Process Biochemistry 44 (2009) 1213–1216

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Partitioning of glycomacropeptide in aqueous two-phase systems

C.A.S. da Silva [a], J.S.R. Coimbra [a,*], E.E.G. Rojas [b], J.A.C. Teixeira [c]

[a] Separation Process Laboratory (LPS), Department of Food Technology, Federal University of Viçosa (UFV), P.H. Rolfs Av., s/n, Zip Code 36570-000, Viçosa, MG, Brazil
[b] Department of Agribusiness Engineering, Federal University Fluminense (UFF), Trabalhadores Av., 420, Zip Code 27225-250, Volta Redonda, RJ, Brazil
[c] Department of Engineering, University of Minho, Largo do Paço, Zip Code 4707-553, Braga, Portugal

ARTICLE INFO

Article history:
Received 25 November 2008
Received in revised form 11 April 2009
Accepted 23 June 2009

Keywords:
Aqueous two-phase systems
PEG molar mass
NaCl addition
Tie line length
pH

ABSTRACT

The partition behavior of glycomacropeptide (GMP) was determined in polyethylene glycol (PEG) and sodium citrate aqueous two-phase systems (ATPS). It was found that the partitioning of GMP depends on PEG molar mass, tie line length, pH, NaCl concentration and temperature. The obtained data indicates that GMP is preferentially partitioned into the PEG phase without addition of NaCl at pH 8.0. Larger tie line lengths and higher temperatures favor GMP partition to the PEG phase. Furthermore, it was verified that PEG molar mass and concentration have a slight effect on GMP partition. The increase in the molar mass of PEG induces a reduction of the protein solubility in the top PEG rich phase, being shown that the use of PEG1500 is beneficial for the extraction of GMP. A protein recovery higher than 85% was obtained in the top phase of these systems, clearly demonstrating its suitability as a starting point for the separation of GMP.

1. Introduction

Glycomacropeptide (GMP) comprises the 64 amino acids in the hydrophilic C-terminal portion of κ-CN. This peptide is formed by the chymosin (or pepsin) cleavage of κ-casein between Phe105–Met106 during the manufacture of cheese [1,2]. Next to β-lactoglobulin and α-lactalbumin, GMP is the most abundant protein/peptide in whey proteins with typical concentrations between 20% and 25% [1]. In recent years, an increasing number of studies show that GMP may exert important biological activities. The literature has highlighted the ability of GMP to bind cholera and *Escherichia coli* enterotoxins, to exhibit growth-promoting effects on bifidobacteria, to suppress gastric secretion and to inhibit viral or bacterial adhesion to intestinal epithelia cells [1,3]. This explains the growing interest in developing techniques for the isolation and purification of GMP. Thus, as an alternative to the first purification step of GMP, a purification process based on aqueous two-phase systems (ATPS) was proposed.

Aqueous two-phase extraction has been used since the mid-1950s as a mild separation method of wide applicability in biochemistry, cell biology and biotechnology [4]. An ATPS is formed when two water-soluble polymers, such as polyethylene glycol (PEG) and dextran, or a polymer and a salt are dissolved in water beyond a critical concentration at which two immiscible phases form [5]. PEG–salt ATPS have certain advantages over PEG–dextran systems such as low viscosity and lower cost [6]. PEG has been combined with sodium citrate to form ATPS, due to citrate's characteristics as a biodegradable and nontoxic compound. PEG + citrate salts form environmentally safe ATPS, which are more suitable for the extraction of biological materials [7].

ATPS have several advantages in comparison with conventional methods for the isolation and purification of proteins such as low cost, nontoxic, the possibility of application on a large scale and the short time required for reaching equilibrium [8]. The selective distribution of ATPS constituents may be affected by different factors like the nature and size of the biocompound, molecular structure and chain size of the polymer, type of salt, pH, initial composition of the system and temperature [9,10]. Thus, the goal of this work is to perform a preliminary study about the partition of GMP using the PEG–sodium citrate system. Relevant parameters such as pH, polymer molar mass, tie line length, NaCl addition and temperature which affect the partitioning behavior of the protein were investigated.

2. Materials and methods

2.1. Chemicals

Glycomacropeptide (GMP) was a kind gift from Davisco Foods International (USA), with purity greater than 83% (w/w). Polyethylene glycol (PEG), with average molar masses of 1500, 2000 and 4000 (PEG1500, PEG2000 and PEG4000), were purchased from Synth (Brazil), Riedel de Haen (Germany) and Isofar (Brazil), respectively. Sodium citrate was obtained from Synth (Brazil). All chemicals were of analytical grade and the polymer and salts were used without further purification. Ultrapure water for the experiments was obtained from a Milli-Q system (Millipore Inc., MA, USA).

* Corresponding author. Tel.: +55 31 3899 2229; fax: +55 31 3899 2208.
E-mail address: jcoimbra@ufv.br (J.S.R. Coimbra).

1359-5113/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2009.06.016

2.2. Preparation of the aqueous two-phase systems

Biphasic systems were prepared with a mixture of PEG (1500, 2000, and 4000) and sodium citrate. To prepare ATPS, stock solutions of PEG 50% (w/w), sodium citrate and water were mixed to obtain a total system composition in accordance with the phase diagrams obtained in the literature [7,11]. Sodium citrate was employed by weighing the solid form; the desired pH (6.0, 7.0 or 8.0) of the ATPS was adjusted by the addition of sodium hydroxide or citric acid. All systems were prepared in graduated centrifuge tubes with a total phase system mass of 10 g. The amount of GMP added to the systems was 20 mg, and it was the last added component. For the systems with sodium chloride, the powdered salt was directly dissolved into the systems, to achieve concentrations of 0.1 or 0.5 M. Each system was then centrifuged at $2000 \times g$ for 20 min, at 25 °C, and transferred to a thermostatic bath where the mixture was allowed to settle for 12 h at the operational temperature (25.0, 35.0, and 45.0) ± 0.1 °C to obtain clear phase separation and to reach equilibrium. After reaching equilibration, estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. In order to determine the concentration of proteins in each of the co-existing phases, separate samples from both the top and bottom phases were collected using a syringe. The tie line length (TLL) was calculated according to Eq. (1):

$$\mathrm{TLL} = \sqrt{[\Delta\mathrm{PEG}]^2 + [\Delta\mathrm{Salt}]^2} \quad (1)$$

where [ΔPEG] and [ΔSalt] are the differences between the concentration of PEG and salt in the top and bottom phases expressed as a percentage (w/w). And the slope of the tie line (STL) was calculated by:

$$\mathrm{STL} = \frac{\Delta\mathrm{PEG}}{\Delta\mathrm{Salt}} \quad (2)$$

2.3. Determination of the protein partition coefficient (K_p)

The partition coefficient (K_p) was defined as the ratio between $[P]_{top}$ and $[P]_{bottom}$, where $[P]_{top}$ and $[P]_{bottom}$ are the equilibrium concentrations of the partitioned protein in the PEG and sodium citrate rich phases, respectively. The partition experiments were carried out in duplicate and the average results are reported. To select the ATPS with the best capability of purifying GMP, the recovery percentage (y, %) in the top phase was calculated according to Eq. (3):

$$y(\%) = \frac{100}{1 + (1/RK_p)} \quad (3)$$

where the phase volume ratio (R) is defined as V_T/V_B; where V_B and V_T are the bottom and top phase volumes, respectively. It is generally desirable to recover the target protein in the top phase, which is better for protein stability; recovery in the bottom salt phase also requires desalination if followed by an ion exchange step [5].

2.4. Protein quantification

Protein quantification in the saline and polymeric phases was conducted using a ÄKTA Purifier® 10/100 chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). The eluent was monitored by UV absorption (UV-900) at 205 nm and conductivity measurements in a flow cell (pH/C-900). Samples were injected by means of a 50 μl loop. The used column was a Superdex® 75 HR 10/30 (Pharmacia Biotech, Uppsala, Sweden) and the mobile phase was a 0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic solution at pH 6.0, with a flow rate of 0.65 ml min^{-1}. Maximum pressure of the column bed was 1.78 MPa. The polymeric phase was diluted before injection in a 2:1 (water:sample) ratio due to its high viscosity.

3. Results and discussion

3.1. Effect of sodium chloride addition and pH on protein partitioning

The ATPS formed by 14.0% (w/w) PEG1500 and 14.9% (w/w) sodium citrate was used to investigate the effect of the addition of sodium chloride (NaCl) and pH on the partitioning and recovery of GMP. The partition experiments were carried out at different pH values (6.0, 7.0, and 8.0) and NaCl concentrations (0.0, 0.1, and 0.5 M). The results are shown in Fig. 1. pH values between 4 and 5 [1] were chosen so that the aqueous phase was neither too acidic nor too basic. This is important since very acidic or basic solutions cannot be discharged in the environment without further treatment.

The partition coefficients of GMP in the systems without NaCl at pH values of 6.0, 7.0, and 8.0 are 5.7, 7.6 and 8.8, respectively, showing that by increasing the pH from 6 to 8, a better partitioning

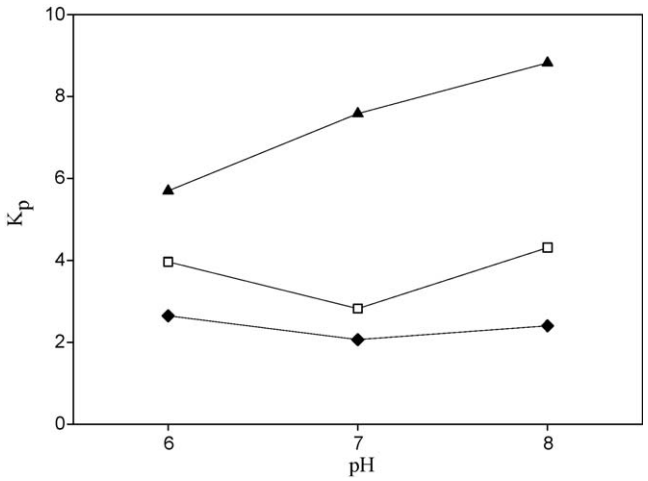

Fig. 1. Influence of pH on GMP partitioning in PEG1500–sodium citrate ATPS at different NaCl concentrations. 0.0 M (▲), 0.1 M (□), and 0.5 M (◆). Temperature: 25 °C.

of the protein in the top phase (PEG rich phase) was achieved. This increase in the partition coefficient value may be due to hydrophobic interaction and net charge effects. According to Mikkelsen et al. [2], the sequence-derived molar mass (MM) of GMP is 6.7 kDa, previous studies suggesting that GMP forms aggregates and that aggregation occurs at pH values greater than 4.5. Kawasaki et al. [12] reported a GMP pH-dependent association/dissociation. At pH 7.0, the apparent molar mass of GMP ranged from 20 to 50 kDa but at pH 3.5 it ranged from 10 to 30 kDa. Mikkelsen et al. [2] verified that tetrameric forms (MM above 35 kDa, determined from size exclusion chromatography (SEC) fractions) of GMP were significantly more hydrophobic than the dimeric and monomeric forms. Moreover, proteins with large hydrophobic surface areas exposed to a solvent have the possibility of interacting with PEG [13]. Consequently, this hydrophobic interaction between PEG and GMP when pH values increase is the factor that drives the GMP partition to the PEG rich phase, which is more hydrophobic than the salt rich phase [14,15].

It is known that salt concentration alters protein partition in aqueous two-phase systems. To obtain a more complete characterization of protein partition, the effect of sodium chloride addition to the system at two concentrations (0.1 and 0.5 M) was measured at pH values of 6.0, 7.0 and 8.0. From Fig. 1, a decrease in the partition coefficient with the increase of the NaCl content in the system is shown. The partition coefficient at pH 8.0 decreases from 8.8 (in the absence of salt) to 2.4 when a 0.5 M NaCl concentration is used. Similar behavior was verified for pH values of 6.0 and 7.0. The occurrence of this effect may be due to an increase in protein solubility as a result of the addition of neutral electrolytes, e.g. NaCl, at a concentration in the order of 0.1–1.0 M; the increased net charge of the protein due to the binding of the ion should increase the electrostatic free energy of the protein and the resulting repulsive forces lead to the decrease in the stability of the protein [16,17]. This electrostatic repulsive force should also prevent protein association or aggregation; in other words, it should increase the solubility of the GMP in the system which is fully in agreement with the known protein salting-in property of NaCl. Thus, we suggest a dissociation of the GMP tetrameric form with the increase of the NaCl content in the system. In addition, the original monomeric form of GMP is highly acidic and hydrophilic [1] and consequently, the affinity between GMP and PEG is lower and a decrease in the K_p values is verified. According to the results, the ATPS formed by 14.0% (w/w) PEG1500 and 14.9% (w/w) sodium citrate systems, at pH 8.0 and without the addition of NaCl, was

Table 1
Top phase recovery and partition coefficients for GMP in PEG–sodium citrate (SC) ATPS at pH 8.0.

Temperature	Phase composition (%, w/w)	TLL	STL	K_p	y (%)
25 °C	14.00% PEG1500–14.89% SC	24.08	1.50	8.82 ± 0.12	85.60 ± 0.28
	14.25% PEG1500–17.23% SC	32.31	1.43	15.60 ± 0.13	90.76 ± 0.08
	15.00% PEG1500–19.25% SC	34.60	1.38	29.74 ± 0.35	94.31 ± 0.06
	14.00% PEG2000–14.53% SC	22.00	1.65	5.16 ± 0.07	81.07 ± 0.26
	14.25% PEG2000–16.21% SC	26.90	1.57	6.09 ± 0.19	86.52 ± 0.05
	15.00% PEG2000–17.40% SC	31.20	1.55	8.86 ± 0.01	86.66 ± 0.05
	14.00% PEG4000–14.85% SC	35.52	1.77	2.31 ± 0.29	60.01 ± 2.99
	14.25% PEG4000–16.81% SC	38.13	1.64	4.14 ± 0.46	72.07 ± 2.40
	15.00% PEG4000–18.36% SC	43.17	1.68	5.01 ± 0.35	73.27 ± 1.39
35 °C	14.00% PEG1500–14.52% SC	26.57	1.53	8.56 ± 0.49	85.92 ± 0.72
	14.25% PEG1500–16.62% SC	31.27	1.49	18.39 ± 0.86	91.51 ± 0.37
	15.00% PEG1500–18.79% SC	36.60	1.41	24.66 ± 0.75	93.55 ± 0.27
	14.00% PEG2000–11.92% SC	18.37	2.04	2.89 ± 0.02	69.12 ± 0.03
	14.25% PEG2000–13.62% SC	25.04	1.85	5.17 ± 0.18	77.93 ± 0.74
	15.00% PEG2000–15.04% SC	30.36	1.81	9.44 ± 0.09	83.92 ± 0.45
	14.00% PEG4000–14.98% SC	37.99	1.88	2.19 ± 0.07	59.80 ± 0.71
	14.25% PEG4000–17.24% SC	41.92	1.78	4.96 ± 0.31	74.33 ± 3.04
	15.00% PEG4000–18.65% SC	44.04	1.64	5.54 ± 0.14	76.30 ± 1.13
45 °C	14.00% PEG1500–14.93% SC	27.93	1.54	10.99 ± 0.40	87.77 ± 1.40
	14.25% PEG1500–16.77% SC	30.54	1.48	20.60 ± 0.50	91.25 ± 0.21
	15.00% PEG1500–18.91% SC	34.96	1.46	33.41 ± 0.10	94.66 ± 0.03
	14.00% PEG2000–11.40% SC	25.40	2.20	3.08 ± 0.30	65.07 ± 2.25
	14.25% PEG2000–12.68% SC	29.81	2.10	3.71 ± 0.30	68.04 ± 2.16
	15.00% PEG2000–13.59% SC	35.46	2.05	5.70 ± 0.08	76.83 ± 0.26
	14.00% PEG4000–14.87% SC	39.32	1.93	2.05 ± 0.18	54.52 ± 2.17
	14.25% PEG4000–16.42% SC	42.84	1.89	3.47 ± 0.04	65.02 ± 0.32
	15.00% PEG4000–18.95% SC	44.61	1.71	6.77 ± 0.13	78.65 ± 0.32

TLL: tie line length expressed as a percentage (w/w); STL: slope of the tie line; K_p: GMP partition coefficient; y: theoretical recovery in the top phase.

chosen for the subsequent studies. In this system, the percentage yield of the extraction in the ATPS was 85.6%.

3.2. Influence of PEG molar mass, tie line length and temperature on protein partitioning

Table 1 shows the effect of the tie line length (TLL), PEG molar mass and temperature on GMP partitioning at pH 8.0, without the addition of NaCl. The results indicate that the system studied is slightly influenced by the PEG molar mass. The effect of polymer molar mass is usually attributed to the excluded volume effects that increase with increasing polymer molar mass. According to this theory, the increase in the molar mass of PEG induces a reduction of the protein solubility in the phase in which the protein is located [18–21]. A decrease in the partition coefficients with the increase of the PEG molar mass was expected and verified by the obtained results. However, it is important to keep in mind that by changing the polymer molar mass, one needs to alter the polymer and salt concentration as well. Consequently, the effect of the tie line length on the partition coefficients of GMP should also be analyzed.

From Table 1, it can be observed that the partitioning of GMP in PEG–sodium citrate systems is dependent on the TLL. When the TLL is increased an increase in the K_p values for studied PEGs molar mass was observed. This effect of TLL on a protein can be due to a fine balance between two factors: the high ionic strength created by the increase in salt concentration, which improves the movement of the protein to the PEG rich phase as a consequence of electrostatic repulsion effects [22] and the PEG–GMP binding through the hydrophobic surface of the protein exposed to the solvent [2].

Obtained results indicate that changes in temperature are capable of increasing the partition coefficient of GMP. Aiming to analyze temperature's effect on the K_p, the slope of the tie line (STL) concept was applied. The STL explains the effect of the operational conditions on system composition. When the temperature increases, the slope of the tie line (STL) tends to increase, reducing the amount of salt needed to form a biphasic system with a given amount of PEG [10]. For a given system composition, tie line length increases with increasing temperature as well as with increasing STL (Table 1). Thus, the effect of increasing temperature in an ATPS may be considered similar to increasing tie line length [23]. Moreover, PEG is a molecule for which each ethylene group in its structure interacts with 14 water molecules being the ordered water formation very sensitive to temperature changes. A temperature increase causes the water molecules to lose their order near the hydrophobic polymer surface, which may improve the protein–PEG interaction and increase the K_p values [24].

4. Conclusions

The GMP partitioning behavior in PEG/citrate ATPS can be controlled by an adequate selection of the system properties. It was verified that the GMP partitioning can be influenced by several factors, including PEG molar mass, TLL, temperature and pH. The ATPS composed of 15.0% (w/w) PEG1500 + 18.9% (w/w) sodium citrate, at pH 8.0 and without the addition of NaCl, showed to have the best capability of recovering GMP. In this system, the GMP partition coefficient was 33.4 and its recovery was of 95% in the top phase. Moreover, the results show that GMP is obtained in the PEG rich phase, where the prevalent non-protein contaminants are PEG and a small amount of sodium citrate (a biodegradable and nontoxic salt), which can be removed from the target protein by means of size exclusion chromatography. This study has clearly shown that ATPS can be successfully used for the recovery and initial purification of GMP.

Acknowledgements

The authors wish to acknowledge the National Council of Technological and Scientific Development (CNPq) and the Research Support Foundation of Minas Gerais (FAPEMIG) for their financial support.

References

[1] Thoma-Worringer C, Sørensen J, López-Fandiño R. Health effects and technological features of caseinomacropeptide. Int Dairy J 2006;16:1324–33.
[2] Mikkelsen TL, Frøkiær H, Topp C, Bonomi F, Iametti S, Picariello G, et al. Self-association is dependent on whether the peptide is free or restricted in κ-casein. J Dairy Sci 2005;88:4228–38.
[3] Abd El-Salam MH, El-Shibini S, Buchheim W. Characteristics and potential uses of the casein macropeptide. Int Dairy J 1996;6:327–41.
[4] Oliveira LA, Sarubbo LA, Porto ALF, Campos-Takaki GM, Tambourgi EB. Partition of trypsin in aqueous two-phase systems of poly (ethylene glycol) and cashew-nut tree gum. Process Biochem 2002;38:693–9.
[5] Gu Z, Glatz CE. Aqueous two-phase extraction for protein recovery from corn extracts. J Chromatogr B 2007;845:38–50.
[6] Balasubramaniam D, Wilkinson C, Van Cott K, Zhang C. Tobacco protein separation by aqueous two-phase extraction. J Chromatogr A 2003;989:119–29.
[7] Muregesan T, Perumalsamy M. Liquid–liquid equilibria of poly (ethylene glycol) 2000 + sodium citrate + water at (25, 30, 35, 40, and 45) °C. J Chem Eng Data 2005;50:1392–5.
[8] Boaglio A, Bassani G, Picó G, Nerli B. Features of the milk whey protein partitioning in polyethyleneglycol–sodium citrate aqueous two-phase systems with the goal of isolating human alpha-1 antitrypsin expressed in bovine milk. J Chromatogr B 2006;837:18–23.
[9] Albertsson PA. Partition of Cell Particles and Macromolecules, 3rd ed., New York: Wiley; 1971.
[10] Carvalho CP, Coimbra JSR, Costa IAF, Minim LA, Silva LHM, Maffia MC. Equilibrium data for PEG 4000 + salt + water systems from (278.15 to 318.15) K. J Chem Eng Data 2007;52:351–3.
[11] Oliveira RM, Coimbra JSR, Minim LA, Silva LHM, Fontes MPF. Liquid–liquid equilibria of biphasic systems composed of sodium citrate + polyethylene(-glycol) 1500 or 4000 at different temperatures. J Chem Eng Data 2008;53:895–9.
[12] Kawasaki Y, Kawakami H, Tanimoto M, Dosako S, Tomizawa A, Kotake M, et al. pH-Dependent molecular weight changes of kappa-casein glycomacropeptide and its preparation by ultrafiltration. Milchwissenschaft 1993;48:191–6.
[13] Zaslavsky BY. Aqueous Two-phase Partitioning: Physical Chemistry and Bioanalytical Applications. New York: Marcel Dekker; 1995.
[14] Spelzini D, Farruggia B, Picó G. Features of the acid protease partition in aqueous two-phase systems of polyethylene glycol-phosphate: chymosin and pepsin. J Chromatogr B 2005;821:60–6.
[15] Rojas EEG, Coimbra JSR, Minim LA, Zuniga ADG, Saraiva SH, Minim VPR. Size-exclusion chromatography applied to the purification of whey proteins from the polymeric and saline phases of aqueous two-phase systems. Process Biochem 2004;39:1751–9.
[16] Arakawa T, Timasheff SN. Preferential interactions of proteins with salts in concentrated solutions. Biochemistry 1982;21:6545–52.
[17] Vojdani F. Solubilty. In: Hall GM, editor. Methods of Testing Protein Functionality. London: Blackie Academic and Professional; 1996.
[18] Nalinanon S, Benjakul S, Visessanguan W, Kishimura H. Partitioning of protease from stomach of albacore tuna (*Thunnus alalunga*) by aqueous two-phase systems. Process Biochem 2009;44:471–6.
[19] Klomklao S, Benjakul S, Visessanguan W, Simpson BK, Kishimura H. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem 2005;40:3061–7.
[20] Reh G, Nerli B, Picó G. Isolation of alpha-1-antitrypsin from human plasma by partitioning in aqueous biphasic systems of polyethyleneglycol-phosphate. J Chromatogr B 2002;780:389–96.
[21] Almeida MC, Venancio A, Teixeira JA, Aires-Barros MR. Cutinase purification on poly(ethylene glycol)–hydroxypropyl starch aqueous two-phase systems. J Chromatogr B 1998;711:151–9.
[22] Saravanan S, Rao JR, Murugesanb T, Nair BU, Ramasami T. Partition of tannery wastewater proteins in aqueous two-phase poly (ethylene glycol)–magnesium sulfate systems: effects of molecular weights and pH. Chem Eng Sci 2007;62:969–78.
[23] Griffin ST, Dilip M, Spear SK, Huddleston JG, Rogers RD. The opposite effect of temperature on polyethylene glycol-based aqueous biphasic systems versus aqueous biphasic extraction chromatographic resins. J Chromatogr B 2006;844:23–31.
[24] Tubio G, Nerli B, Pico G. Relationship between the protein surface hydrophobicity and its partitioning behaviour in aqueous two-phase systems of polyethylene glycol–dextran. J Chromatogr B 2004;799:293–301.