Determination of Wheat Proteins in Solution by Dye Binding in Flour, Dough, and Bread Crumb

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ABSTRACT

Color development by the Bradford reagent with soluble wheat proteins has been examined so as to allow an easy and reliable application. The range examined was 0.3–3.5 mg/ml of protein. Second-order regressions fully interpret the nonlinearity in the color response. The reasons for nonlinearity were investigated. Commercial gliadin, standard gluten, water and an acid extract of wheat flour, and bovine serum albumin were assayed. The absorbance developed per unit weight differed. The spectral response depended on the polypeptide composition of the samples assayed. Dye binding proved to be a rapid and reliable technique for the routine assay of soluble proteins in wheat flour, in dough, and after baking. Data obtained is in full accordance with total nitrogen determinations.

Some commonly used techniques for measuring proteins in solution are based on dye-binding capacity. Interaction with the protein modifies the absorption spectrum of the dye, and this allows quantitation of the complex. This type of assay has the advantage of being rapid and highly reproducible (Bradford 1976, Sedmak and Grossberg 1977, Esen 1978, Amhad and Saleemuddin 1985).

Coomassie Brilliant Blue stain is the absorption reactant most frequently used. Compton and Jones (1985) reported that the Coomassie Blue G dye exists in three states: an anionic blue form that absorbs maximally at 595 nm, a neutral green form with maximum absorption (λ_{max}) at 650 nm, and a cationic red form that peaks at 470 nm. The dye interacts with: 1) basic amino acid residues; 2) the terminal amino group in the polypeptide chain; and 3) also, according to some authors, with aromatic residues (Amhad and Saleemuddin 1985, Compton and Jones 1985). Binding shifts the equilibrium toward the species absorbing at 595 nm, and the increase of absorption at this wavelength is proportional to protein content (Compton and Jones 1985). Using Coomassie Blue, Bradford developed a highly sensitive method for protein analysis that does not suffer from the typical interferences of the Lowry and biuret tests (Bradford 1976) except when used in the presence of detergents, especially sodium dodecyl sulfate (SDS) and Triton (Sedmak and Grossberg 1977, Esen 1978, Stoscheck 1990). According to these authors, 4*M* urea does not interfere with color development. A detailed study aimed at optimizing the assay conditions was performed by Read and Northcote (1981).

Two major limitations were reported: 1) the amount of protein estimated depends upon the protein used (Bradford 1976, Esen 1980a, Read and Northcote 1981, Stoscheck 1990); 2) color development is not linear over wide ranges of protein concentrations (Bradford 1976, Esen 1978, Read and Northcote 1981, Stoscheck 1990). Sedmak and Grossberg (1977) reported that the response is linear when protein quantity is plotted against the ratio A_{620}/A_{465}.

Esen (1978) spotted the protein solution on Whatman chromatography paper before staining with Coomassie Blue. The test became more rapid and sensitive but displayed considerable variability at low protein concentration, the very range where linearity was best observed.

Color variability among different proteins is one of the most troublesome aspects of protein assay by dye binding using Coomassie Blue. Binding of different proteins to Coomassie Blue is attributed to different amino acid compositions (Read and Northcote 1981, Amhad and Saleemuddin 1985, Stoscheck 1990). In part, this problem can be overcome by using conditions that favor the binding of the dye to basic residues. This can be achieved by adding NaOH to the reagent, which increases the anionic form of the dye. Under these conditions, proteins poor in basic residues give a more pronounced color increase than do proteins rich in basic residues (Stoscheck 1990). A similar result is obtained by decreasing the amount of phosphoric acid in the reagent or increasing the level of Coomassie Blue (Read and Northcote 1981). Nonetheless, these modifications do not completely eliminate the differential reactivity.

Direct quantitation of dissolved cereal proteins is used relatively rarely. This is probably because the availability of automatic equipment makes the assay of total nitrogen easy. Also, in studies using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), quantitation of the separated bands by laser scanning has become rather common. A ready and reliable test for the direct assay of dissolved proteins is quite desirable.

Reduced reactivity of cereal proteins in dye binding with respect to bovine serum albumin (BSA) was noted (Esen 1980a, Skerrit and Hill 1990), as well as a somewhat lower reproducibility in dye binding compared to that of the micro-Kjeldahl assay (Esen 1980a). Ratios of values determined by the two procedures depended upon the corn variety. This effect was attributed to presence of free amino acids (Esen 1980a,b). Every (1987) used the absorbancy developed at 595 nm by dye binding to assess residual soluble protein in heat-damaged wheat but did not correlate the color response to the protein content.

In the course of studies on the protein component of wheat products, it appeared necessary to understand the conditions of color response more thoroughly than literature data allows. We therefore studied the behavior of color development by dye binding in gluten, gliadins, water, and acid-soluble wheat proteins. We investigated which approach out of the several proposed was best reproduced. The conditions fixed provided a reliable routine assay for wheat protein in flour, dough, and bread crumb.

MATERIALS AND METHODS

Materials

Coomassie Brilliant Blue G-250 stain was obtained from Bio-Rad; commercial gliadins, orthophosphoric, lactic, and acetic acids were obtained from Fluka; 2-mercaptoethanol was obtained from Merck; and ethanol was a Naker Analyzed reagent. BSA and gluten were obtained from Sigma. Commercial flour from mixed soft wheat cultivars, 00 grade, was supplied by Vigevano Milling Co., Italy.

The dough was produced by mixing flour, water, and commer-

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cial compressed yeast (61:27:2) in an arm mixer (Hobart) for 10 min; it was then kept at 35–37°C for 75 min. An aliquot, frozen in liquid nitrogen, dried, ground, and sieved (particle size = 45 mesh) was stored for the tests. The dough was cooked in a forced-convection oven at 220°C for 25 min. After cooling, crumb was taken from the middle of the loaf and was treated like the dough. Flour, freeze-dried dough, and crumb contained, respectively, 14.3, 7.2, and 5.9% water and 13.2, 14.3, and 14.1% (dwb) protein.

Protein Assay with Coomassie Brilliant Blue

Protein assay with Coomassie Blue followed the procedure of Bradford (1976). A 100-μl protein sample was mixed at room temperature with 5 ml of water containing 0.12 m*M* Coomassie Brilliant Blue, 1.02*M* ethanol, and 0.87*M* orthophosphoric acid. The absorbance at 595 nm was read on a Varian DMS 90 spectrophotometer after 5 min and within 1 hr after mixing, against a reference that omitted the protein.

According to Read and Northcote (1981), under the conditions mentioned the dye-protein interaction was complete, even with poorly reactive proteins. As observed also by the above authors, no aggregation occurred and highest values of A_{595} developed. Duplicate samples were assayed. The spectrophotometric data were processed using the program Sigma Plot from Jandel Scientific.

The spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer interfaced with a computer and PECSS software (Perkin Elmer).

Total nitrogen was determined in an automatic analyzer (NA 1500, Carlo Erba). Samples were assayed in triplicate. The nitrogen standard was atropine (Carlo Erba). The conversion factor was 5.7 (Tkachuk 1969).

Polyacrylamide Gel Electrophoresis

SDS-PAGE was run using 12% polyacrylamide gels in the presence of 0.17*M* 2-mercaptoethanol (2-ME) according to Laemmli (1970). The sample was solubilized in the denaturing mixture of 31 m*M* Tris-HCl (pH 6.8); 66 m*M* SDS; 0.17*M* 2-ME; and 2.9 m*M* glycerol. After electrophoresis, the gels were stained with Coomassie Blue and processed using a video-image computer system and the software Cream (Kem-en-Tech).

The apparent molecular mass (M_r) of the separated polypeptides was determined by comparison with a Sigma standard mixture of proteins (myosin, β-galactosidase, phophorylase b, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme).

Preparation of Standard Gluten

Commercial gluten (15 g) defatted for 5 hr in a Soxlhet apparatus was extracted with water (1:20, w/v) at pH 6.5, and the suspension was centrifuged for 15 min at 5,900 × *g* at 20°C. The pellet was solubilized in 5 m*M* acetic acid (1:20) and centrifuged as above. The supernatant was dialyzed against water, freeze-dried, ground, and sieved (45 mesh). For assay, the powder was dissolved in either 5 m*M* lactic acid or 0.05*M* acetic acid.

Proteins (as total N) represented 86 ± 1% of dry weight and 64% of total nitrogen before extraction.

Extraction of Proteins

Extraction of proteins from flour, dough, and bread was done sequentially; first in water and then at acid pH by a modified Shogren procedure (Shogren et al 1969). Samples (250 mg) were mixed with 5 ml of water containing 0.1 m*M* dithiothreitol (DTT) and 1 m*M* ethylene diaminotetracetic acid (EDTA), and brought to pH 6.5, stirred for 1 hr at room temperature, and centrifuged for 15 min at 5,900 × *g* at 20°C; the supernatant formed the water extract. The pellet was suspended in 5 ml of 5 m*M* lactic acid (pH 3.0–3.5) and treated as above. The final supernatant was the acid extract. Water and acid suspensions from bread were heated at 100°C for 1 hr before centrifuging. This treatment improved protein recovery. For nitrogen analysis, the extracts were freeze-dried (Christ Alpha 1-4 equipment, B. Braun) and ground.

RESULTS AND DISCUSSION

Monitoring the Dye-Binding Response

Figure 1 shows the spectra of the dye without protein added and with BSA, commercial gliadin, standard gluten, water, and acid extracts of flour. It appears that with all proteins, the absorbance of the neutral dye (λ_{max} 650 nm) significantly overlaps that of the protein-bound anion (λ_{max} 595 nm), whereas the 460 nm absorbance is due only to the cation.

On a weight basis, BSA and, to a lesser extent, the water extract from flour absorb significantly more at 595 nm than do gluten, gliadin, and the acid extract from flour. The absorbance spectra of these three preparations practically overlap.

Absorbance values at 595 nm of gluten samples and of water extracts from flour in the range of 0.3–3.5 mg/ml of protein, determined as total nitrogen, are given in Figure 2. Lack of linear response confirmed literature reports. It was thus necessary to establish which regression best interpreted the spectrophotometric data and allowed the absorbance of unknown samples to be compared with standards. This was done by using first-order and second-order regression analysis. First-order regression was applied both to the 595 nm absorbance of samples subtracted of dye absorbance and to the ratios of such values to readings at 460 nm against water (Fig. 2, A and B). Readings at 595 nm are more specific for the protein-bound dye anion than those at 620 nm, the wavelength used by Sedmak and Grossberg (1977). At 620 nm, under our conditions, the unreacted neutral dye overlaps more heavily with the anion (Fig. 1, see also Compton and Jones 1981).

Figure 2 gives the mean, the 95% confidence interval at each protein value ($t \times SD/\sqrt{n}$, where $t = 3.18$ and $n = 4$), and the 95% confidence interval of the data distribution of four gluten acid extracts and four water extracts of flour. Gluten proteins and albumins are thus separately considered. First-order regression required three equations yielding the 95% confidence intervals indicated in Figure 2A. The gradient decreased upon increasing the protein, and the intercepts on the *y* axis significantly differed from zero. The distribution of ratios A_{595}/A_{460} could be interpreted by a single linear equation, with the 95% confidence

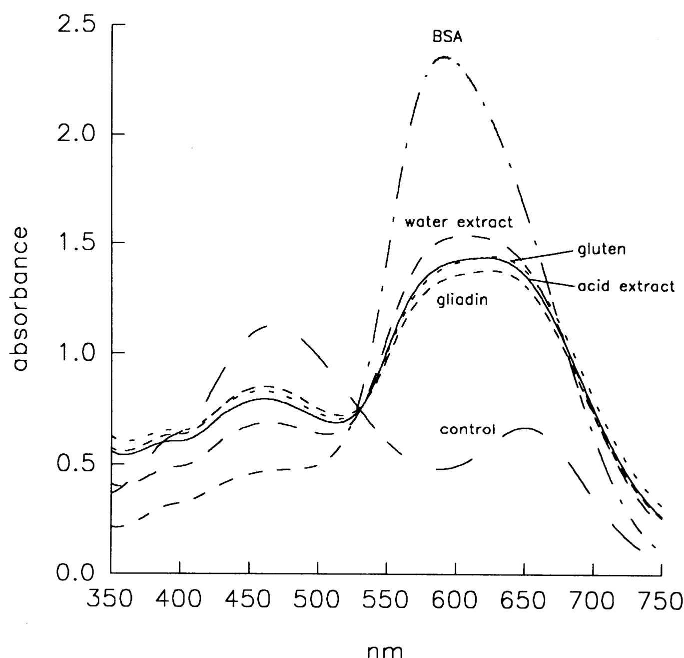
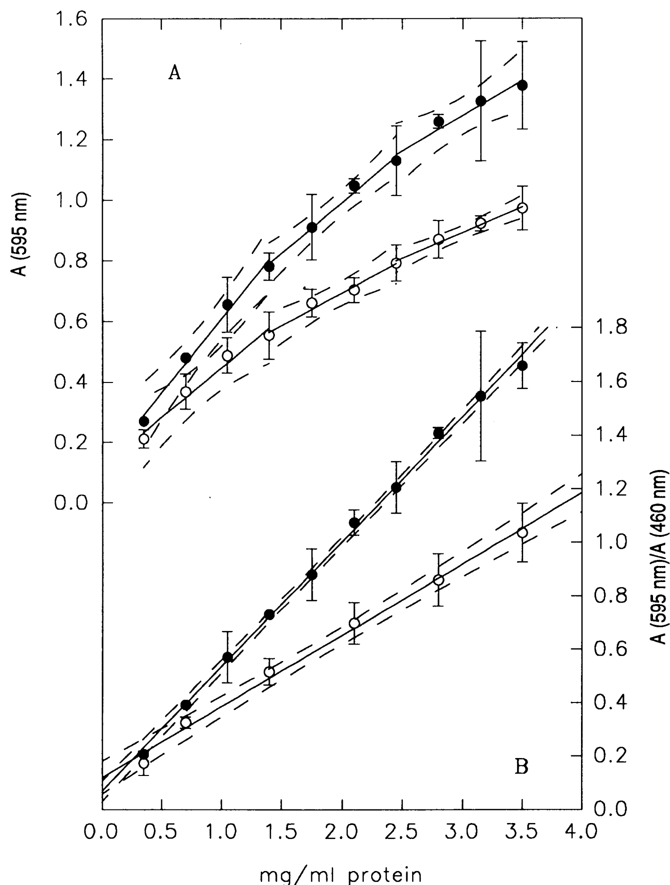


Fig. 1. Spectra of Bradford reagent with and without (control) protein added. The mixtures assayed contained 5 ml of Bradford reagent and 0.1 ml of protein solution (3 mg/ml) of bovine serum albumin (BSA), gluten, gliadin, water extract, and acid extract of wheat flour. All spectra were recorded against water. Spectra of proteins dissolved in 0.05*M* acetic or 5 m*M* lactic acid were equivalent.

interval shown (Fig. 2B). A second-order regression curve was best suited to experimental data (Fig. 2C); the 95% confidence interval was smaller than that for first-order regressions. The assay at 595 nm requires spectrophotometric readings at a single wavelength. Therefore it is simpler and is thus preferred. Under these circumstances, the use, advocated by Stoscheck (1990), of conditions that enhance dye reactivity becomes less necessary.



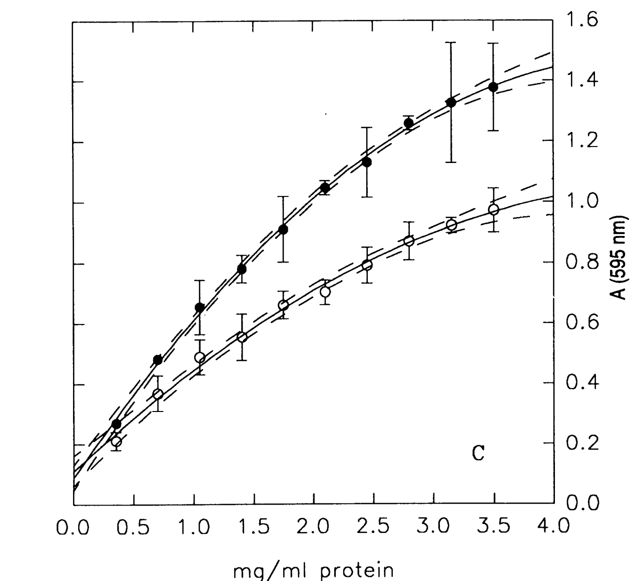


Fig. 2. Regression analysis of the dye-binding assay of standard gluten (○) and of the water extract of flour (●). Average values of the 595 nm absorbance of four samples were interpreted with linear (A) and second order (C) regression. The ratios between averages of absorbance at 595 and at 460 nm were interpreted with linear regression (B). The 95% confidence interval for the data distribution (dotted lines) and the one of the means ($t \times$ standard deviation/$\sqrt{n}$) at each protein concentration (vertical lines) are indicated. Protein concentration was measured by total nitrogen assay.

Bradford (1976) suggested that the source of nonlinearity lies in the fact that the background absorbance at 595 nm due to unreacted Coomassie Blue decreases as more dye is bound to protein, whereas a constant blank value is subtracted. As can be inferred from Figure 1, both cation (λ_{max} 460 nm) and neutral form of dye (λ_{max} 650 nm) contribute the background absorption at 595 nm. In the reaction, the cation follows nonlinear kinetics with respect to protein concentration (Fig. 3). Its quantity can be determined as 460 nm absorbance, but it does not represent the real background, because the contribution of the neutral form cannot be established.

As shown in Figure 4, the calculated curves relating A_{595} to gliadin and gluten protein, the 95% confidence intervals of mean absorbance values, and data distribution are practically coin-

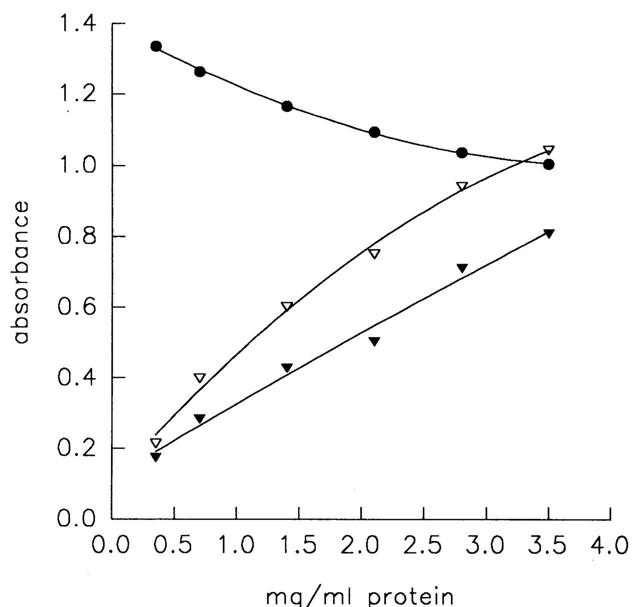


Fig. 3. Absorbance of standard gluten solutions upon addition of 5 ml of Bradford reagent at: 460 nm (●), 595 nm (△), and 650 nm (▲).

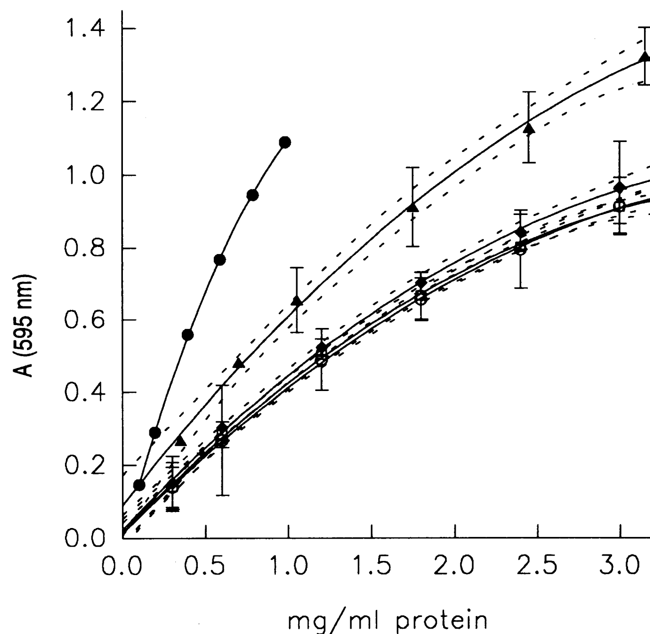


Fig. 4. Reactivity of different proteins with Coomassie Blue. Absorbance values of bovine serum albumin (●), gluten (○), gliadin (□), water (▲) and acid (◆) extracts of wheat flour were interpreted with second-order regressions. The 95% confidence interval of means (vertical lines) and of the data distribution (dotted lines) are shown.

cidental. All mean-absorbance values in the acid extract from flour are included in the same range. A gluten standard was therefore routinely used for all these preparations.

The 95% confidence intervals of the mean absorbance values in water extract from flour overlapped with those of other preparations up to 1.0 mg/ml of protein, but not for higher amounts. Also the data distribution and its 95% confidence interval were different. Therefore, a standard made of the same protein fraction, quantified by total nitrogen determination, was used for comparison.

The SDS-PAGE profiles of standard gluten, gliadins, and the acid extract of flour were similar and were mainly composed of glutenin- and gliadin-like polypeptides. This justifies their similar reactivity with Coomassie Blue. The water extract displays large amounts of polypeptides in the 15–10 kDa and 60 kDa regions (Fig. 5), which are attributed to albumins in accordance with other well-known albumins (CRC 1970). These albumins may be the cause of the special spectral behavior of the water extract.

The content of amino acids reactive to Coomassie Blue in the proteins considered is given in Table I. The color developed by the various proteins in our assay conditions best complies with the sum of all the Coomassie Blue-reactive amino acids or with the sum of lysine and arginine residues. A recent study by Congdon et al (1993), indicates that the different color developed by various proteins may primarily depend upon numbers of arginine and lysine residues. However, using synthetic polyaminoacids, Compton and Jones (1985) showed that the color response by poly(arg), poly(tyr), poly(trp), poly(his), and poly(phe) relative to poly(lys) made equal to 1.0, were 36.0:4.7:4.4:4.2:1.9. The diversity in color response of proteins underlines the importance that structural features may have in determining the binding and absorptivity of the dye.

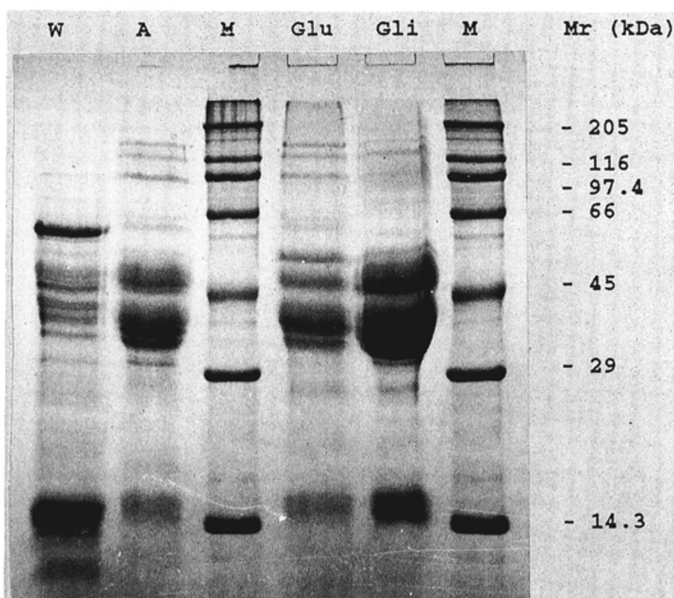


Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12% acrylamide gel in the presence of 0.17*M* 2-mercaptoethanol. W = water, A = acid extracts of wheat, M = marker proteins, Glu = gluten, Gli = gliadin. Molecular mass (M_r) of marker proteins is indicated.

TABLE I
Amino Acid Content (g/16 g of N) in Wheat Proteins and Bovine Serum Albumin (BSA)[a]

	Arg	Lys	His	Tyr	Trp	Phe	Arg+ Lys	Arg+ Lys+ His+ Tyr+ Trp+ Phe
Albumin[b]	5.1	3.2	2.0	3.4	1.1	4.0	8.3	18.8
Gliadin[c]	1.9	0.5	1.6	2.2	0.7	6.0	4.5	12.9
Gluten[d]	3.0	1.5	1.7	3.4	2.2	4.1	4.5	15.9
BSA	6.0	13.0	4.0	5.2	0.6	6.5	19.0	35.3

[a] Bushuk and Wrigly 1974 and Dayhoff 1976.
[b] Water-soluble proteins.
[c] 70% Ethanol-soluble proteins.
[d] 0.5*M* acetic acid-soluble proteins.

Routine Spectrophotometric Assays of Wheat Products

Protein extraction and the spectrophotometric assay of unknown samples and the standard was performed as described above. Second-order reference regressions were established in the range of 0.3–3.5 mg/ml of protein using proper standards, i.e., gluten for the acid extracts of flour, dough, and bread (flour-water extract determined by total nitrogen after freeze-drying was the standard for the water extracts). Unknown values were referred to standard curves by the equation:

$$ax^2 + bx + c - d = 0$$

where d is the 595 nm absorbance of the unknown sample; a, b and c are the coefficients of the reference parabola; and x is the unknown amount of protein.

The spectrophotometric and nitrogen assay of protein content in the various extracts from wheat flour, dough, and bread crumb are compared in Table II. Spectrophotometric assays were run in duplicate. Nitrogen determinations were made in triplicate on two separate extractions of the same material. Standard deviations, therefore, reflect the reliability of the extraction and that of the assay procedures.

Standard deviations indicate that the dye-binding assay and the extraction method were highly reproducible. Dye binding was more reliable than the total nitrogen determination. This difference may depend upon the more troublesome procedure (freeze-drying, weighting, etc.) involved in nitrogen assay.

Water-soluble proteins increased slightly in the dough, while bread displayed a much lower overall extraction compared to flour and dough. These results are in accordance with literature reports (Collar et al 1989, Pomeranz 1989, Westerlund et al 1989). Dye-binding assays were not made on insoluble residues because, for this, the poorly soluble material makes total nitrogen determination preferable and easiest.

Given the equivalence of acetic and lactic acids in the assay, the method can also be applied to determining acid-soluble proteins of isolated gluten; 70% ethanol and 50% propanol, which are generally used for gliadin extraction, modify the spectrum of blank and protein. More experiments are needed before reproducible conditions for assay are established.

The results of the present study provide a detailed analysis of color development in the dye-protein interaction and thereby

TABLE II
Soluble Protein in Wheat Products[a]

	Spectrophotometric Determination (%)	Nitrogen Determination (%)
Flour		
Total protein (mg/100 mg, dwb)		13.2 ± 0.2
Water extract	19.4 ± 0.2	21.9 ± 2.9
Acid extract	57.7 ± 0.4	56.5 ± 0.7
Dough		
Total protein (mg/100 mg, dwb)		14.3 ± 0.1
Water extract	22.3 ± 0.1	18.9 ± 1.4
Acid extract	58.2 ± 0.4	59.4 ± 2.8
Bread crumb		
Total protein (mg/100 mg, dwb)		14.1 ± 0.5
Water extract	6.9 ± 0.8	9.7 ± 0.9
Acid extract	8.3 ± 0.1	7.2 ± 1.2

[a] Extractions and assays were as described in the text. The results are given as percent of total protein, measured by nitrogen determination in the unfractioned sample. Two separate extraction were measured in each case: spectrophotometric determinations were in duplicate, nitrogen determination in triplicate.

give additional information for the understanding of the mechanism of the reaction. The rapid and reliable assay conditions established for the colorimetric assay will be of great use whenever determination of wheat proteins in solution is needed. The procedure represents a considerable advance with respect to total nitrogen determinations, which, even with automatic equipment, are more time-consuming, and do not distinguish between protein and other nonprotein nitrogen compounds.

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