SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR RAPID EXAMINATION OF SIZE DIFFERENCES OF CEREAL PROTEINS

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INTRODUCTION

Considerable research has been carried out on proteins of wheat and other cereals to determine relations between composition and effect on quality. Because quality is often associated with the occurrence of large protein aggregates, it is essential to assess the size range of gluten proteins and to determine the proportions of aggregating and monomeric proteins in flour or grain. SDS-PAGE can be used to separate prolamins or subunits of glutelins according to molecular size. However, because the reduction of S-S bonds is a prerequisite to investigate large size aggregates, SDS-PAGE studies cannot avoid a loss of information on how the individual polypeptides interact to form large glutenin polymers. Therefore, techniques that could perform high-resolution separations of proteins in a non-denaturing manner would have a decisive advantage to investigate protein functionality in the various applications of wheats. Size-exclusion chromatography, later adapted to high-performance methods as size-exclusion high-performance liquid chromatography (SE-HPLC), is the technique most commonly used to retain information at the level of protein aggregates and to give insight into structure and interactions between components. This chapter is an attempt to review methods of SE-HPLC

analysis of cereal proteins, especially for rapid examination of size differences. The first part presents basic principles, and considers the equipment and procedures. Practical information, to assist investigators, is not described in detail since it is largely common to all types of HPLC, but indications relating to specific problems of SE-HPLC fractionation of cereal proteins are given where relevant. I then consider the application of SE-HPLC to studies of wheat proteins (characterization of molecular size, discrimination of genotypes, quality prediction).

BASIC PRINCIPLES

In size-exclusion chromatography (also called gel permeation or gel filtration), the fractionation support is a gel column. The principle of separation is based on a restricted molecular diffusion in the gel granules depending on their porosity. If molecules or aggregates are larger than the average diameter of support pores, they are not able to diffuse into the pores of the particles of the stationary phase: they are excluded and elute at the void volume. If they are smaller, they are retarded as a consequence of their greater or lesser penetration into the porous stationary phase (Fig. 1). Because solutes penetrate the pores differentially, elution time is a function of dynamic volume, so that the separations observed are mainly based on molecular size (although some ionic adsorption may also occur).

Since gel filtration media permitting good separations have been developed, such as Sephadex G-100 or G-200 (loosely woven strands of dextran polymer), Sepharose (beaded agarose), Biogel (polyacrylamide gel), or controlled pore glass, several outstanding studies of wheat proteins have been reported. For instance, ethanol-soluble fractions could be divided into HMW-gliadin, ω-gliadin and LMW gliadin on Sephadex G-100 (Bietz and Wall, 1980), whereas reduced and alkylated glutenin could be separated into three distinct groups of subunits on Sephadex G-200 by Khan and Bushuk (1979). In addition, Huebner and Wall (1976) could observe for the first time the distribution of molecular weights of native glutenin on Sepharose 4B, with the largest peak having a possible mol wt of more than 20 million.

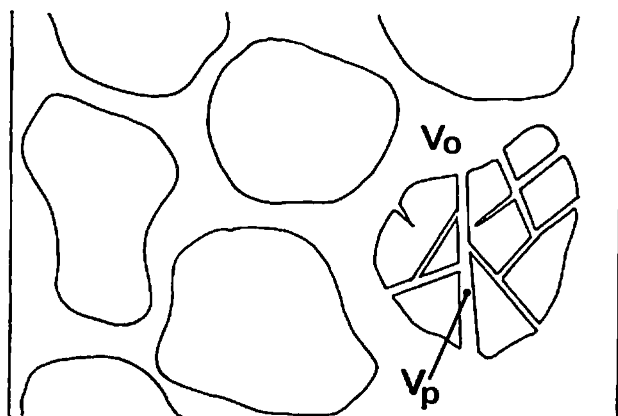

Fig. 1. Schematic representation of the path of various protein molecules during size-exclusion chromatography. The chromatographic partition coefficient is defined as $K = (V_e - V_0)/(Vt - V_0)$, where V_e is the measured elution volume, V_0 is the column void volume or exclusion volume and Vt is the total column volume, i.e., the sum of V_0 and the pore volume V_p.

However, conventional chromatographic methods suffer from disadvantages: they are tedious, lengthy, and difficult to reproduce or to quantitate. They fit quite well to preparative fractionations and purifications, not to rapid screening of many wheat samples, making it necessary to investigate high performance supports that could allow high mobile phase velocities and therefore very short runs.

Size-exclusion chromatography was the first mode of liquid chromatography to be adapted to high performance methods for protein analysis due to the development of highly resistant packings that were assumed to minimize interactions with the support. However, two major deficiencies initially associated with SE-HPLC methods prevented attainment of separations that were at least comparable to those of classical gels. All supports investigated, including silica and polymers, still more or less adsorbed proteins by ionic and hydrophobic interactions, respectively, making it necessary to deactivate the support surfaces by stable neutral layers thin enough to allow access to 60–1000 Å pores. Also, swelling properties of new supports

were inferior to those of carbohydrate gels, making them unable to resolve large size aggregates (Gooding, 1986).

Methods development has progressed quickly and improved columns, coupled with better equipment that significantly improved chromatographic separations, were made available. New silica-based packings, prepared from 3–5 micron-sized ultrapure silica bonded with a patented hydrophilic coating, were largely developed that reduced interactions and approximated ideal conditions of size-exclusion. In addition, various improvements such as selection of proper mobile phase, appropriate adjustment of pH and ionic strength, and especially use of detergents counteracting hydrophobic interactions made resolution and analysis time in high performance systems obviously superior and generally allowed any protein that could be separated on carbohydrate columns to be analyzed by SE-HPLC.

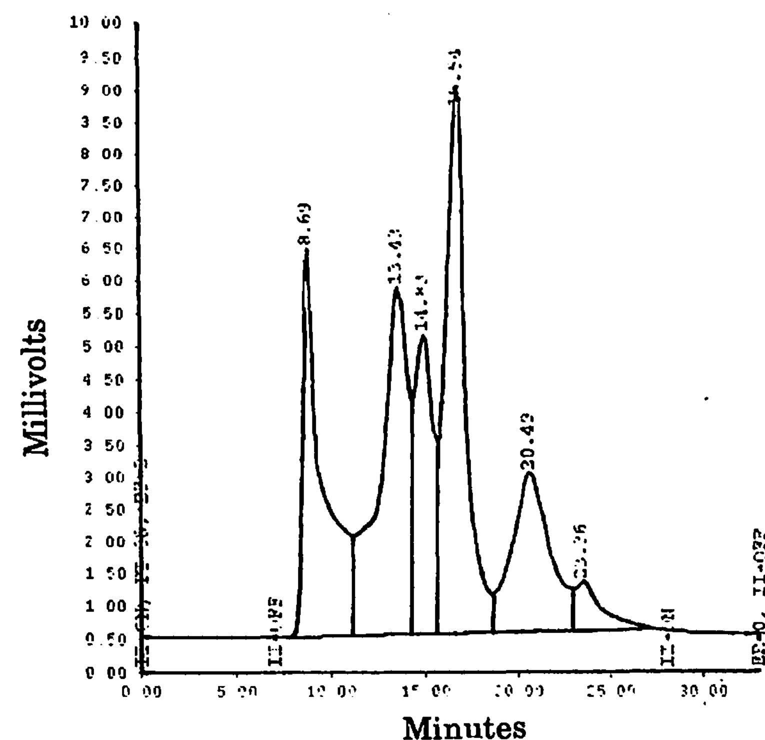

Fig. 2. Typical elution pattern of barley storage proteins extracted by phosphate-SDS buffer (pH 6.9) (from Benetrix, unpublished results).

In the case of cereals, although RP-HPLC has been much more developed for various uses (see other chapters of this book), SE-HPLC has become an invaluable tool for rapid examination of size differences of proteins (Fig. 2). Despite specific difficulties associated with insolubility and aggregative behavior of storage proteins, especially glutenin, a number of reports were presented, following pioneer studies from Bietz's group (Bietz, 1984*a*,*b*, 1985; Huebner and Bietz, 1985), that could make SE-HPLC work in good conditions, and were a breakthrough in the investigation of protein functionality and quality assessment.

ANALYTICAL CONDITIONS AND METHODS

Columns

While a major limitation of conventional gel-type media is their lack of mechanical strength, it is obvious that the high-mobile-phase velocities used in HPLC require packing materials that are rigid, physically and chemically stable, uniform and inert. Such columns, generally silica-based, suitable for proteins, are now available. Unlike the types of columns that are used in reversed-phase HPLC in which silica is modified to incorporate hydrophobic groups, or in ion-exchange HPLC in which silanols are derivatized with compounds having ionizable groups, SE-HPLC requires, in ideal conditions, that the stationary phase does not interact with proteins. Consequently, in SE-HPLC columns, residual silanols are usually blocked by "end-capping" to minimize adsorption and allow sample recoveries usually greater than 95%.

While a number of columns are available for various types of polymers, including oligosaccharides, polysaccharides, or cationic polymers, those specifically designed for proteins are few. These last years, many SE-HPLC studies of cereal proteins used TSK columns from Toyo Soda, marked by various suppliers (Spherogel from Beckman, Protein Pak from Waters, Ultro Pak from Pharmacia/LKB, Biosil from Biorad), all made from ultrapure silica bonded with a hydrophilic group that removes activity of residual silanols. For instance, the use of

TSK-3000SW columns (10 ± 2 µm of particle diameter, with 250 Å pores) was reported by Bietz (1984*a*) and Örsi and Békés (1986), whereas the use of TSK-4000SW (13 ± 3 µm particle diameter, with 400 Å pores) was more widely used (Huebner and Bietz, 1985; Bietz, 1986; Seilmeier *et al.*, 1987; Dachkevitch and Autran, 1989). The use of Protein-Pak 300 (diol-bonded 10 µm silica gel, mol wt range 10,000 to 300,000) has been also reported by Singh *et al.* (1990*a*,*b*) and Batey *et al.* (1991). These columns allow fractionation over a wide range of mol wt which depends, in fact, on the type of solvent that more or less lines or blocks pores. For instance, the range for TSK-3000SW is assumed to be 1,000 to 300,000 when a protein is injected in a salt solution, and only 1,000 to 100,000 when in a 0.1% SDS solution, 1,000 to 70,000 when in 6*M* GuHCl solution. The same is true for TSK-4000SW (5,000 to 1,000,000 when in salt solution).

Very recently, polyether-bonded 5 µm silica packings (UltraSpherogel™-SEC columns SEC 2000, 3000, 4000) were developed by Beckman to avoid non-specific interactions. These columns have increased reproducibility and efficiency from 500,000 to 2 million Da, pH 2.5–8.0, and 0 to 40°C at 2000 psi. They withstand denaturing agents such as 8*M* urea or 6*M* GuHCl, but to our knowledge, have not been used on cereal proteins.

Alternatively, Superose 6 or 12 (agarose-based) have been used with Pharmacia FPLC (Lundh and MacRitchie, 1989; Huebner *et al.*, 1990; Pasaribu *et al.*, 1992). However, although specifically designed for the study of proteins and enzymes (lower pressure, glass pumps and Teflon tubing, no stainless steel), such systems are usually not strictly classified as HPLC.

All analytical SE-HPLC columns are generally larger (7.5 × 300 mm, or 7.5 × 60 cm) than analytical RP-HPLC columns. Preparative columns (25 × 300 mm) are also available.

SE-HPLC columns are usually much more expensive ($600–1,000) than RP-HPLC columns. On the other hand, their lifetime is generally shorter [a loss of resolution after 250–300 injections was reported by Méritan (1990) and by Batey *et al.* (1991)], and they are less resistant to certain solvents and pHs. For instance, TSK can operate in the 2.0–7.5 pH range only

because the silica melts over pH 7.5, irreversibly destroying the column. Therefore, when using SE-HPLC columns, buffers must remain below the pH limit of silica melting, which may constrain the investigation of cereal storage proteins, as indicated in the section below.

Apparatus and Solvent Conditions

SE-HPLC systems are identical to those for RP-HPLC. However, because no solvent gradient is necessary (isocratic elution), only one pump is required and a solvent programmer is not essential. Flow rates of about 1.0 mL/min are common, although higher resolution at lower flow rates (0.5–0.7 mL/min) has been reported. To minimize band spreading, extracts are applied in small volumes, e.g., 20 μL.

Many elution solvents can theoretically be used provided they are compatible with the column packing as far as pH range and viscosity are concerned. They must also have a low absorbance in the 210 nm region and they must have a moderate but non-zero ionic strength to reduce affinity of proteins to the column and allow elution.

Because of the absolute condition of operating at a pH lower than the limit of silica melting, it is impossible to directly transfer to HPLC systems certain procedures of conventional size-exclusion chromatography in which solvents with extreme pHs or with high viscosity were used to keep all proteins in solution (e.g., Tris buffer: Hamauzu *et al.*, 1979; ammonium hydrate buffer: Godon, 1969; AUC solvent: Huebner and Wall, 1976).

In recent years, several solvents have been reported for elution in SE-HPLC including detergents (SDS) or hydrogen bond dissociating agents (urea, DMF). In studies on cereal proteins, 0.1*M* sodium phosphate, pH 6.9, containing 0.05 or 0.1% SDS, has been the most frequently used since it allows good compromise between low absorbance, low viscosity and protein extractability. It also keeps in solution a relatively high proportion of medium-size native aggregates, and gives a good relationship between molecular size and elution volume (Bietz, 1984*a*, 1985, 1986; Huebner and Bietz, 1985).

Because hydrophobic interactions often play an important role in the formation of aggregates, detergents such as SDS are acceptable. They help counteract hydrophobic interactions, although certain manufacturers state that they tend to shorten column life. Batey *et al.* (1991) clarified this question and, after comparing various other solvents including ethanol/water (70:30 v/v), isopropanol/water (50:50) and acetonitrile/water (50:50), the latter both with and without trifluoroacetic acid (TFA) (0.1%), they demonstrated that the loss of resolution was caused by the amount of SDS passing through the column. They investigated, therefore, the possibility of removing SDS from the elution buffer to extend the column life. Whereas either 70% aqueous ethanol or 50% aqueous isopropanol had to be abandoned because very high back pressures were necessary to maintain the flow rate, Batey *et al.* (1991) finally recommended the use of 50% (v/v) aqueous acetonitrile containing 0.1% TFA, which, without calling into question again the presence of SDS in the sample extract, resulted in a reduction by a factor of 200 in the amount of SDS to which the column was exposed and extended the column life to at least 2,000 injections, while further improving resolution (Fig. 3).

Sample Extraction and Stability of Extracts

One of the obstacles to characterizing the protein composition of cereals is the difficulty of solubilizing the whole proteins. Except in a few studies in which protein aggregates were reduced to subunits (Huebner and Bietz, 1985; Belitz *et al.*, 1987), most investigators tried to achieve complete extraction of proteins while retaining the integrity of the native complexes of flour. To satisfy these requirements, various non-reducing agents have been tried including acids, detergents and dissociating agents. Until 1990, however, no one extracted more than 60–90% of total proteins using solvents compatible with chromatographic conditions (e.g., solvent absorption in the UV region and distortions in the elution curve must be avoided). Finally, the optimal extracting agent found in the comparative studies by Dachkevitch (1989) and Méritan (1990) was 0.1*M* phosphate

buffer, pH 6.9, containing 2% SDS, as proposed in the earliest reports from Bietz (1984*a*, 1985) and Huebner and Bietz (1985).

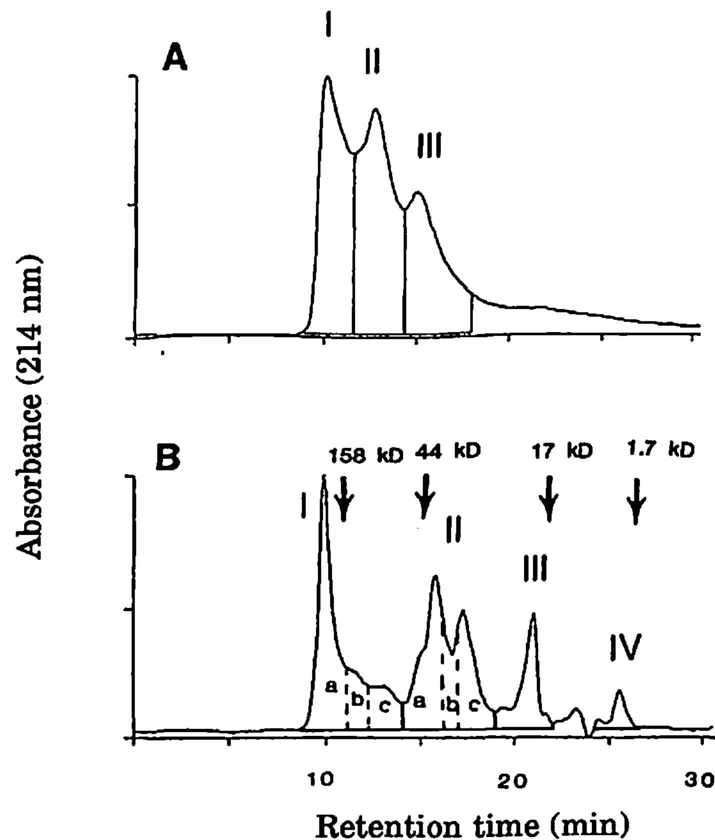

Fig. 3. SE-HPLC profiles of the wheat cv. Cook, extracted with phosphate buffer, pH 6.9, containing 2% SDS. A, the old elution procedure (phosphate buffer with 0.1% SDS); B, the new elution procedure (50% acetonitrile and water containing 0.1% TFA) (reprinted, with permission, from Batey *et al.*, 1991).

However, in earlier SE-HPLC fractionations, a dramatic instability of the protein extracts was noticed, resulting in a continuous decrease of the percentage of excluded peak during the first hours after extraction. Reproducible and comparable results could be obtained only upon storage of the extracts for one day, which allowed an equivalent stability of the mol wt distribution for samples extracted at different times. This brought Huebner and Bietz (1985) to the conclusion that further studies were necessary to improve the method's accuracy and to the choice of RP-HPLC in most of their subsequent studies of wheat proteins.

Dachkevitch and Autran (1989) speculated that such an instability could be related to the dissociating effect of SDS, resulting in a relatively slow disruption of large noncovalently bound aggregates until the extract contains only the more stable S-S-bonded complexes. Alternatively, as proposed by Huebner and Bietz (1985), it could not be ruled out that proteases remain active in phosphate-SDS buffer and are involved in the decrease of the excluded peak. Whereas no clear improving effect was found by adding various protease inhibitors in the extracting solution, it was reported by Dachkevitch (1989) that a higher extraction temperature (e.g., 60°C for 2 h) could totally overcome the problem of instability and make the extracts ready for SE-HPLC analysis without any equilibration or other treatment. This minor change yielded an extremely stable elution curve, even with supernatants that had been stored for 48 h after extraction, making possible the comparison of samples extracted at different times and full use of an automatic sampler for injection (Dachkevitch and Autran, 1989).

However, the difficulty of completely dissolving the storage proteins from flour without using conditions that chemically alter them still remained unresolved. Until 1990, no chromatographic solvent had been developed that would extract more than 90% of total proteins without scission of disulfide bonds and, in addition, protein extractability was quite variable, proteins from strong wheat flours being much less extractable than those from weak flours (Danno *et al.*, 1974; Bietz, 1984*a*). Considering that dough mixing could allow more efficient solubilization of unreduced proteins without affecting their size-based fractionation into polymeric glutenin, monomeric gliadin and albumin/globulin, Singh *et al.* (1990*a*) tried to achieve similar shear degradation of large gluten polymers using ultrasonic probes in order to solubilize total proteins from small flour samples and allow a more reliable pattern of protein aggregates to be obtained through SE-HPLC fractionations.

Using a sonifier generating ultrasonic vibrations with a frequency of 20 KHz in 1.5 mL Eppendorf tubes, Singh *et al.* (1990*b*)

clearly demonstrated that complete dissolution of unreduced proteins from strong and weak flours was possible in a 2% SDS solution (pH 6.9), with the following major advantages: (i) a very short time (30 sec) is needed to completely extract proteins, (ii) a very small quantity of flour (11 mg) is required, (iii) only very large glutenin polymers—that require much less energy for their shear degradation—are degraded and the resulting products elute from the column without affecting the size-based fractionation into polymeric and monomeric groups.

Troubleshooting

Care of SE-HPLC columns is similar to that of any type of HPLC and is that recommended by manufacturers. Protein extracts must be centrifuged (at least 25,000 g for 10 min) and elution solvents must be filtered through a 0.45 or 0.22 µm filter to remove particulates. In-line filters and/or guard columns are also to be used (Bietz, 1990).

The major problem with SE-HPLC is the loss of resolution and increased pressure that occur after a few hundred injections, sometimes even in less than one hundred injections when the protein sample is extracted from complex mixtures such as wholemeal wheat, rye or sorghum flours or hulled barley kernels. While in other types of HPLC it is sometimes possible to restore column performance and normal operating pressure by washing according to manufacturer's recommendations (often in the reverse direction and using alcohols, acetonitrile, diluted sodium hydroxide solutions, or proteases), there are few reports of durable regeneration of a silica-based SE-HPLC column, either by washing, or by local repacking. As stressed by Bietz (1986), once problems occur, lost resolution or increased pressure can seldom be reversed.

The origin of these troubles is manifold: clogging by large aggregates that do not elute from the column, irreversible adsorption interactions between the stationary phase and proteins or impurities of the extract (soluble starch, polyphenols?), or SDS that binds to the support and affects the apparent pore size and resolution.

These factors make SE-HPLC more expensive than other HPLC modes, and limit the use of SE-HPLC as a routine procedure. To extend column life and to achieve acceptance of SE-HPLC as a routine procedure, Batey *et al.* (1991) proposed either finding an alternative column packing or changing the sample preparation and running conditions. As indicated above, these authors especially recommend completely removing SDS from the elution solvent and using 50% (v/v) aqueous acetonitrile containing 0.1% TFA, which is likely to extend the column life to at least 2,000 injections. In connection with this, Pasaribu *et al.* (1992) recently suggested adding 0.08*M* NaCl to the phosphate-SDS buffer in fractionations using Superose columns. This, associated with sonicated extracts, allowed NaOH washing of the columns, and led to higher protein recovery, extended column life and improved resolution.

As a general rule, because slow changes in selectivity and resolution generally occur that result from type and age of SE-HPLC columns, it is highly recommended that system performance be monitored. This can be achieved by analyzing at regular intervals standard proteins of known molecular weight as shown in Fig. 4.

APPLICATIONS

Characterization of Molecular Size of Protein Polypeptides and of Protein Aggregates in Wheat

The knowledge of the size range of proteins is likely to provide a better insight into the aspects of protein composition that determine quality (functional properties) of various cereal products. It is essential, therefore, to determine routinely the proportions of the various classes of monomers and aggregates that occur in breeding lines, in harvested grains, or in processed foods.

Since proteins are sorted on the basis of their Stokes radii or hydrodynamic volumes during SE-HPLC fractionation, mol wt can be estimated from elution volumes. To determine the molecular size of proteins separated by SE-HPLC, it is necessary

first to calibrate columns using proteins of known mol wt (Fig. 4).

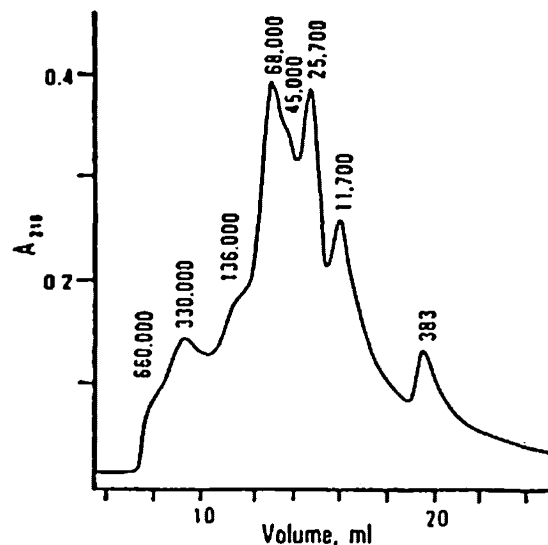

Fig. 4. SE-HPLC of native standard proteins on TSK-4000SW. Thyroglobulin (mol wt 660,000 plus 330,000), bovine serum albumin (68,000 plus a 136,000 dimer), ovalbumin (43,000), chymotrypsinogen A (25,700), cytochrome c (11,700) and adenosine-5'-phosphate (383), complexed to SDS, were analyzed at 1.0 mL/min. The column effluent was monitored at 210 nm (reprinted, with permission, from Bietz, 1984*a*).

When the logarithm of mol wt for standard proteins in either native or reduced states is plotted against elution time (or volume) for each protein, a straight line is obtained under ideal conditions. The equation for this line

$$\log \text{mol wt} = aV_e + b$$

where V_e is the elution volume, determined by linear regression analysis, can then be used to estimate the mol wt of unknown proteins on the basis of elution time (Bietz, 1984*a*). Computer programs can be used with this information to indicate start, maximum and end time, relative area and relative % and apparent mol wt of each peak.

The molecular size distribution of the main groups of wheat proteins has been reported by Bietz (1984*a*). For instance, when

whole gliadin is chromatographed on TSK-3000SW, a major peak (containing α-, β- and γ-gliadins) is observed with an apparent mol wt of ≈28,000, preceded by minor peaks with mol wt 63,000 and 105,000 corresponding to ω-gliadins and aggregated gliadins, respectively. On the other hand, using TSK-4000SW, several peaks were observed by Bietz (1984*a*, 1986) from native glutenins, with apparent mol wt of 821,000, 142,000, 66,000, 26,000, 13,000 and 2,000 (Fig. 5).

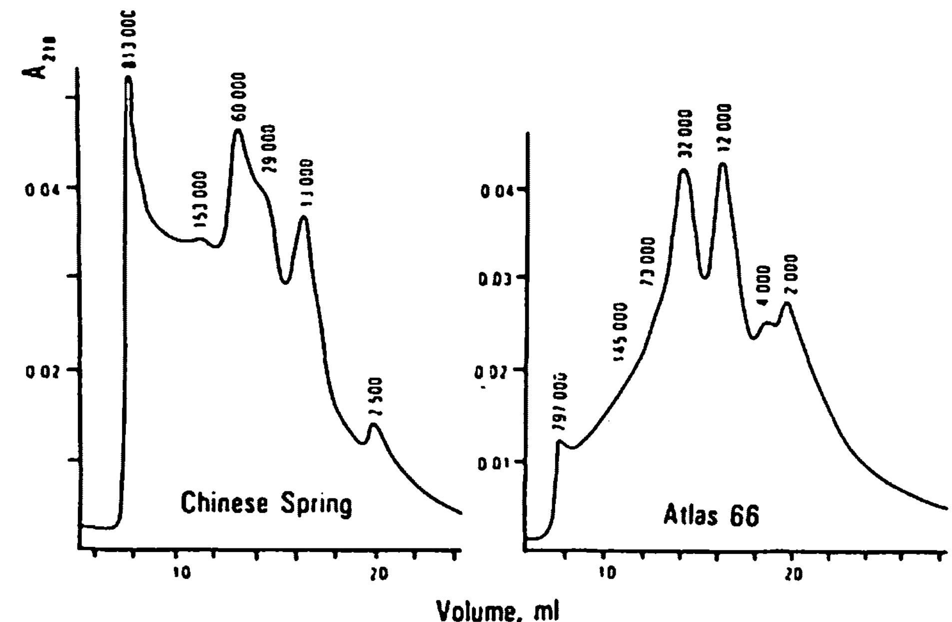

Fig. 5. Comparison of native glutenin proteins, extracted with solvent containing SDS, from single kernels of the varieties Chinese Spring and Atlas 66 by SE-HPLC on TSK-4000SW (reprinted, with permission, from Bietz, 1984*a*).

Examples of SE-HPLC of total unreduced proteins were shown above (Figs. 2 and 3). Insofar as a reliable calibration is used, these results permit unbiased comparison of protein compositions in routine procedures (e.g., breeding lines, cultivars, stages of maturity, stages of technological processes, etc.).

However, the validity of these results suffers a number of limitations, mainly because the mol wt of native aggregates may range up to 20,000,000 (Huebner and Wall, 1976) and are as yet

beyond the range of commonly used large pore-size columns, such as TSK-4000SW, which has a fractionation range of only one to two million (Bietz, 1986). Thus, the information obtained relates only to the soluble fraction. This is of interest in various applications, but only partially describes the protein composition, since information on highly aggregated fractions (that do not elute from the column) is not accessible. Moreover, such results can be misleading when comparing cultivars or lines whose protein extraction rate is variable. It was clearly demonstrated by Singh *et al.* (1990*a*) that both the proportions of the main peaks and absolute areas strongly depend on % of protein recovery (Table I).

TABLE I
Extractability of Unreduced Proteins in 2% SDS Solution
(Reprinted, with permission, from Singh *et al.*, 1990*a*)

Treatment	% Protein Recovery[a]	HPLC Area (%)			Absolute HPLC Area[b]		
		P1	P2	P3	P1	P2	P3
Stirring only							
120 min	65.4	20.2	53.5	26.3	56	148	73
Sonication							
Setting 10							
2 min	97.1	37.4	41.4	21.3	135	149	77
4 min	97.6	35.8	42.7	21.5	130	155	78
Setting 7							
2 min	94.6	37.9	41.2	21.0	130	142	72
4 min	95.6	36.4	41.7	21.9	129	148	78
Setting 3							
10 min	94.9	36.6	41.7	21.8	131	149	78

[a] Based on Kjeldahl N in the residues.
[b] Arbitrary unit for HPLC peak area $\times 10^{-5}$.

The quantitative distribution deduced from the HPLC curve is also strongly influenced by the wavelength used to monitor the effluent, especially if fixed-wavelength detectors are used.

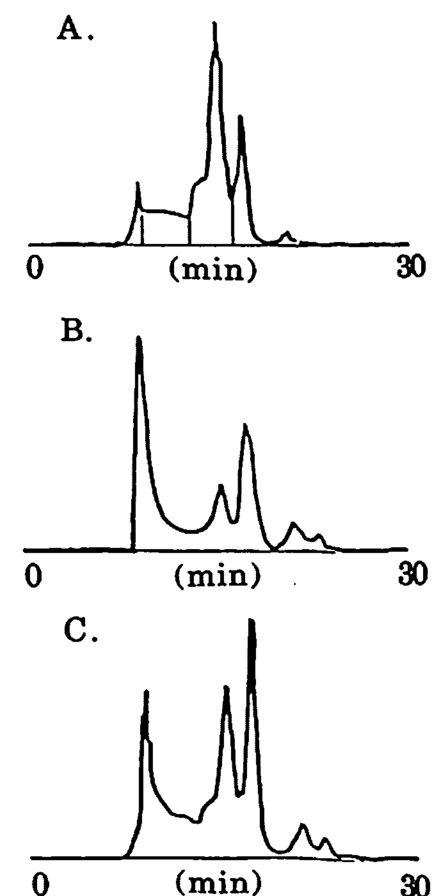

Fig. 6. Influence of the wavelength used to monitor effluent on apparent distribution of SE-HPLC protein peaks. A, 214 nm; B, 254 nm; C, 280 nm.

As shown on Fig. 6, when comparing the same wheat protein extract at 280, 254 and 214 nm, respectively, completely different curves are usually observed, leading to disagreeing results for protein distribution. This is due to significant differences in amino acid composition between the protein fractions eluted in the various peaks. Peak 1 (excluded) is usually overestimated at 254 nm, probably because of the prevalence of Phe over Tyr in this fraction (Méritan, 1990). An opposite situation is found in peak 3 (α-, β- and γ-gliadins). Use of a 214 nm wavelength (to

detect peptide bond carbonyl groups at maximum sensitivity) is likely to give a more reliable pattern of protein distribution. Care is also necessary to avoid overestimating the excluded peak that is frequently turbid, so light scattering and UV absorption influence absorption.

Alternatively, gel permeation (GP-HPLC in Ultro-Pak TSK-G4000SW) was used by Belitz *et al.* (1987) and Seilmeier *et al.* (1987) to fractionate glutenin reduced by SDS and DTE. Three major fractions and five minor fractions were obtained (Fig. 7), which were analyzed for amino acid composition (Table II).

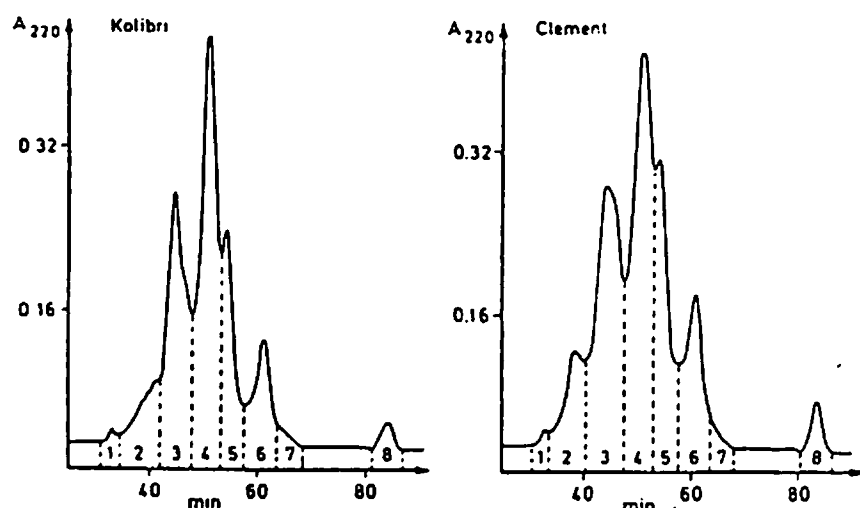

Fig. 7. Gel-permeation HPLC of reduced glutelins from the wheat varieties "Kolibri" and "Clement" (reprinted, with permission, from Belitz *et al.*, 1987).

These results clearly show that compositions of the various fractions are significantly different. Fractions 3 and 4 obviously contain HMW subunits (high content of Gly and Tyr and low content of hydrophobic amino acids) and LMW subunits (high content of Glx and Pro and low content of Gly and Ala), respectively. In contrast, high molecular weight fractions 1 and 2 and low molecular weight fraction 6 have compositions not typical of glutenin.

TABLE II
Amino acid composition of reduced glutenin fractions from the variety "Kolibri" obtained by gel permeation HPLC (Fig. 7) (reprinted, with permission, from Belitz *et al.*, 1987)

Fraction	1	2	3	4	5	6
Asx	6.2	4.1	2.8	2.7	4.5	6.1
Thr	4.7	4.8	3.2	2.7	3.6	5.4
Ser	7.0	8.2	6.5	7.6	6.8	6.6
Glx	14.7	20.9	32.3	35.3	29.2	15.8
Pro	6.5	10.5	12.4	13.6	11.6	8.9
Gly	11.7	10.9	12.8	4.2	5.6	7.9
Ala	9.5	5.1	4.1	3.7	5.3	7.4
Val	7.9	7.3	4.2	4.9	5.8	9.2
Met	0.7	1.5	1.1	1.6	1.7	1.5
Ile	5.7	5.8	2.8	4.4	4.9	6.5
Leu	9.6	6.7	5.9	7.8	7.8	8.0
Tyr	1.8	3.7	3.8	1.7	2.1	2.5
Phe	4.5	2.7	2.3	4.2	3.9	3.5
His	1.8	1.6	1.4	1.6	1.6	1.7
Lys	3.3	3.8	2.8	2.7	3.7	5.4
Arg	4.4	3.8	2.8	2.7	3.7	5.4

Genetic and Physiological Studies

Discrimination of Genotypes

Wheat kernel quality parameters such as yield, disease resistance and baking strength are largely genotype-specific. Because the price paid for grain often varies among various cultivars, the ability to identify grain varieties is essential. Since gliadins or glutenin subunits are nearly invariant expression products of the wheat genome, their composition is specific of variety and can thus serve to identify grain lots unsuitable for specific purposes.

Although electrophoresis is very successful for varietal identification, it is only one of many procedures, including reversed-phase high-performance liquid chromatography (see Chapter 5 in this book).

However, in contrast to RP-HPLC, very few studies attempted cultivar identification from SE-HPLC curves because the technique yields only a few large peaks with apparently little possibility for discriminating many cultivars.

For instance, Bietz (1984*a*) extracted glutenin from four cultivars with an SDS solution (without ME) after previous extraction of albumins, globulins and gliadins, and observed that the glutenins differed markedly in SE-HPLC pattern. Atlas 66, a high-protein soft red winter wheat used in breeding programs, had relatively little HMW glutenin in the extract, but was rich in LMW proteins, which apparently incorporate noncovalently into glutenin. In contrast, Chinese Spring and Red Chief, wheats of poor quality, have large amounts of HMW proteins. Centurk, a good bread wheat, has a balance of HMW and LMW species. Thus, SE-HPLC may differentiate genotypes by determining the mol wt distributions of HMW proteins (Bietz, 1986).

Another attempt took into account not only Rf and intensity of peaks, but the information of the whole elution curve (including the shape of the peaks and shoulders), using an application of classical discriminant analysis of digitalized signals (Bertrand *et al.*, 1990).

Such applications, however, present certain difficulties. The number of data points is often very large. Chromatograms may include several hundred variables, depending on the measurement intervals. Digitalized signals are often redundant (two adjacent data points give almost the same information). In an attempt to overcome these problems, Bertrand *et al.* (1990) combined the advantages of factorial analyses with a stepwise procedure which introduces only the more relevant pieces of information. The procedure can be presented as a succession of two principal component analyses (PCA), the first being performed directly on the raw data and giving PC scores, the second being applied on the gravity centers of each qualitative group assessed on the normalized PC scores. The method was tested on a

collection of 165 SE-HPLC chromatograms of wheat proteins, consisting of 55 genotypes grown in three areas of cultivation.

While direct observation of the chromatograms gave little information on differences between samples, the use of stepwise canonical discriminant analysis yielded significant classification of samples. This was due to the identification of regions of the curve presenting either positive (peak at 16 min, i.e., 30–45 kDa, corresponding to α-, β- and γ-gliadins), or negative (peak at 15 min, i.e., 60–65 kDa, corresponding to ω-gliadins) values about their first discriminating scores (Fig. 8). For instance, the discrimination of growing locations was performed using seven to nine PC scores and gave more than 86% accurate classifications of samples both in training sets and verification sets. The genotypes were also rather well identified, with more of 85% of the samples correctly classified. Accordingly, analysis of the shape of SE-HPLC chromatograms may be as efficient as RP-HPLC for discriminating wheat genotypes. Also, SE-HPLC runs are three times faster than those typical of RP-HPLC.

Effect of Rye Chromosome

Some sets of wheat contain rye chromatin due to the wide use of 1BL/1RS translocation lines as breeding parents. Dough stickiness and weakness may result.

SE-HPLC comparisons of rye translocation and HMW null lines and interchange experiments allowed MacRitchie *et al.* (1989) and Dhaliwal and MacRitchie (1990) to demonstrate that dough strength decreases for both sets of lines as the proportion of glutenin (peak 1) is lowered. However, dough strength appeared to decrease to a greater extent with loss of HMW glutenin subunits (null lines) than with loss of LMW subunits (rye translocation lines).

Since rye also contains interchain disulfide-linked proteins, including 75k γ-secalins controlled by the *Sec-2* locus on chromosome arm 2RS, SE-HPLC elution profiles of total protein revealed that 2BL-2RS translocation lines had significantly higher amounts of polymeric proteins than normal 2BL-2BS lines, suggesting that the higher protein levels and the increase in Extensigraph resistance in 2BL-2RS lines are largely due to

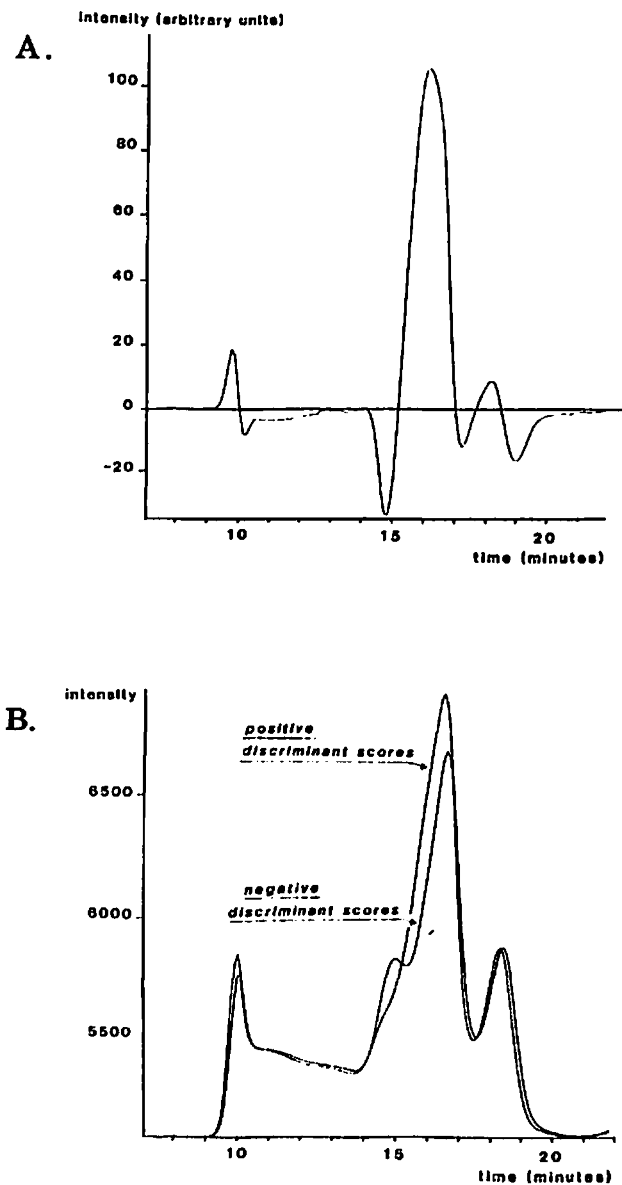

Fig. 8. Discrimination of genotypes using discriminant analysis on digitalized SE-HPLC signals. A, First discriminant pattern of genotypes; B, Averages of ten chromatograms of cultivars having positive and negative values of their first discriminant score (reprinted, with permission, from Bertrand *et al.*, 1990).

increased amounts of polymers which originate from 75k γ-secalins (Gupta *et al.*, 1989).

Effect of Environmental Factors

Considerable variation exists in flour end-use depending on when and where its parent wheat is grown. Because the bases of phenotypic quality, which govern the stability/instability of quality expression, are not clearly understood, it is still extremely difficult to predict behavior of a genotype with regard to the variation of environmental factors.

In a recent study, Scheromm *et al.* (1992) used SE-HPLC (TSK-4000SW columns) to investigate changes in mol wt distribution in phosphate-SDS extracts of two contrasted wheat cultivars as a function of level of nitrogen supply. Certain cvs such as Camp Rémy contained a constant amount of protein aggregates (F1) irrespective of the level of nitrogen supply, while an opposite trend was found in other cvs such as Fandango. Because the former are known to be stable wheats as far as phenotypic quality is concerned, and the latter are unstable wheats whose quality undergoes variation under the influence of agroclimatic conditions, it was suggested that SE-HPLC be used as a tool to evaluate stability of quality in response to changes in nitrogen level or other environmental factors.

Stages of Maturity

Because there is considerable variation in grain quality and protein composition depending on environmental and physiological factors, HPLC fractionations have been performed by Huebner *et al.* (1990) to follow changes induced by stages of maturity, kernel size and spike location. From glutenin samples extracted at various stages of maturity and redissolved in phosphate buffer containing 5% acetonitrile, 2% SDS and 0.01% dithiothreitol, two major peaks were observed in SE-HPLC, corresponding to high (peak B) and low (peak C) mol wt glutenin subunits, with some albumins and globulins present (peak D) and unreduced material also present at the void volume (peak A). Only fraction C increased as wheat matured, indicating that the accumulation of LMW subunits may parallel synthesis of gliadins and formation of protein bodies. In contrast, the

amount of HMW subunits and of other constituents remained nearly constant and may be largely non-storage proteins. Thus, although both types of subunits of glutenin are present early during kernel development, they differ in rates of accumulation.

Similar studies of SE-HPLC profiles of unreduced protein aggregates were carried out in developing bread wheat cultivars by Dachkevitch (1989) and durum wheat cultivars by Benetrix *et al.* (unpublished results) to better understand phenotypic expression and stability of quality. A general trend in kernels sampled from 5 days post-anthesis to maturity was an increase of the fraction insoluble in phosphate-SDS and of the largest protein aggregates (peak F1). However, the behavior of wheat cultivars differing in quality was not identical. For instance, among durum wheats, the increase in % F1 was much greater in cv. Capdur (having a type 45 γ-gliadin) than in cv. Tomclair (having a type 42 γ-gliadin). In addition, the slope of the SE-HPLC curve reversed in the F2 region after the 37th day post-anthesis (phase of kernel dehydration) in cv. Capdur, but not in cv. Tomclair, suggesting that mobilization of low and medium size aggregates into large size complexes apparently did not occur in the poor quality cultivar. The same is true among bread wheats (Fig. 9).

Germination

Biochemical changes occur in wheat with increased sprout damage, ultimately rendering the flour produced from it unsuitable for breadmaking. SE-HPLC investigations have been used to monitor changes in protein components during germination of wheat kernels and to determine whether chromatographic profiles can indicate stage or germination or proteolytic activity.

Using proteins extracted by 0.05*M* sodium phosphate buffer, pH 7.0, containing 0.5*M* NaCl, Kruger (1984) demonstrated that the major change in SE-HPLC profiles (TSK-3000SW column) upon germination was formation of low molecular weight species, in agreement with previous studies indicating that amino acids increase progressively during germination. Because the area of low molecular weight components increased linearly

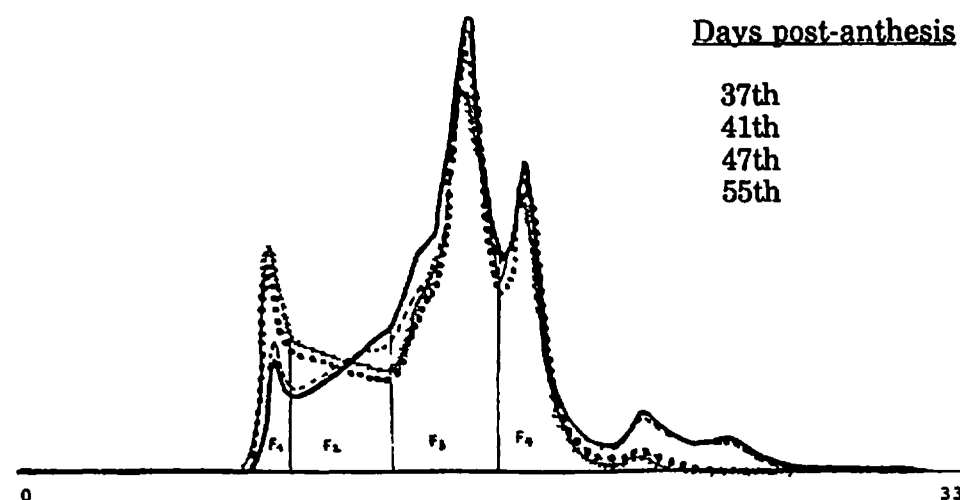

Fig. 9. Changes in SE-HPLC profiles (inversion of the slope in the F2 region of the curve) of phosphate-SDS extracts from a bread wheat (cv. Capitole, good baking quality) upon grain development (from Dachkevitch, 1989).

with germination time, Kruger and Marchylo (1985) also suggested that SE-HPLC profile could be used as a guide to the stage of wheat kernel germination. The method, however, seemed unsuitable for detecting incipient sprouting since few changes were observed in protein components during the two first days of germination, although levels of enzymes increased.

Prediction of Potential Baking Quality of Wheat Genotypes in Breeding Programs

Baking quality is difficult to assess in a varietal breeding program. Especially in early stages, breeders need rapid and small-scale microtests for predicting the intrinsic value of genotypes (Feillet, 1980; Branlard and Autran, 1986; Bietz, 1987). Several biochemical tests have a high potential for analyzing large series of samples consisting of small amounts of seeds. For instance, microtests based on protein solubility or allelic

variation at loci coding HMW-glutenin subunits yield results generally independent of the agronomic record of samples, and are suitable for screening genotypes in early generations of breeding programs.

However, to evaluate breadmaking potential and use such information intelligently in breeding and during processing, further studies on flour proteins were necessary, not only at the level of monomers or subunits (e.g., using SDS-PAGE), but using methods capable of assessing protein structure and interactions.

The advent of HPLC techniques for wheat protein analyses, with their automation, reproducibility and quantitation, have made possible the rapid screening of large numbers of breeders' samples. This was not possible by low pressure conventional liquid chromatography (Bietz, 1983, 1984*a*, 1985, 1986; Autran, 1987).

Major advances in this field have been obtained using reversed-phase HPLC (RP-HPLC). Several reports have applied RP-HPLC to quality prediction because of relationships of some specific peaks or chromatographic regions to quality characteristics (Huebner and Bietz, 1985, 1986, 1987; Burnouf and Bietz, 1987; Lookhart and Albers, 1988; Huebner, 1989).

Conversely, although it has been reported that baking strength is primarily determined by the occurrence of large protein aggregates (Huebner and Wall, 1976; Field *et al.*, 1983; Miflin *et al.*, 1983; Bushuk, 1987), SE-HPLC has been, until these very last years, rarely attempted for quality prediction.

Pioneering studies by Bietz (1984*a*) and Huebner and Bietz (1985) attempted to extend to HPLC systems the previous results of Huebner and Wall (1976), who related the mol wt distribution of AUC-soluble proteins on agarose columns to breadmaking quality. Higher quality wheats contained more proteins of higher molecular weights. Some promising results, obtained from a limited number of samples, confirmed that dough mixing time was correlated with the amount of high-molecular-weight glutenin, or with the ratio of aggregative to monomeric proteins, indicating possible use in breeding (Huebner and Bietz, 1985; Örsi and Békés, 1986; Örsi *et al.*, 1987; MacRitchie *et al.*, 1989).

However, whereas some early results indicated that the magnitude of the first peak (> 800 kDa) from unreduced (phosphate-SDS) extracts was directly related to mixing time, later results tended to show the opposite trend because of (i) instability of extracts and (ii) poor control of protein extraction. For instance, Autran (1987) showed from 30 French genotypes that the peak 1 (excluded)/peak 2 (size: 115–650 kDa) ratio was negatively related to dough strength and to the elastic recovery of gluten. Further attempts to overcome the instability of protein extracts were carried out by Dachkevitch and Autran (1989) who noticed that a higher extraction temperature (60°C for 2 h) could make the phosphate-SDS extracts ready for SE-HPLC on TSK-4000SW columns without any other equilibration or treatment. Such conditions yielded an extremely stable elution curve, even between supernatants stored for 0, 12, 24 or 48 h after extraction. This allowed an automatic sampler to be used with unreduced extracts.

Dachkevitch and Autran (1989) extended this study three years, and assessed not only correlations with quality data, but also ability to discriminate between genotypes, inheritance, and respective influence of genetics and growing location, year, or protein content.

This study suggested that the *negative* relationship observed between the proportion of excluded peak and baking strength (Fig. 10) resulted from the use of a mild extraction, which left in the residue a high percentage (up to 30%) of total proteins. It was indicated that use of very efficient solvents (generally not compatible with SE-HPLC columns) would show a positive correlation whereas intermediate conditions (e.g., an acetic acid solvent) would show no correlation at all.

Using extractions by phosphate-SDS (pH 6.9) containing 2% SDS, it was demonstrated that either the percentage of peak 1 or the peak 1/peak 2 ratio has a good ability to discriminate between genotypes (as indicated by the high values of the ratio σ^2_G/σ^2_L of the variances respectively assignable to genotype and to growing location) and may be more reliable parameters for breeding programs than many other criteria (Table III).

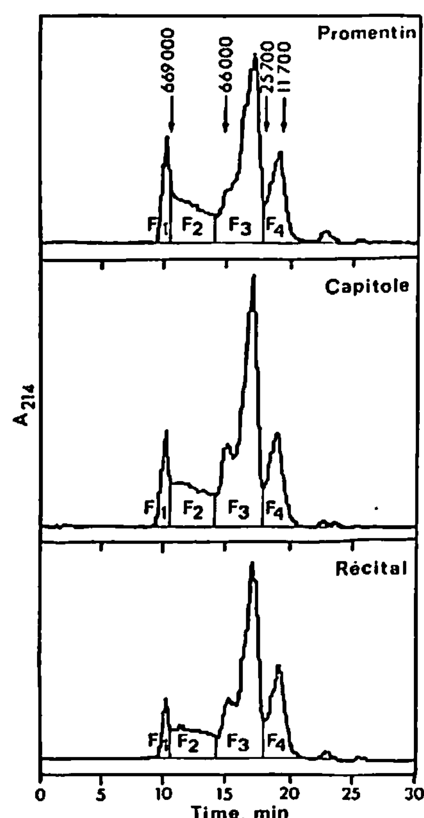

Fig. 10. Elution profiles obtained by SE-HPLC on a TSK-4000SW column of unreduced flour proteins extracted with phosphate-SDS from three bread wheat cultivars, Promentin, Capitole, and Récital. Column solvent was 0.1*M* sodium phosphate containing 0.1% SDS. Elution positions of the molecular weight standards are indicated. Alveograph W indices for the three cultivars are 105, 110, and 200, respectively. The characteristics of the chromatograms of the three cultivars are as follows: %F1 = 11.5, 8.8, and 6.4; F1/F2 ratio = 0.49, 0.39, and 0.29 (from Dachkevitch and Autran, 1989).

On the other hand, correlations obtained from 65 genotypes grown in three locations in 1985, 1986 and 1987 (Table IV) suggested the following observations:

- the flour protein content is much more associated to the soluble fraction *Fs* (r = 0.78***) than to any of the fractions F1 to F4.
- both % F1 and % F2 are negatively correlated with Alveograph W index, to Mixograph index, and Zeleny volume, and, to a lesser extent to Alveograph P index, while % *Fi* and % F3 are positively correlated with these criteria.
- the highest (negative) correlation coefficients with the different baking strength criteria are observed for the F1/F2 ratio (for instance: r = -0.80*** with both W index and Mixograph index).
- although the insoluble residue (*Fi*) may contribute to baking strength, the correlations involving % F1 or F1/F2 ratio are still significant whether the variation in *Fi* is or is not taken into account.
- a positive correlation is observed (from a restricted number of samples) between the % F2 and both loaf volumes in French baking technology and dough extensibility, suggesting possible importance of intermediate aggregates as a basis of these technological characteristics.

TABLE III
Analysis of Variance of Some SE-HPLC Chromatographic and Technological Characteristics for 15 Genotypes Grown in Five Locations in 1985 (from Dachkevitch and Autran, 1989)

	Percentage of Variability Assignable to			
Characteristic	Genotype	Growing Location	Residue	σ^2_G/σ^2_L
% F1	76.4	2.9	20.7	large
% F2	70.4	7.8	21.8	9.0
% F3	67.9	14.6	17.5	5.0
% F4	39.5	45.4	15.1	0.8
F1/F2 ratio	62.4	2.9	34.7	large
% *Fs*	17.3	70.2	12.5	0.2
% *Fi*	9.1	79.3	11.6	0.1
W (Alveograph)	77.1	9.3	13.6	8.0
Mixograph Index	70.3	18.5	11.2	4.0

TABLE IV
Correlation Coefficients for Relationships Between SE-HPLC Characteristics and Technological Data of 65 Wheats Grown in Three Locations in 1987 (from Dachkevitch and Autran, 1989)

	Flour Protein	Alveograph W	Alveograph P	Zeleny Volume	Mixograph Index
% F1	-0.52***	-0.72***	-0.49***	-0.55***	-0.67***
% F2	-0.55***	-0.56***	-0.38**	-0.52***	-0.44***
% F3	0.59***	0.52***	0.43**	0.34**	0.40**
% F4	-0.20	0.11	-0.05	0.19	0.23
F1/F2	-0.44***	-0.80***	-0.53***	-0.65***	-0.80***
% *Fs*	0.78***	0.12	0.26*	0.29*	0.01
% *Fi*	0.50***	0.63***	0.41**	0.67***	0.66***

The study of Dachkevitch and Autran (1989) stressed that SE-HPLC is a powerful tool for studying native protein aggregates and the physicochemical basis of baking strength, and has potential for rapid assessment of baking quality of bread wheat genotypes in breeding programs. Two limiting factors were identified, however: (i) the effect of the year on absolute values of SE-HPLC data (due to variations in protein extractability) made it necessary to calibrate the prediction equation of baking strength from samples of each new crop year, and (ii) the short column life made the analysis expensive for routine use.

In recent years, attempts to overcome these two difficulties have been made. As reported above in the Analytical Conditions and Methods Section, the introduction of sonication of the extracts by Singh *et al.* (1990*a,b*) has allowed consistent solubilization of total flour proteins (> 95%) from wide-ranging wheat cultivars, resulting in a much better ability to discriminate between genotypes, either on the basis of the percent area of peak 1, or of the ratio of aggregating to monomeric protein, as suggested by MacRitchie *et al.* (1989). For instance, very highly significant (positive) correlations were found between percent area of peak 1 and quality attributes related to loaf volume, or to

Extensigraph or Mixograph parameters (Table V). In contrast, relative quantity of gliadin (peak 2) did not show very strong correlations with quality attributes (negative correlations with loaf volume and parameters of dough resistance), which agrees with flour and gluten reconstitution studies. On the other hand, the relative quantity of albumin/globulin (peak 3) (very negatively correlated with flour protein content) followed an inverse trend, which may be indirectly due to an effect of protein content.

TABLE V
Correlation Coefficients Between High-Performance Liquid Chromatography Measurements and Quality Attributes (reprinted, with permission, from Singh *et al.*, 1990*b*)

Attribute	Percent Area Peak 1	Percent Area Peak 2	Percent Area Peak 3
Flour protein	0.51	0.14	-0.92***
Loaf volume	0.72**	-0.52*	-0.49
Extensibility	0.84***	-0.48	-0.72**
Max. resistance	0.84***	-0.63*	-0.56*
Extensigraph area	0.89***	-0.59*	-0.68**
Dough devt. time	0.72**	-0.14	-0.92***
Dough breakdown	-0.57	0.17	0.68**
Water absorption	0.14	0.44	-0.70**
Mixograph devt. time	0.84***	-0.66**	-0.52*

On the other hand, introduction of 50% acetonitrile as a new elution solvent (Batey *et al.*, 1991), considerably increased column life and resulted in much improved resolution of different protein classes. This is likely to make SE-HPLC a routine procedure.

Alternatively, SE-HPLC was reported by Huebner and Bietz (1985) as a rapid method for quality assessment through examination of the two main groups of glutenin subunits upon reduction of disulfide bonds, alkylation of resulting sulfhydryl groups and fractionation on TSK-4000SW columns. Although resolu-

tion (Fig. 11) is much lower than in RP-HPLC or in SDS-PAGE of reduced proteins, this presents an interesting possibility of rapidly screening samples for their respective proportions in HMW (apparent mol wt 71,000 to 108,000) and LMW (≈41,000) subunits.

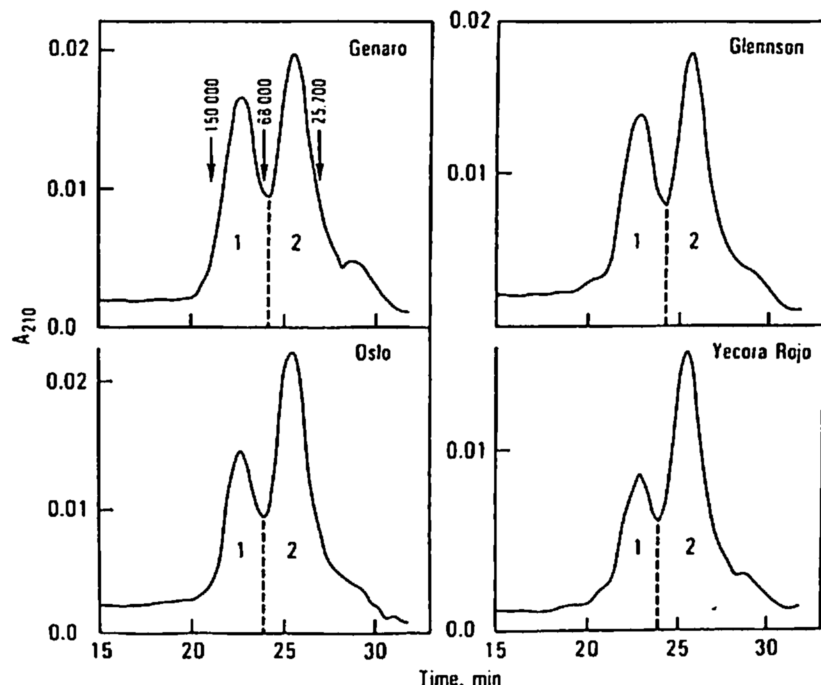

Fig. 11. SE-HPLC of reduced and alkylated insoluble residue protein. Wheat varieties are Genaro, Glennson, Oslo, and Yecora Rojo. Elution positions of unreduced protein standards (immunoglobulin F, 150,000; bovine serum albumin, 68,000; chymotrypsinogen A, 25,700) are indicated (reprinted, with permission, from Huebner and Bietz, 1985).

Characterization of Specific Protein Fractions that Determine Functional (Breadmaking) Properties

In recent years, functional (breadmaking) properties of wheats have been associated with occurrence of large proteins. On the other hand, various protein markers have been identified by simple correlation between the presence or amount of specific monomers (e.g., γ-gliadins) or subunits (e.g., HMW-glutenins) with quality data. It is essential, therefore, to link these two approaches by demonstrating the physicochemical basis for correlations between markers and quality data. SE-HPLC (especially in a preparative version) can be used to this end through identification of specific proteins that contribute to aggregates of various sizes. A first type of application was control of purity (i.e., absence of monomers) of glutenin-like unreduced fractions obtained by SDS after DMSO and ethanol extractions (Gupta and MacRitchie, 1991), or the demonstration of the aggregative behavior of HMW albumins (Gupta *et al.*, 1991) using a Protein-Pak 300 size-exclusion column.

On the other hand, in an attempt to more specifically identify wheat protein subunits related to breadmaking potential, an original procedure based on preparative SE-HPLC was used by Méritan (1990). Phosphate-SDS extracts of flour were fractionated on a preparative TSK-G4000SW column and the various peaks and the insoluble residue were collected to allow further quantitation of all reduced subunits from SDS-PAGE patterns (Fig. 12) and investigation of their distribution into various fractions (Table VI). From the behavior observed for each glutenin subunit, i.e., its tendency to be distributed either in the insoluble residue, or in the aggregates of various sizes such as peaks F1, F2, or F3, a potential aggregative index was inferred (Table VII). Interestingly, there was good agreement between such a quality index based on physicochemical data and those proposed following allelic studies by Payne *et al.* (1987) or Pogna and Mellini (1988), which can make the method very powerful for predicting the quality potential of any individual protein subunit from either material available only in very small amounts, or newly identified subunits in germplasm collections.

Assessment of Quality in Cereal-Based Flours or End-Products

Baking Quality of Wheat Flours

In the previous section, it was shown that SE-HPLC seemed much more adapted to predict the potential quality of wheat genotypes than the effect of environment. Although there is often a considerable variation in flour quality depending on

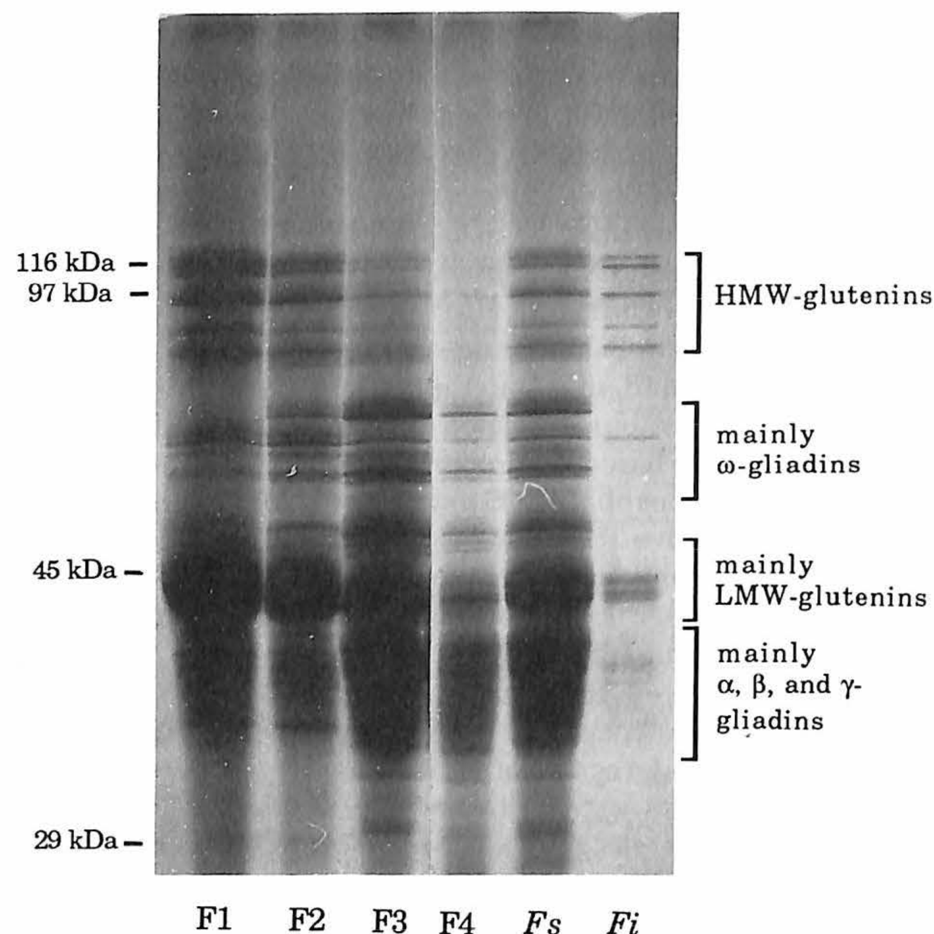

Fig. 12. SDS-PAGE characterization of reduced SE-HPLC fractions (numbered as in Fig. 10) of a phosphate-SDS extract from the wheat cv. Aubaine. F1, F2, F3 and F4 correspond to the four main regions of the diagram as in Fig. 10; *Fs* and *Fi* are controls of respectively reduced supernatant and residue of the phosphate-SDS extraction (reprinted, with permission, from Autran and Morel, 1991).

environmental conditions, the exact causes of variation are still poorly understood. Because differences in quality exist among different flour samples within a given genotype, Méritan (1990) investigated SE-HPLC characteristics of several hundred flour samples produced at the pilot scale and attempted to use SE-HPLC to assess baking quality in flour samples to enable traders

TABLE VI

Distribution, in % of Total Proteins, of HMW Subunits of Glutenin of cvs. Aubaine and Corin into the Various SE-HPLC Fractions (reprinted, with permission, from Méritan, 1990)

	# HMW subunit	Residue *Fi*	F1	F2	F3
cv. Aubaine	2*	23.0	42.6	31.7	2.7
	5	47.5	31.2	19.3	2.0
	7	25.0	35.4	34.2	5.4
	8	27.1	42.4	28.2	2.3
	10	36.5	35.9	22.6	5.0
cv. Corin	3	35.4	36.6	24.3	3.7
	6	6.2	46.5	38.0	9.3
	8	12.7	46.2	32.0	9.1
	12	14.6	40.5	34.1	10.8

and millers to select flours adapted to specific end-uses. This study failed, however, to show significant intravarietal differences, confirming that SE-HPLC patterns were essentially variety-dependent. A prediction of flour quality within genotypes seems therefore unreliable, all the more so because the harvest year parameters interact with the results. However, new data have been obtained by Scheromm *et al.* (1992) which identify cultivars whose expression of flour quality is more stable under the influence of nitrogen supply.

Cooking Quality of Durum Wheat Semolina and Processed Pasta

Assessment of cooking quality of semolina has been attempted using the same methodology as for bread wheat flours (Autran *et al.*, 1988). In contrast with bread wheat flours, however, proteins of durum wheat semolina are almost entirely extractable using phosphate-SDS buffer, so that a significant *positive* correlation was found between the percentage of fraction F1 (or F1+F2) and gluten strength or viscoelasticity determined by a Viscoelastograph.

SE-HPLC has also been used, beside several other techniques (protein solubility, circular dichroism, RP-HPLC, etc.) to monitor changes in the aggregative state of the protein complexes subsequent to thermal modification in various food processes such as high-temperature pasta drying.

TABLE VII
Quality Index Attributed to Each HMW Subunit of Glutenin, Based on its Distribution into Various Molecular Sizes of the SE-HPLC Diagram [the index was calculated from the sum of the percentages observed for each subunits in Table VI, using arbitrary coefficients of +0.2, +0.1, -0.1, and -0.2 for their occurrence in fractions *Fi*, F1, F2, and F3, respectively (reprinted, with permission, from Méritan, 1990)]

# HMW Subunit*	Quality Index Based on SE-HPLC
5	10
10	7
3	7
8 (cv. Aubaine)	6
2*	5
7	4
8 (cv. Corin)	2
12	1
6	0

* According the nomenclature of Payne *et al.* (1987).

For instance, Feillet *et al.* (1989) and Chardard (1991) investigated the respective effects of moisture content, duration and temperature of durum wheat pasta drying using SE-HPLC. Proteins were extracted from semolina and pasta by a phosphate-SDS buffer without reducing agent. This solubilized mainly LMWG aggregates along with gliadins and salt-soluble proteins. Fractionations were carried out using TSK-4000SW columns. Four peaks were obtained having mol wt from 800,000 to 13,000. SDS-PAGE showed that peaks 1 and 2 were mainly composed of aggregated LMWG with most of the extracted HMWG also present in peak 1. Autran *et al.* (1989) and Pollini *et al.* (1990) showed that, during pasta drying, all protein peaks decreased as a function of the intensity of heat treatments, but the phenomenon especially affected peaks 1 and 2, which rapidly disappeared from the elution curves (Fig. 13), confirming the heat sensitivity of LMW subunits of glutenins. However, because the magnitude of the decrease was similar in both good and poor durum wheat cultivars, this may not explain all parameters of cooking quality, suggesting that interactions with other protein fractions (e.g., sulfur-rich proteins) should be considered to explain functional properties (thermal behavior) of LMWG aggregates (Feillet *et al.*, 1989).

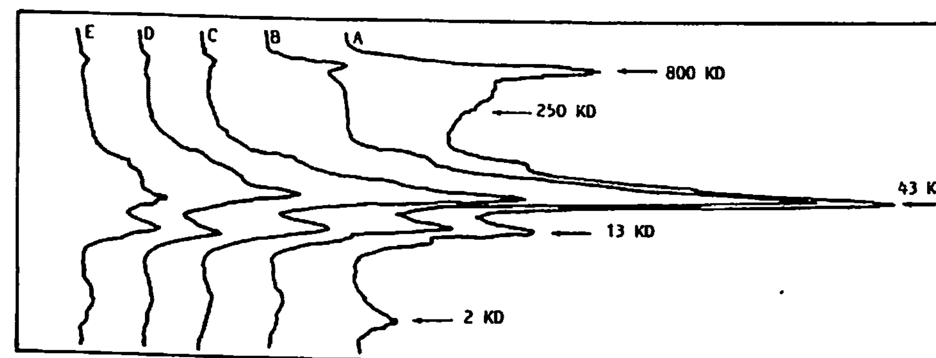

Fig. 13. SE-HPLC of phosphate-SDS extracts from durum wheat semolina (A), pasta dried at 55°C (B), pasta left for 2 h at 90°C at 13% (C), 18% (D), and 24% (E) moisture content, respectively (reprinted, with permission, from Feillet *et al.*, 1989).

In another study aimed at understanding the mechanism of dough formation, the distribution of protein aggregates was investigated from pieces of dough sampled along the extrusion screw (Autran and Courajoud, unpublished results). A dramatic change (drop in fraction F1 from 7% to 1%, on a total protein basis, that paralleled an increase of fraction F3) was observed in the SE-HPLC profile in regular dough (without additive), in the place (third thread of the screw) where the change

from hydrated semolina into continuous network is assumed to occur. In contrast, in dough containing 0.06% sodium sulfite, no major drop was observed. Because the presence of this additive was associated with significant improvements in cooking quality, it was assumed that a reducing agent may help maintain the protein aggregates of the dough in a less highly polymerized state which, under the high pressure conditions of the extruder, allowed possible SH/S-S interchanges and recombining of the dough system, leading to a protein network more resistant to disintegration during cooking.

Quality of Industrial Glutens

Wheat gluten, or *vital gluten,* is the concentrated protein prepared from wheat flour, usually by washing the starch from a flour-water dough. It is used primarily as a bread additive to increase flour protein content and functionality. Several predictive tests of gluten vitality have been investigated, including blending gluten samples to commercial flours, or dough reconstitutions from gluten and starch, but all have been only partially successful. Because it is acknowledged by the gluten industry that a rapid and accurate method to test gluten quality and suitability for the various end-uses would be extremely useful, recent studies based on SE-HPLC have been carried out by Méritan (1989).

Interestingly, a much higher protein solubilization was observed (≈ 90%) by using phosphate-SDS buffer than in the above-mentioned SE-HPLC studies on proteins extracted from bread wheat flours. Whether because of the absence of soluble proteins that may limit protein solubilization when present in flour, or because proteins become more accessible to the phosphate-SDS solvent after gluten preparation (dough formation, washing and drying). Because more protein aggregates go into solution, the SE-HPLC curves of gluten contain a higher proportion of excluded peak F1 (15–20% of the surface under the curve) than in flours. From the analysis of 13 industrial glutens, a significant *positive* correlation (r = 0.80) was found between the percentage of F1 and the improvement of loaf volume in a reference baking test.

On the other hand, as reported by Godon (1991), it was shown from another set of samples including industrial and pilot samples of glutens, that each SE-HPLC peak carries information on effects of the various treatments. For instance, a higher proportion of peak 1 or a lower proportion of peak 4 is characteristic of freeze-dried glutens compared with industrially-dried glutens.

Malting Quality of Barley

The amount and composition of barley storage proteins (hordeins) play a major role in malting and brewing. For instance, the tendency of some hordein groups (B and D) to form a persisting aggregated matrix around starch granules may restrict hydrolysis of starch during mashing (Baxter, 1980; Millet *et al.*, 1991). The possibility of assessing the mol wt distribution in barley or malt proteins may therefore allow prediction of malting or brewing quality.

In a recent study, Millet (1991) demonstrated that the characteristics of the mol wt distribution of barley proteins extracted with a phosphate-SDS buffer, as shown by SE-HPLC patterns, and especially parameters such as % F1 (aggregated B-hordeins), % F2 (mainly C-hordeins) and F1/F2 ratio, were essentially variety-dependent. However, when extracted from wholemeal barley samples, only a poor prediction of malting quality seemed attainable. On the other hand, when different histological layers of the barley kernel (or successive fractions obtained by progressive pearling of the kernel) were separately examined, major differences in mol wt distribution were observed with a higher proportion of F2 in the center. Instead of explaining malting quality on the basis of highly aggregated proteins, it was hypothesized that a higher proportion of hydrophobic fractions contributed to limit water intake, whereas a higher proportion of fraction F1 makes grain imbibition easier.

On this basis, Millet (1991) suggested use of the F1/F2 ratio as a malting quality index. For instance, barley cvs. such as Carina or Triumph (F1/F2 ratio higher than 0.9) have a higher malting potential than cvs. such as Menuet or Kym (F1/F2 ratio lower than 0.7). Moreover, the quality potential seemed higher when there is a concentration of aggregated B-hordein (fraction

F1) in the subaleurone layers, whereas an opposite trend (high concentration of intermediate size and hydrophobic fraction F2 in the outer parts) seemed to determine a lower quality potential.

CONCLUSION

Although SE-HPLC has only recently been applied to cereal proteins, it has already been adopted and used for numerous problems. This chapter has examined some specific SE-HPLC methods and their application to cereal proteins.

SE-HPLC is ostensibly the simplest chromatographic mode because, under ideal conditions, there is no solute interaction with the support. First of all, SE-HPLC allows examination of size distribution of protein polypeptides and protein aggregates (e.g., estimation of aggregating vs. monomeric proteins), and assessment of various characteristics related to size distribution.

For instance, analytical (and preparative) SE-HPLC is an invaluable tool for characterizing the protein fractions or subunits that contribute to aggregates of various sizes. It thus has the potential of providing information on baking quality and mixing requirements useful for breeding and genetic improvement of wheats.

Whereas conventional size-exclusion chromatography is tedious, lengthy, and difficult to reproduce or to quantitate, the introduction of rigid SE-HPLC columns allowed reduction of sample size and analysis time (30 min per analysis), while increasing reproducibility and giving better quantitative data due to its computer capabilities. SE-HPLC thus has significant potential for the analysis of large series of samples.

SE-HPLC can be recommended for identifying wheat proteins related to breadmaking potential. Whereas many other biochemical techniques (SDS-PAGE, RP-HPLC) are based on reduced or dissociated proteins, resulting in loss of much information concerning structure, interactive aspects and stability of the protein complexes, SE-HPLC allows studies of protein composition of native unreduced aggregates, and also quantitation that has often been neglected in SDS-PAGE. A major advantage of SE-HPLC, therefore, is its potential to keep relatively large

aggregates in a quasi-native state, to retain information on the aggregate level, and to be more likely to approach the physicochemical and structural basis of wheat quality.

Besides applications for quality prediction, SE-HPLC methods are highly useful in genetic and physiological studies, and give new insights into discrimination of genotypes, effect of environmental factors, and changes in storage proteins during grain development or germination. SE-HPLC methods are applicable to proteins extracted from cereal kernels or flour, as well as those extracted from processed foods such as dough, gluten, pasta or malt.

Although SE-HPLC columns are more expensive, SE-HPLC may have some advantages over RP-HPLC. SE-HPLC equipment is much simpler: a single pump and no gradient controller are needed since an isocratic elution is used. The time of analysis is shorter (55 min in RP-HPLC). Fewer reagents are necessary (phosphate buffer and SDS), and their purity seems less critical. Also, in view of routine use for predicting baking quality in breeding programs, the interpretation of the elution curve is simpler, since it consists of four major fractions very easy to identify (instead of 20–30 in RP-HPLC), two of them being related to quality.

SE-HPLC methods are still evolving, and improvements may be expected in the resolution and pore size of the columns, permitting analysis of larger and larger protein aggregates. We are also learning better ways to use these methods. For instance, as stressed by MacRitchie *et al.* (1989), it is no longer acceptable to work on only partially solubilized material, as in all studies reported between 1985 and 1990. It is essential that measurements are based on a complete protein extract. Because methods for complete solubilization are now available (Singh *et al.*, 1990*a*), size distribution for the range of molecular species thus extracted can be determined quantitatively by SE-HPLC and more reliable correlations with potential quality of genotypes or end-use quality parameters can now be determined.

In conclusion, SE-HPLC has become an essential method for investigation of cereal proteins, and is a significant step toward better use of wheats and other cereals. Thus, SE-HPLC will remain a powerful and indispensable method to isolate, charac-

terize and compare proteins of grains, flours, or processed cereal products. It will help us explore and further improve the full potential and the industrial use of cereals, assuring good quality of cereals and cereal-based products.

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HPLC ION-EXCHANGE CHROMATOGRAPHIC SEPARATIONS OF CEREAL AND LEGUME PROTEINS

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INTRODUCTION

For many years, ion-exchange chromatography has been one step in methods for the preparative fractionation of cereal proteins. The advent of consistent ion-exchange media meant that protein separation could be effected reproducibly not only within a particular laboratory, but also in others. Despite this long history of use for the purification of cereal storage proteins, high performance ion-exchange chromatography (IE-HPLC) has not become a common technique in cereal protein separations. The introduction of reversed-phase HPLC as a tool for protein chemists has left the development of ion-exchange methods for cereal proteins lagging far behind. It was only with the preparation of mono-disperse polymeric beads (Ugelstad *et al.*, 1983) that high-performance ion-exchange liquid chromatography made a real leap forward. Columns made from these beads, with wide pore diameter and regular bead size, gave a far higher consistency of column performance in comparison with silica-based columns of the day (Janson, 1985). Methods utilizing these columns are frequently referred to as fast protein liquid chromatography, or FPLC (a registered trademark of

HPLC

High-Performance Liquid Chromatography of Cereal and Legume Proteins

Edited by James E. Kruger and Jerold A. Bietz