ELSEVIER

Journal of Chromatography B, 711 (1998) 245–254

JOURNAL OF CHROMATOGRAPHY B

Fractionation of wheat gliadins by counter-current distribution using an organic two-phase system

Helle Truust, Göte Johansson*

Department of Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

Abstract

A liquid–liquid two-phase system based on *N,N*-dimethylformamide and the two polymers, poly(ethyleneglycol) and Ficoll, useful for partitioning of hydrophobic proteins, has been developed. The system has been applied to a counter-current distribution process in 56 steps for analysing the heterogeneity of proteins extracted with *N,N*-dimethylformamide from wheat flour. The counter-current distribution patterns of proteins, extracted from eight kinds of wheat, have been analysed. The minimum number of hypothetical proteins necessary to describe the patterns was found to be seven. The relative amount of these hypothetical components varied among the wheats. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Organic two-phase system; Partitioning; Counter-current distribution; Gliadins; Proteins

1. Introduction

The bread-making quality of wheat flour results from a balance between proteins, lipids, starch and water, and the interactions between them [1,2]. Both the concentration and the composition of protein are most important in determining the bread-making properties [3–6] of a specific wheat flour. Fractionation of wheat proteins is therefore the basis of further studies on the relation between their molecular structure and baking-related properties. The standard extraction procedure, according to Osborne [7], divides the wheat proteins into five groups: Albumins, soluble in water; globulins, soluble in salt solutions; gliadins, soluble in organic solvents; glutenins, soluble in diluted acids; with the remainder being insoluble proteins. The glutenins and gliadins are described as gluten proteins or wheat storage proteins that are only found in the endosperm, where they are the primary proteins [8]. Gliadins and glutenins confer extensibility and elasticity to the dough, respectively [3,9], and they have molecular mass distributions from 30 000 to about $20 \cdot 10^6$ Da. The gliadins make up 30–35% of the total protein present. Variations in baking performance can be related to the properties of the flour's gluten proteins. Several explanations have been put forward: (1) The gliadin/glutenin ratio [10,11], (2) the mean molecular mass distribution of glutenins [10] and (3) the presence of certain gliadin subunits [12].

In this work, partition between two liquid phases, in combination with centrifugal counter-current distribution, has been applied to the fractionation of wheat proteins that are soluble in organic solvents. One of the advantages of using liquid–liquid extraction for the fractionation of storage proteins is that the proteins can be separated in their native form

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00051-6

instead of being fractionated (by electrophoresis) into their subunits, obtained by reduction and denaturation. The isolation of native proteins also offers the opportunity to study the influence of intact proteins on baking performance by adding them to a standard dough.

Two-phase systems composed of two immiscible liquids, both consisting primarily of the same solvent, can be used to separate biological material, for example, proteins, by making use of differences in the distribution of the components between the two phases [13]. The actual partitioning between the top and bottom phases depends on the molecular structure and surface characteristics of the partitioned protein molecules. The two-phase systems used here are prepared by dissolving two polymers, poly(ethyleneglycol) (PEG) and Ficoll, in *N*,*N*-dimethylformamide (DMFA). In these two-phase systems, PEG is concentrated in the upper phase and Ficoll in the lower phase [14]. The partitioning of a substance is usually expressed by a partition coefficient, *K*, defined as the ratio between concentrations, *C*, of partitioned material in the two phases, that is, $K = C_{top}/C_{bottom}$.

2. Experimental

2.1. Chemicals

Poly(ethylene glycol) (PEG, $M_r = 8000$) was supplied by Union Carbide (New York, NY, USA). Ficoll 70 ($M_r = 70\,000$) was obtained from Pharmacia Biotech (Uppsala, Sweden). DMFA, analytical grade, was obtained from British Drug House (Poole, UK). Bicinchoninic acid (BCA) protein assay reagent was received from Pierce (Rockford, IL, USA). All other chemicals were of analytical grade.

2.2. Flour

Commercial durum flour was obtained from Hvendurum (Hven, Sweden). Flours from seven varieties of wheat were supplied by Svalöf Weibull (Svalöv, Sweden).

2.3. Analysis of phase composition

The composition of the phases was analysed by gel permeation chromatography of samples diluted with water on a Sephacryl S-300 column (45×1.5 cm I.D.) using water as the eluent. The eluate was analysed with a refractive index monitor (Multiref 902B; Optilab, Vällingby, Sweden) [14]. The Ficoll concentration in the phases was also analysed by polarimetry [15].

2.4. Density and viscosity of phases

The density was determined by weighing 10 ml of phase in a pyknometer that had been calibrated with water. The viscosity of phases was measured with the aid of a capillary U-tube viscometer, which had a flow time for water of 29 s [16].

2.5. Extraction of proteins

Wheat flour (10 g) was treated with DMFA (25 ml) by mechanical stirring at room temperature for 2 h. The mixture was then centrifuged for 15 min at 3500 *g*. The gliadins were present in the supernatant, which was used for the counter-current distribution.

2.6. Assays

Protein concentration was determined photometrically [17], using the combination of the biuret reaction with a selective detection reagent for Cu^+, BCA. The absorbance of the purple reaction product was measured at 562 nm after 2 h of incubation at room temperature.

2.7. Two-phase systems and partitioning of proteins

Organic two-phase systems containing 9% (w/w) PEG 8000, 11% (w/w) Ficoll 70, 80% (w/w) DMFA and 2.5 m*M* lithium chloride were prepared by dissolving the polymers and solid salt in DMFA, with or without extracted sample. The partition of proteins was determined by equilibrating the systems (8 g total mass) at 23°C by careful mixing for 2 min followed by centrifugation at 3000 *g* for 5 min.

Samples (25 μl) were withdrawn from each phase and their protein content was determined.

2.8. Counter-current distribution

The settling time of the phases was reduced by centrifugation. A centrifugal counter-current distribution (C-CCD) apparatus [18] containing 60 chambers and having 0.81 ml bottom phase cavities was used. Mixed systems containing 0.75 ml of bottom phase and 1.35 ml of top phase were added to each chamber. Systems with sample were applied in chambers zero and one. Sample was prepared by dissolving the polymers and the LiCl in the flour extract (in DMFA) to obtain the systems. The protein concentrations were roughly 7 mg/ml of phase system. Fifty-six distribution steps were carried out at 23°C. Each cycle included mixing of the phases at unit gravity for 2 min and centrifugation for 10 min at 100 *g*. Finally, 2 ml of DMFA were added to each chamber to obtain homogeneous solutions, and the obtained fractions were collected and analysed for protein content. The diagram obtained by plotting absorbance values as a function of tube number, the distribution diagram, shows the fractionation of protein components.

3. Results

3.1. Counter-current distribution

Counter-current distribution (CCD) can be used to fractionate complex mixtures of macromolecules, e.g. proteins. The CCD process can be seen as a stepwise chromatographic process. The principle is that two sets of liquid phases (upper and lower phases, respectively) come into contact with each other until equilibrium is achieved and then, all upper phases are transferred one step relative to the lower phases (see Fig. 1). The sample to be analysed is initially contained in one or several of the first systems (numbered zero and upwards). The CCD experiment consists of repeated cycles of shaking (to disperse the phases), settling (to separate the phases) and a transfer, where the top plate moves one step, so every top phase comes into contact with the bottom phase of the next adjacent cavity. The partitioning of a homogeneous compound between

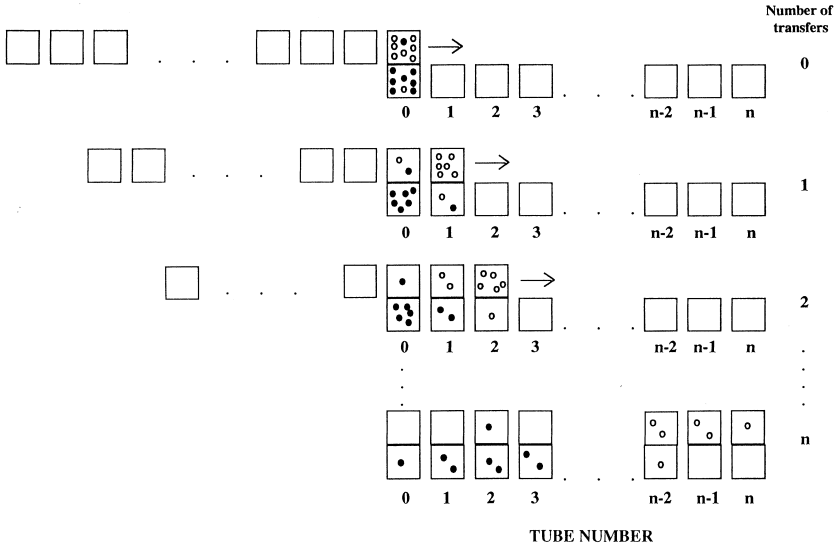

Fig. 1. Principle of CCD for two substances with partition ratios, *G*, of 1/7, filled circles, and 7, open circles. The upper phases are transferred stepwise from left to right. Between each transfer, the two-phase systems are equilibrated by shaking and the phases are allowed to settle.

the mobile and the stationary part of the two phase system is expressed by its partition ratio, *G*. The amount of substance in each tube, numbered from zero to *n*, where *n* is the number of transfers, is given by Eq. (1) for the case of sample applied to tube number zero only [19]:

$$T_i = \frac{i!}{n!(n-i)!} \cdot \frac{G^i}{(1+G)^n} \quad (1)$$

The result of the CCD is presented as a distribution curve (CCD diagram) in which the quantity of material in each cavity is plotted versus the tube number. To estimate the heterogeneity of the proteins, the CCD patterns were analysed by determining the lowest number of components (with different *G* values) necessary to give a reasonable fit by visual inspection. This was done by using a computer program based on Microsoft Excel.

3.2. Properties of the two-phase system used

The two polymers, Ficoll and PEG, in DMFA at 23°C give a liquid–liquid two-phase system where the volume of the upper phase is 1.66 times the volume of the lower phase. Both liquid phases contain mainly DMFA; PEG is concentrated in the upper phase and Ficoll in the lower phase (Table 1). The viscosities of the phases are moderate, 1.3 and two times the viscosity of water, and their densities are close to unity (Table 1). The phases are quickly dispersed in each other, even with very gentle mixing. The time for separation is relatively long, 20–25 min, at a fluid depth of 3.5 cm, due to the similarity in density of the phases and also probably due to a low interfacial tension. The time for separation in bulk phases can be reduced to less than 2 min by centrifugation at 1000 *g*. The time required to reach partition equilibrium for proteins was checked by mixing the two-phase systems, present in sealed centrifugation tubes, by slow inversions (1/s), after the addition of concentrated protein solution. Equilibrium was reached within 10 s of mixing, followed by 1 min of incubation. The results are shown in Fig. 2, with the apparent partition coefficient (ratio of protein concentrations in the upper and lower phases) as a function of the mixing time. To provide stable partition conditions for the protein, some salt must be included in the system. The salt used here, due to its solubility in DMFA and its steering effect on the cereal proteins, was lithium chloride [20]. Dextran, which is often used together with PEG to obtain aqueous two-phase systems, cannot be used here because it is insoluble in DMFA.

3.3. Choice of two-phase system for CCD

When a two-phase system is to be used for CCD, it is important to be able to steer the partition in the systems, because the resolution of the CCD diagrams is related to the average *G* value, i.e. the ratio between the mass of partitioned material in the top and bottom phases. The best separation of a mixture is normally obtained when approximately equal amounts of partitioned material are present in the transferred portion (primarily top phase) and in the stationary portion (primarily bottom phase) in the sample system, or average $G = 1$ [18]. This can be achieved by either adjusting the overall *K* value of the mixture, or by choosing the phase volume ratio, $V_{top}/V_{bottom} = 1/K_{average}$. The salt used here, LiCl,

Table 1
Content of DMFA, Ficoll and PEG in the phases of the liquid–liquid two-phase system, together with their viscosities and densities at a temperature of 23°C

Phase	DMFA % (w/w)	Ficoll % (w/w)	PEG % (w/w)	Viscosity relative to water	Density (g ml^{-1})
Top	82	4.1	13.5	1.3	0.986
Bottom	76	22.0	2.1	2.0	1.030

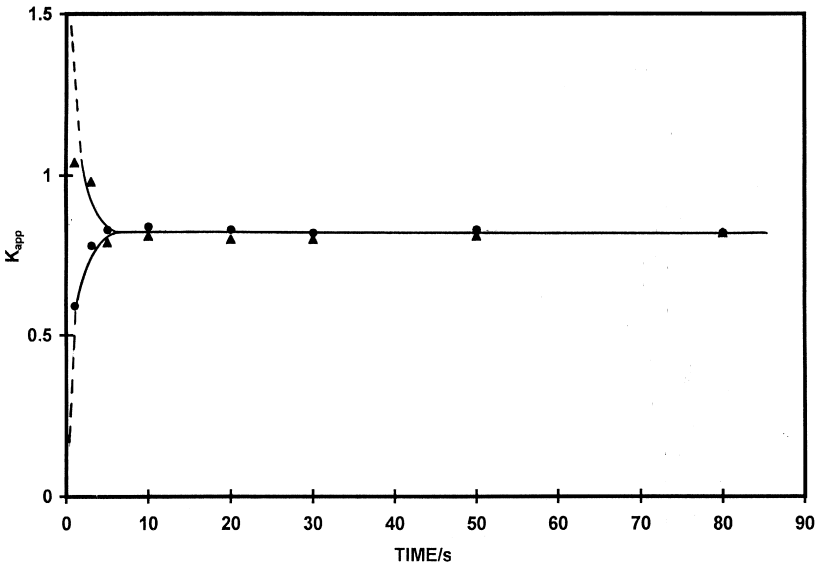

Fig. 2. The apparent partition coefficient, K_{app}, as a function of time used for mixing the two-phase systems. The wheat protein was first dissolved either in the upper phase, ▲, or in the lower phase, ●. After mixing, the systems were left for 1 min before being centrifuged.

gives an average K value of around 0.8 for cereal proteins (Fig. 2).

3.4. CCD of protein extract

Fig. 3 shows some examples of CCD distribution curves of DMFA-soluble proteins when different varieties of wheat were analysed. The proteins soluble in DMFA were a mixture of many components, which gives the experimental CCD curves a complex appearance. Complete resolution could not be obtained with the number of steps used (56 transfers) and, therefore, the proteins overlap. The distribution curves can, however, be analysed by calculating the theoretical curves for the various components and subsequent fitting of the weight sum of these theoretical curves to the observed data. By fitting the sum of the theoretical curves to the experimental curve, one can estimate the minimum number of components, and their relative amounts can be determined. A minimum of seven components have to be assumed to obtain a reasonable fit with the experimental curve. This is the case for all of the eight kinds of wheat tested, indicating that the flour samples contain at least seven, but probably many more, proteins that are soluble in organic solvent.

The wheats studied with CCD were Durum, Urban A9, Dragon A9, Turbo A6, Cadenza B5, Contra B4, Haven C2 and Hussar C2, which vary in their baking characteristics. Here they are given in the order of decreasing hardness of the gluten. Table 2 shows the amount of each theoretical component as a percentage of the sum of all of the assumed protein components. The seven (theoretical) components differ in their relative amounts. Subfraction 2 for the three varieties of flour with quite strong gluten strength (Durum, Urban A9 and Dragon A9) is more dominating than in the other five flours with weaker gluten. Durum shows a characteristic subfraction 6, which is twice as large by percent as for the other wheats. By visual inspection, this fraction (tubes numbered 36–45) was found to have a distinct yellow colour compared with the other subfractions. This may be due to the high level of carotenoid pigments in Durum flour [21]. The endosperm of Durum wheats contains about twice the concentration of yellow carotenoid pigments that are present in the endosperm of normal bread wheats. The

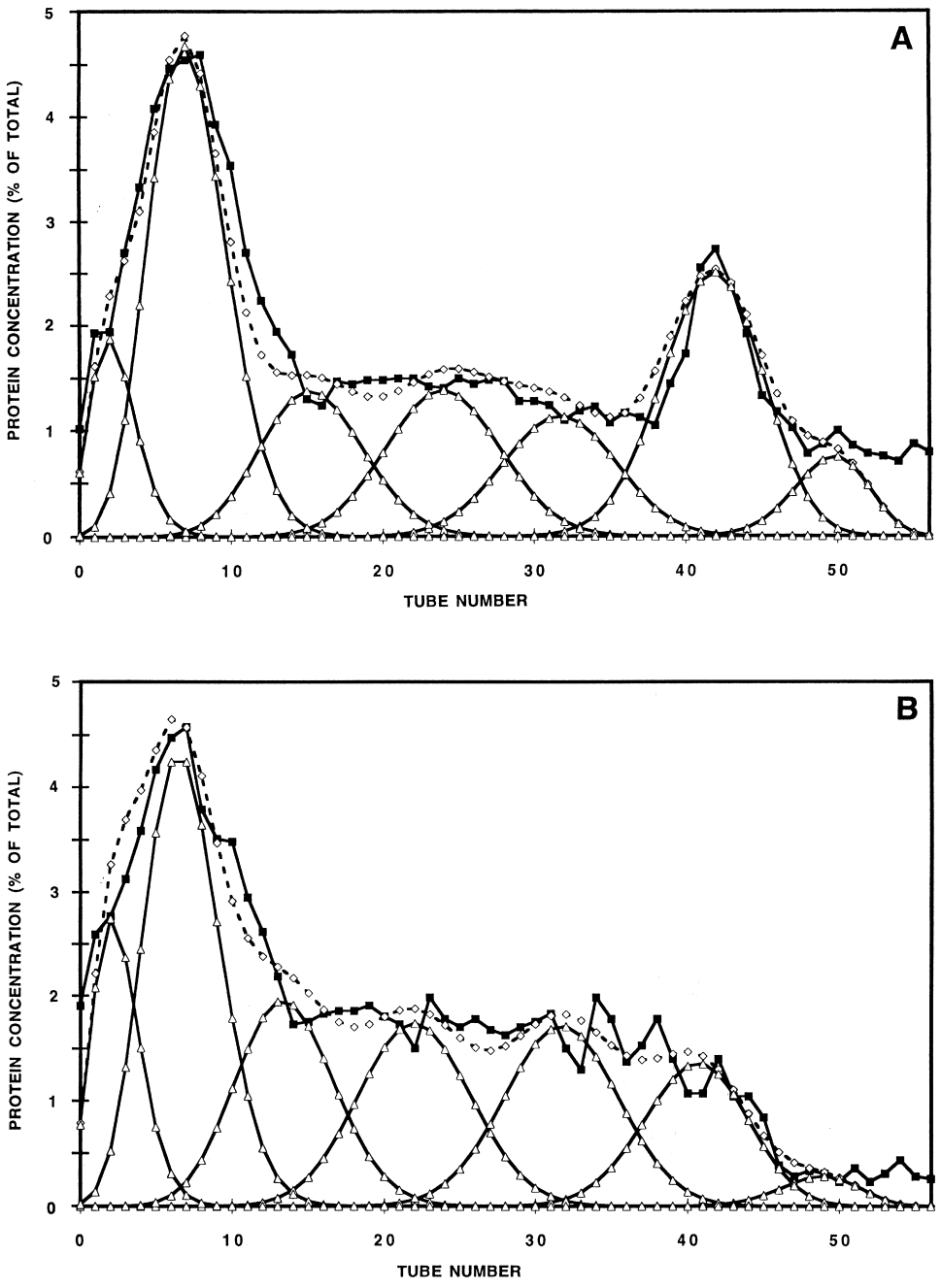

Fig. 3. CCD diagrams of eight varieties of wheat: (A) Durum; (B) Urban A9; (C) Dragon A9; (D) Turbo A6; (E) Cadenza B5; (F) Contra B4; (G) Haven C2 and (H) Hussar C2. (■) represents the experimental curve, (△) represents the theoretical curves and (◇) represents the sum of the theoretical curves.

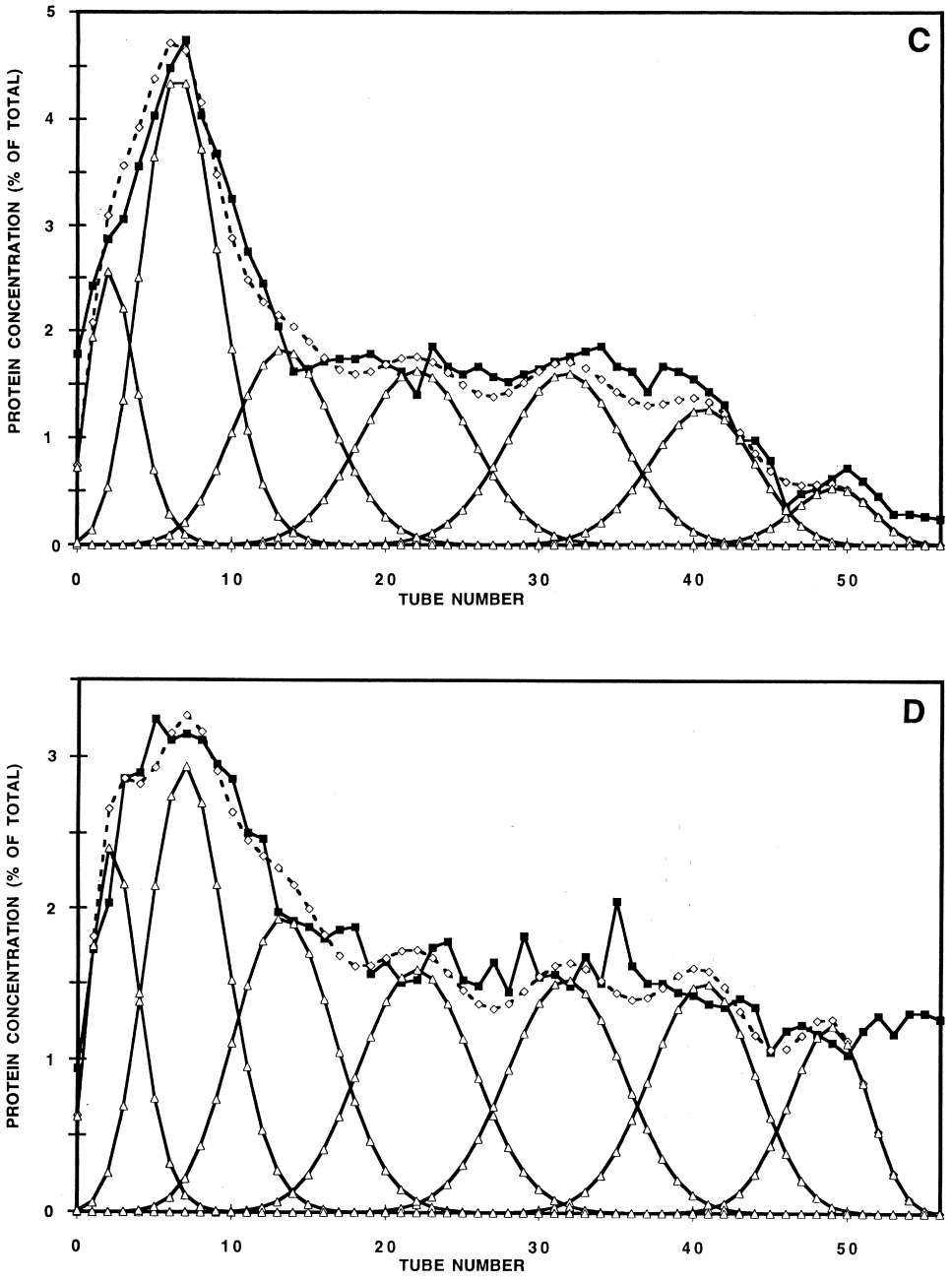

Fig. 3. (*continued*)

yellow colour of this protein component indicates that proteins of this peak bind these carotenoid pigments. For the softest wheats (Haven C2 and Hussar C2), component 5 dominates.

4. Discussion

The experiments show that proteins that are soluble in organic solvents can be fractionated in

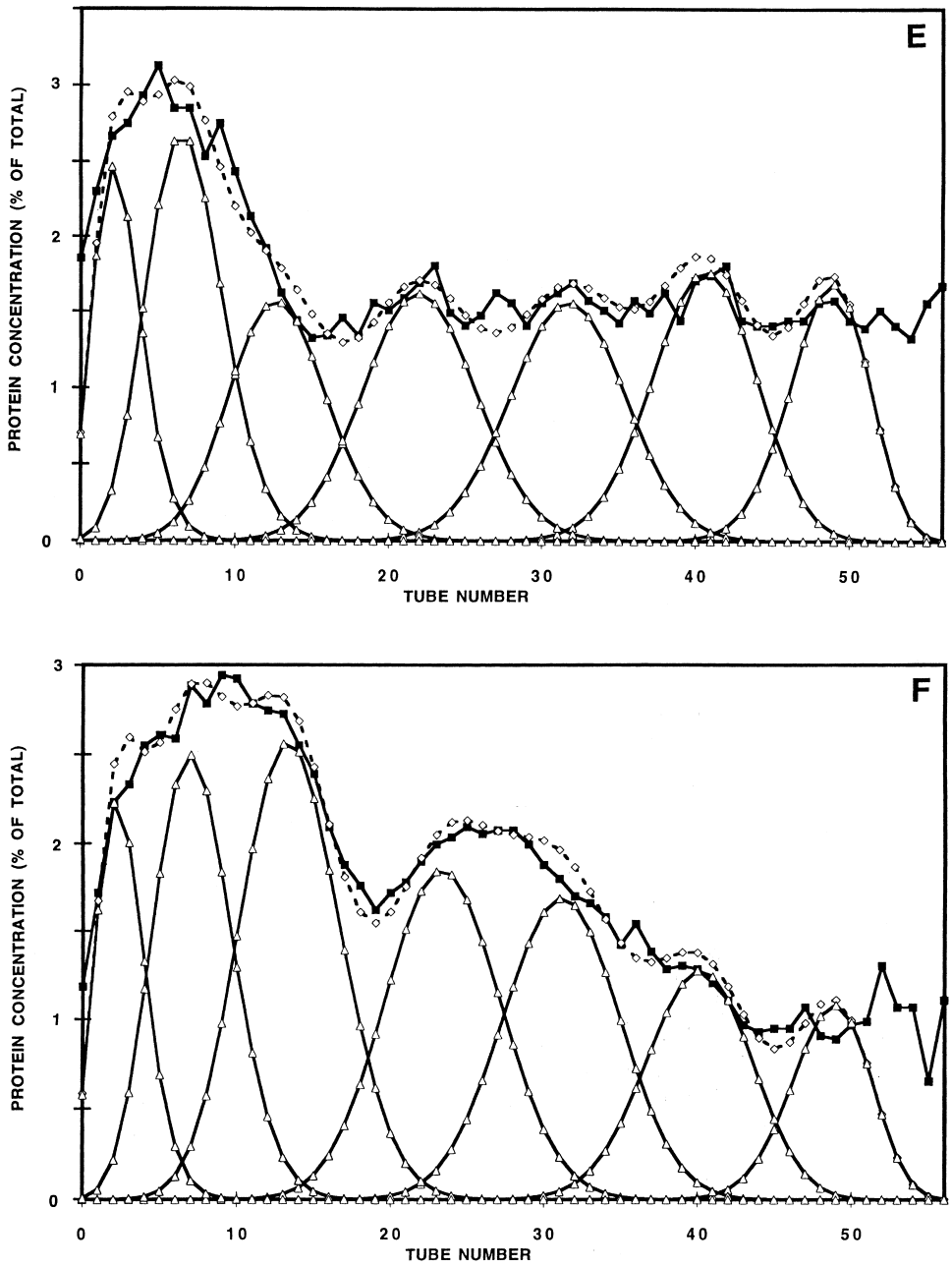

Fig. 3. (*continued*)

two-phase systems based on DMFA. The separation is enhanced by using CCD.

Counter-current distribution shows marked differences in the distribution pattern for proteins of flour made from various wheats, which can be correlated to the hardness of the gluten. This indicates that it may be possible to relate the patterns with other properties of the wheat. For example, the patterns could be compared with those from sodium dodecyl sulphate (SDS) gel electrophoresis, which is often

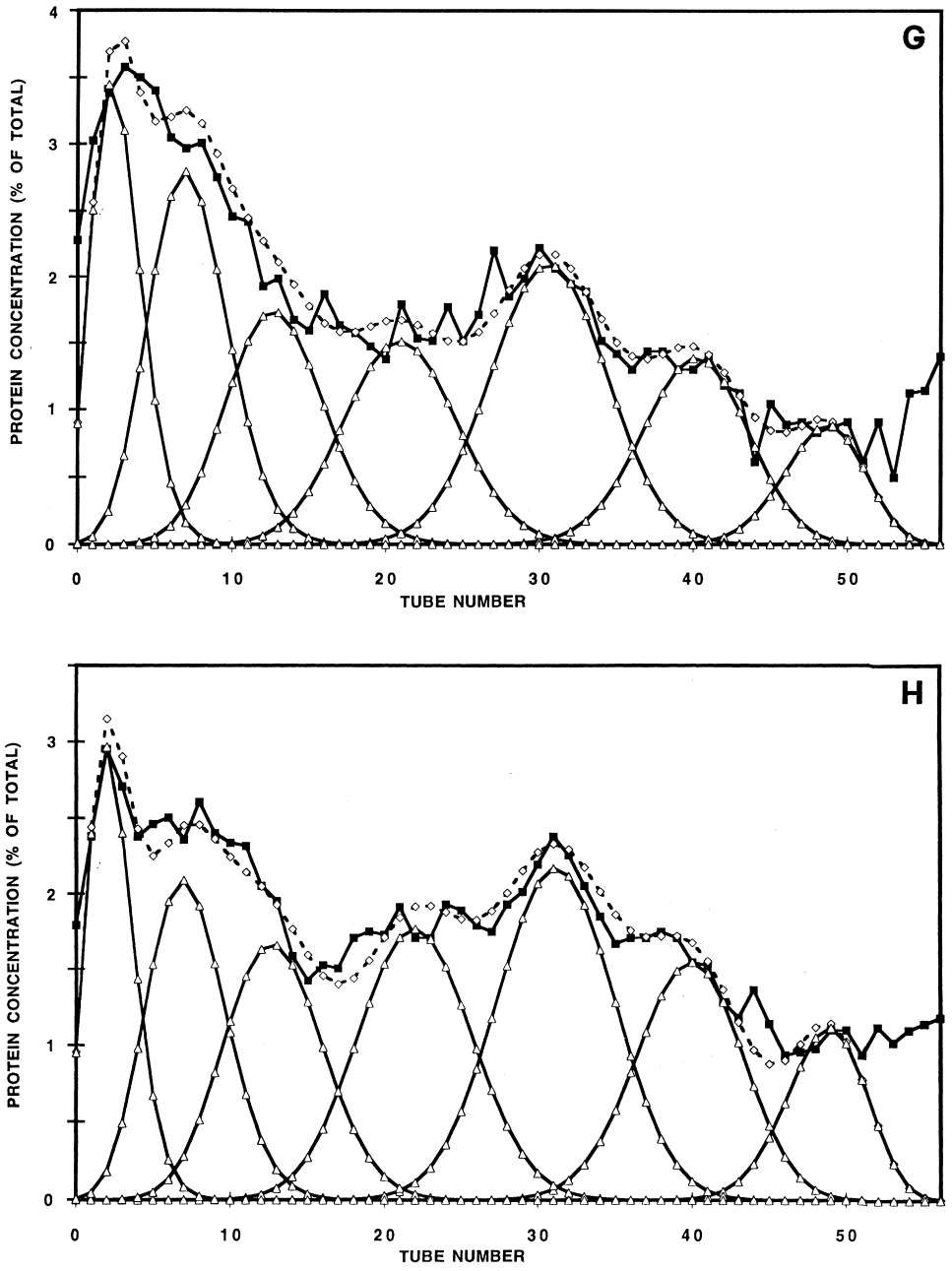

Fig. 3. (*continued*)

used as a standard method for the analysis of pattern recognition for wheat proteins, while the CCD technique, using DMFA-based systems, gives rise to other patterns that are based on the surface properties of the intact proteins.

One of the advantages of using two-phase systems for the fractionation of gluten proteins is that the proteins can be separated in their native forms instead of having to reduce them to their subunits. This should make it possible to add purified intact

Table 2
Percentage distribution of the seven hypothetical proteins, represented by the theoretical curves, for eight types of wheat

Wheat type	Hypothetical protein component						
	1 $G=0.05$	2 $G=0.1–0.2$	3 $G=0.3–0.4$	4 $G=0.6–0.8$	5 $G=1.2–1.3$	6 $G=2.4–2.6$	7 $G=6.3–7.5$
Durum	7.3	30.6	12.0	13.3	10.9	21.3	4.7
Urban A9	10.9	27.1	16.6	16.3	16.3	11.6	1.8
Dragon A9	10.4	28.5	15.4	15.6	16.6	11.4	2.4
Turbo A6	10.3	19.9	16.8	15.7	15.3	13.6	8.4
Cadenza B5	10.2	17.7	13.3	15.9	15.5	15.9	11.3
Contra B4	9.2	16.4	21.6	17.8	16.4	11.3	7.2
Haven C2	14.3	18.4	14.3	14.3	20.4	12.3	5.9
Hussar C2	11.8	14.0	14.0	17.2	21.5	14.0	7.5

These protein components are numbered from left to right in the CCD diagrams.

proteins to flours and thus study their influence on baking performance.

The resolution of the separation can be enhanced by increasing the number of transfers. Alternatively, the two-phase system may be used for liquid chromatography, by adsorbing one of the phases to a carrier and using the opposite phase as the mobile phase. This approach has successfully been realized with water-based polymeric two-phase systems [22].

Acknowledgements

We thank Dr. Gunnar Svensson, Svalöf Weibull AB, for samples of flours from a number of wheats. We also thank Dr. József Medve for his help with the computer work and Dr. Deboura Hawker-Schneider for suggestions and linguistic corrections. This work was supported by The Foundation Cerealia Research and Development.

References

[1] Y. Pomeranz, in Y. Pomeranz (Editor), Wheat: Chemistry and Technology, Vol. II, American Association of Cereal Chemists, St Paul, 1988, p. 201–381.
[2] F. MacRitchie, Adv. Food Res. 29 (1984) 201–207.
[3] J.S. Wall, in D.L. Laidman, R.S. Wyn Jones (Editors), Recent Advances in the Biochemistry of Cereals, Oxford University Press, Oxford, 1979, pp. 275–311.
[4] K.F. Fenney, M.A. Barmore, Cereal Chem. 25 (1948) 291–312.
[5] K.H. Tipples, R.H. Kilborn, Can. J. Plant Sci. 54 (1974) 231–234.
[6] G. Branlard, M. Dardevet, J. Cereal Sci. 3 (1985) 329–343.
[7] T.B. Osborne, The Proteins of the Wheat Kernel, Carnegie Institute, Washington, DC, 1907, Publ. No 84.
[8] A.-C. Eliasson, K. Larsson, Cereals in Breadmaking, Dekker, New York, 1993, pp. 241–250.
[9] P.I. Payne, L.M. Holt, E.A. Jackson, C.N. Law, Phil. Trans. R. Soc. London B 304 (1984) 359–371.
[10] F.R. Huebner, J.S. Wall, Cereal Chem. 53 (1976) 258–269.
[11] H.D. Belitz, W. Kieffer, W. Seilmeier, H. Wieser, Cereal Chem. 63 (1986) 336–341.
[12] G. Branlard, M. Dardevet, J. Cereal Sci. 3 (1985) 329–343.
[13] H. Walter, G. Johansson, Anal. Biochem. 155 (1986) 215–242.
[14] G. Johansson, M. Joelsson, J. Chromatogr. 464 (1989) 49–59.
[15] P.-Å. Albersson, Partition of Cell Particles and Macromolecules, Almqvist and Wiksell, Stockholm, and Wiley, New York, 2nd ed., 1971, pp. 250–252.
[16] D.E. Brooks, R. Norris-Jones, Methods Enzymol. 228 (1994) 14–27.
[17] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[18] H.-E. Åkerlund, P.-Å. Albertsson, Methods Enzymol. 228 (1994) 87–99.
[19] P.-Å. Albertsson, B. Andersson, C. Larsson, H.-E. Åkerlund, Methods Biochem. Anal. 28 (1982) 115–150.
[20] H. Truust, G. Johansson, J. Chromatogr. B 711 (1998) 117–126.
[21] Y. Pomeranz, in Y. Pomeranz (Editor), Wheat: Chemistry and Technology, Vol. II, American Association of Cereal Chemists, St. Paul, 1988, pp. 777–783.
[22] W. Müller, Methods Enzymol. 228 (1994) 100–112.