flour weight unit;. the shearing stress underwent by protein would increase as solvent volume decrease. Considering this finding, we tested various locations (top, bottom and middle) for the sonicator probe tip within the test tube, but no change was observed on SE-HPLC profiles (results not shown).

Reproducibility

Reproducibility of the protein extraction and SE-HPLC measurement was estimated according to two standardized procedures, NF V 03 110 (AFNOR 1998) and NF ISO 5725 (AFNOR 1994) For the NF V 03 110 procedure, a set 30 flour samples showing a wide range of protein content and baking quality was run three times in random order. For the NF ISO 5725 procedure, a flour sample with standard characteristics was run in four replicates for 18 consecutive days. Variance analysis was conducted for each SE-HPLC fractions. Results (Table IV) showed that a very satisfactory reproducibility was obtained for fractions F3 to F5. The higher standard deviations calculated for fractions F1 and F2 remained within a reasonable range.

SE-HPLC Profiles from SDS-Soluble, SDS-Insoluble, and Total Protein Extracts

An example of typical SE-HPLC profiles from total, SDS-soluble, and SDS-insoluble protein extracts obtained from the same flour is given in Fig. 5. These three types of extracts were obtained from a set of 27 flour samples and analyzed by SE-HPLC. Surface areas from the total protein profile on one hand, and from the sum of SDS-soluble and SDS-insoluble profiles on the other hand (sum profile), were considered. Total areas from both types of profiles were strongly related (r = 0.94) and on average differed by 2 ± 4%. Also, the total amounts of polymeric protein (Gpol) estimated from the sum of F1 and F2 areas, either from total protein profile or from the sum profile were in close agreement (r = 0.97) and on average differed by 1.6 ± 3.4%. Nevertheless, from the typical profiles presented in Fig. 5, it is obvious that F1 area from the sum profile will be larger than the one obtained from the total protein extract. On average within the flour sample set, F1 area estimated from the sum profiles was 25 ± 4.3% larger than the area calculated from total protein profiles, both still highly related (r = 0.96). This result suggests that sonication could shift the size distribution of SDS-soluble polymeric protein toward smaller species or that oversonication could be less marked on a protein pellet than on a dispersed flour sample.

TABLE III
Effect of Solid-to-Solvent Ratio on Extractability of Total Protein from Size-Exclusion (SE) HPLC Measurements

			Total SE-HPLC Area[a]		F_1 Area (%)		F_2 Area (%)	
Flour (mg)	Solvent (mL)	Ratio (mg/mL)	Soissons	Thésée	Soissons	Thésée	Soissons	Thésée
80	20	4	22,790,856	20,706,825	16.67	15.18	21.71	23.54
80	10	8	22,987,574	20,569,092	14.35	13.04	24.46	25.78
160	20	8	22,866,002	20,688,749	16.39	14.77	22.21	23.71
160	10	16	22,654,978	20,207,820	14.26	12.18	24.82	26.61

[a] Injection volume was 40 µL (80 mg/20 mL), 20 µL (80 mg/20 mL and 160 mg/20 mL), and 10 µL (160 mg/10 mL). Total protein (db) was 11.6% from Soissons and 10.3% from Thésée. SE-HPLC areas given in µV/sec.

TABLE IV
Reproducibility Data (within laboratory) from Size-Exclusion (SE) HPLC Analysis

	NF V 03 110[a]		NF ISO 5725[b]	
	S(R)[c]	*R*[d]	S(R)[c]	*R*
%F1	0.36	1.00	0.37	1.04
%F2	0.37	1.04	0.47	1.32
%F3	0.17	0.48	0.24	0.67
%F4	0.17	0.48	0.24	0.67
%F5	0.11	0.31	0.27	0.76
Gpol[e]	0.21	0.59	· · ·	· · ·
Monomer[f]	0.21	0.59	· · ·	· · ·

[a] 30 flour samples (3×).
[b] 1 flour sample (18 days × 4 replicates).
[c] Reproducibility standard deviation (residual standard error from random model variance analysis)
[d] Reproducibility R (95%), (2.8·S(R))
[e] Glutenin polymeric protein (sum of F1 and F2 areas),
[f] Sum of F3, F4, and F5 areas.

Usefulness of SE-HPLC Profiles for Predicting Rheological Properties of Flour Dough

Tables V and VI give correlation coefficients between flour protein content and *W* alveographic index and absolute or relative amount of SE-HPLC fractions from SDS-soluble, SDS–insoluble and total protein extracts obtained from the 27 flour samples.

Flour protein content was strongly correlated with total SE-HPLC area for each of the three extracts, the highest coefficient was obtained for the total protein extract. This indicated consistent extraction of flour protein by sonication from diverse cultivars, showing large variations in protein content and technological quality. Within the SE-HPLC fractions, the highest coefficient of correlation was obtained with fraction F4 for all the three extracts. Absolute amount of gliadin (F4) would be a good indicator of the flour protein content. Polymeric proteins (F1 and F2) from total protein extract were also highly correlated to flour protein content, but lower coefficients of correlation were found considering F1 and F2 fractions from both SDS-insoluble protein and SDS-soluble protein extracts. This suggests that the balance between insoluble protein polymers and their soluble counterparts is not controlled by the flour protein content and would vary with wheat genotype (Gupta et al 1993, Ciaffi et al 1996, Bean et al 1998).

As a general trend, the relative proportions of SE-HPLC fractions were not strongly related to flour protein content except for the %F5. In terms of absolute amount, F5 showed the narrowest range of variation in relation with flour protein content. Consequently, the relative amount of F5 fraction significantly decreased with increasing flour protein content.

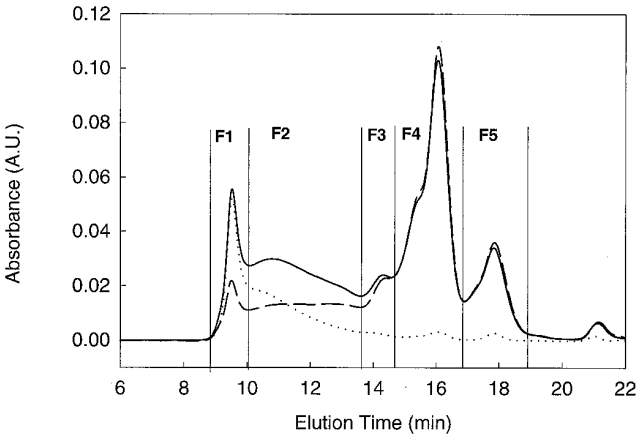

Fig. 5. Size-exclusion (SE) HPLC profiles from SDS-insoluble, SDS-soluble, and total protein extracts. All protein extracts obtained from cultivar Tigre (8.89% protein, db. SE-HPLC profiles adjusted to solvent-to-solid ratio of 160 mg/20 mL. SDS-soluble protein extract (– – – –),SDS-insoluble protein extract (·····), and total protein extract (——).

For total protein extract, similar correlation coefficients with *W* index were obtained for total polymeric protein (Gpol) and total SE-HPLC area. Gpol showed that the total polymeric protein in flour was not a better criterion for predicting flour dough strength than total protein content, which is in agreement with previous findings of Gupta et al (1993). *W* index was highly positively correlated with the total amount of SDS-insoluble proteins, which is in accordance with the finding of Ciaffi et al (1996). A very significant coefficient of correlation ($r = 0.71$) was also found between the area of fraction F1 from total protein extract and the *W* index, demonstrating that fraction F1 provided a good estimate of the amount of SDS-insoluble proteins in flour.

Weaker relationships were found between the relative amount of SDS-insoluble protein in total protein or the SDS-insoluble polymer in total polymer, and the *W* index ($r = 0.66$ and 0.67, respectively) (not shown). Thus, within these flour samples, it appears that *W* index was more highly correlated with the amount of SDS-insoluble protein in flour than with the relative amount of SDS-insoluble protein in total protein. Similar findings were found by Ciaffi et al (1996) and Bean et al (1998) for wheat flour quality attributes relative to dough strength (Alveographic *P* index and loaf volume or mixing tolerance).

As judged from the absolute values of the correlation coefficients calculated with *W* index, %F1 and F1/F2 ratio from SDS-soluble protein extract were good indicators of SDS-insoluble polymer in total polymer and of relative amount of SDS-insoluble polymers in total protein, respectively. Dachkevitch and Autran (1989) previously discussed similar findings. By comparison, lower coefficients of correlation with *W* index were found for %F1 ($r = 0.33$) and for F1/F2 ratio ($r = 0.45$) from total protein extract. This was logical because SDS-insoluble polymers are partly contaminated by SDS-soluble polymers within fraction F1 from total protein extract.

It was supposed that glutenin polymers become insoluble when the polymer size become larger than some not-yet-defined threshold (MacRitchie 1992, Kasarda 1999). Shift of the size distribution of polymers toward larger insoluble species results in flour dough strength increase as illustrated by the negative relationship between *W* index and %F1 or F1/F2 ratio from SDS-soluble proteins. Should sonication shift the size of SDS-insoluble polymers below the solubility threshold, a shifted profile of the initial distribution would be expected. This was not disclosed by

TABLE V
Correlation Coefficients for Absolute Amounts of Size-Exclusion (SE) HPLC Areas from SDS-Soluble, SDS-Insoluble, and Total Protein Extracts[a]

	SDS-Soluble Protein							SDS-Insoluble Protein							Total Protein						
Flour	**T[b]**	**F1**	**F2**	**F3**	**F4**	**F5**	**Gpol[c]**	**T[b]**	**F1**	**F2**	**F3**	**F4**	**F5**	**Gpol**	**T[b]**	**F1**	**F2**	**F3**	**F4**	**F5**	**Gpol**
Protein content	0.95	0.34	0.70	0.72	0.88	0.51	0.60	0.71	0.65	0.70	0.62	0.79	0.65	0.70	0.99	0.87	0.90	0.83	0.92	0.57	0.94
W index	0.43	–0.34	0.01	0.10	0.48	0.02	–0.10	0.84	0.73	0.87	0.85	0.85	0.50	0.83	0.57	0.71	0.38	0.31	0.62	0.15	0.53

[a] Samples set (27 flours) analyzed for linear correlation. Correlation coefficients >0.38 and 0.48 are significant at $P < 0.05$ and 0.01, respectively. Values of SE-HPLC fractions from SDS-soluble protein and SDS-insoluble protein are averages of duplicate extracts; those from total protein are averages of triplicate extracts.
[b] Total SE-HPLC area.
[c] Glutenin polymeric protein, sum of F1 and F2 areas.

TABLE VI
Correlation Coefficients for Relative Amounts of Size-Exclusion (SE) HPLC Areas from SDS-Soluble, SDS-Insoluble, and Total Protein Extracts[a]

	SDS-Soluble Protein						SDS-Insoluble Protein						Total Protein					
Flour	**%F1**	**%F2**	**%F3**	**%F4**	**%F5**	**F_1/F_2**	**%F1**	**%F2**	**%F3**	**%F4**	**%F5**	**F_1/F_2**	**%F1**	**%F2**	**%F3**	**%F4**	**%F5**	**F_1/F_2**
Protein content	–0.21	–0.04	0.32	0.40	–0.77	–0.11	0.16	–0.12	–0.39	0.46	–0.53	0.15	–0.14	0.00	0.30	0.44	–0.71	–0.10
W index	–0.67	–0.53	–0.21	0.41	–0.54	–0.69	0.07	0.04	–0.14	0.32	–0.70	0.03	0.33	–0.30	–0.13	0.48	–0.56	0.45

[a] Samples set (27 flours) analyzed for linear correlation. Correlation coefficients >0.38 and 0.48 are significant at $P < 0.05$ and 0.01, respectively. Values of SE-HPLC fractions from SDS-soluble protein and SDS-insoluble protein are averages of duplicate extracts; those from total protein are averages of triplicate extracts.

SE-HPLC analysis; no relationship was found between %F1 and F1/F2 ratio from SDS-insoluble protein and *W* index. Nevertheless, protein polymers (Gpol) from SDS-insoluble fraction might present contrasting size-distribution ranges which could be assessed by light-scattering measurements or by fractionation on a column with a larger pore size packing.

CONCLUSIONS

Ultrasonic energy delivered to the flour sample was the main variable affecting protein extractability and size distribution. Our results show that oversonication (i.e., breakdown of soluble polymers) occurs while total protein extraction is not fully achieved. Polymeric proteins excluded in fraction F1 were very sensitive to ultrasonic disruption. We delivered limited ultrasonic energy to the sample to maximize the F1 area, while achieving total protein extraction. Thus, F1 area from total protein extract would mainly consist of polymers resulting from the disruption of the unextractable polymeric fraction. Dispersion of flour sample at 60°C allows the inactivation of proteases responsible for SE-HPLC profile instability. SE-HPLC analysis of the size distribution from total protein extract obtained according to our procedure provides at the same time, reliable estimates of flour content in total protein, gliadin, total, and unextractable glutenin polymers.

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