Physical and rheological characterisation of polyethylene glycol–cashew-nut tree gum aqueous two-phase systems

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Abstract

The characterisation of the polyethylene glycol–cashew-nut tree gum aqueous two-phase system is described. Factors which affect the phase diagram including polymer molecular mass, pH and temperature were analysed. The physico–chemical properties of the system such as density, viscosity, volume ratio and phase separation times were also described. The characteristics of the system studied indicate it to be very attractive as a separation technique. © 2002 Elsevier Science B.V. All rights reserved.

1. Introduction

Aqueous two-phase systems are based on water soluble polymers and salts and/or two different water soluble polymers. Phase separation occurs over certain concentrations of the phase components. The binodial line is the boundary condition of concentrations for phase separation. Above the binodial line, the phase component separates to the upper phase and the lower phases. However, below the binodial line, one mixed phase exists [1,2]. The overall composition of phase-forming components has been exploited to influence the phase preference of proteins [3]. Although other methods have been

suggested [4], composition may be usefully characterized by tie-line length [5], since systems sharing a common tie-line have the same composition of top and bottom phase and exhibit similar partition behaviour [3].

Two-phase systems made by organic solvent and aqueous solution are being used for separation. Two-phase systems sometimes cannot be applied to the separation of active biological materials, which require mild aqueous environments. On the other hand, aqueous two-phase systems have low osmotic pressure, high water activity, buffering effects of added salts and very low interfacial tension between the upper and the lower phases [1,2]. Aqueous two-phase systems have been used for separating plant and animal cells, cell organelles, membranes, enzymes, nucleotides and other biological materials

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[1,6]. The aqueous two-phase systems have been designed for scale up of downstream processes for biomaterial separation [7–10]. Brooks et al. [11] established methods of stock solution preparation and experiments to deal with viscous solutions and biomolecules.

Partition characteristics depend on the surface properties of the biological materials which are to be separated [12–14]. The surface charge of biological materials is one of the use of partitioning [15]. Molecular mass, shape, surface hydrophobicity and specific binding sites of biological materials also affect the partition profiles [14,16].

The main advantages of using such systems [1], may be summarized as follows:

- Scale-up can be predicted easily and reliably from small laboratory experiments.
- Rapid mass transfer and equilibrium is reached by relatively little input of energy in the form of mechanical mixing.
- Continuous processing is readily achievable.
- The polymers stabilize the enzymes.
- Separation can be made selective and rapid
- Separation can be carried out at room temperature due to the rapid separation.
- It has proven to be more economical than other separation processes.

Albertsson [1] has investigated several two-phase aqueous systems and provided the relevant phase diagrams. He has stabilised the factors which affect the phase diagram including types of polymer, molecular mass of the polymer, pH and temperature. Although the effects and mechanisms by which they influence phase separation are still not completely understood, they are of relevant importance to the knowledge of characteristics and applications of a system.

Most industrial-scale aqueous two-phase separations use polyethylene glycol (PEG)–salt systems, which may damage fragile proteins [17] and which present waste disposal problems [18]. Two-polymer systems can overcome these drawbacks. However, the high cost of the fractionated dextrans has prevented their use on a large scale [19]. A number of low-cost alternatives have been investigated, including crude dextran [20], maltodextrins [21], hydroxy-

propyl starch derivatives [22,23] and arabinogalactan [24].

Despite the success of the aqueous two-phase separation technique, data on the properties of phase systems that are necessary for the design of extraction processes and for the development of models that predict phase partitioning are few [3].

We have shown recently an economic water-soluble acidic heteropolysaccharide gum from *Anacardium occidentale* L. (cashew-nut tree) found in Brazil [25] that has the potential to act as an alternative to fractionated dextran in aqueous two-phase systems.

In this work we have studied the factors which influence the phase diagram including polymer molecular mass, pH and temperature and some physico–chemical properties (density, viscosity and volume ratio) of the PEG–cashew-nut tree gum systems with the aim to obtain a more complete characterisation of this new system for application in separation processes.

2. Experimental

2.1. Chemicals

Crude gum was collected as natural exudate from cultivated *Anacardium occidentale* trees of various localities in Pernambuco State, Brazil. Plants (20 years old) producing yellow cashews were used. PEG 8000 and PEG 4000 were obtained from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade.

2.2. Purification of gum

Clear nodules free of bark were selected to be purified via ethanol by use of the Rinaudo–Millas method as previously described [26]. Precipitation with ethanol permitted the isolation of the polysaccharide from the monosaccharides and oligosaccharides, which remained in solution.

2.3. Phase diagrams

Phase diagrams at 25 ± 2 and $40\pm2°C$ were determined according to Albertsson's [1] procedure. The binodial of the phase diagram, the demarcation

between PEG–cashew-nut tree gum compositions showing monophasic and biphasic behaviour was obtained by direct observation of two-phase formation for a large number of solutions containing varying concentrations of PEG and cashew-nut tree gum (Table 1). Systems that displayed a distinct phase/phase interface were considered biphasic. The polymer composition of the top and bottom phases were then analysed. PEG concentration was determined according to Skoog [27]. Polysaccharide concentration was determined by measuring reducing sugars (DNS method, Miller [28]) concentration after a hydrolysis step with sulfuric acid.

A constant temperature bath was employed to maintain the temperature at the desired value. Systems were prepared and transferred to a glass beaker and the beaker was placed in the constant temperature bath. The mixture was mechanically stirred for some minutes to ensure equilibrium conditions. The phases generated were then allowed to separate in the constant temperature bath for 24 h.

2.4. Two-phase systems

The systems (total mass 10 g) were prepared from stock solutions of the polymers in water, 30% (w/w) cashew-nut tree gum and 50% (w/w) PEG. The polymers solutions were weighed out and mixed with phosphate buffer (15 mM) according to the desired pH (6.0, 7.0 and 8.0).

2.5. Rheological properties (viscosities and densities)

The densities of the top phases were determined by weighing 1 ml of phase with a micropippete in an analytical balance, in quadruplicate. The densities of the

Table 1
Compositions (w/w) of PEG–cashew-nut tree gum systems with PEG 4000 and 8000

System	Tie-line	PEG (%)	Cashew-nut tree gum (%)
PEG 4000	1	9	18
	2	11	20
	3	13	22
PEG 8000	1	9	16
	2	11	18
	3	13	20

bottom phases were determined by a mass balance. For systems at $40 \pm 2°C$, densities were determined in the same way, using a constant temperature bath.

Phase viscosities were measured using a torque measuring viscometer (Viscometers UK – model Cole Parmer Rotational) using a constant temperature bath.

2.6. Phase separation times

The time required for phase separation was determined by a set of four parallel experiments performed in test tubes containing 10 g of a two-phase system. The systems were allowed to separate under gravity until a clear interface was noticed. For systems at $40 \pm 2°C$, time of phase separation was determined the same way, using a constant temperature bath.

2.7. Volume ratio

Visual estimates of the volumes of the top and bottom phases (10 g) were made in graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio. For systems at $40 \pm 2°C$, the volume ratio was determined the same way using a constant temperature bath.

3. Results and discussion

3.1. Effect of polymer molecular mass on binodials

The phase diagrams for the systems PEG 4000– and PEG 8000–cashew-nut tree gum at $25 \pm 2°C$ are shown in Figs. 1 and 2. For comparison the binodial curves of the systems PEG 4000–dextran 500 and PEG 8000–dextran T500 are also shown. The curves, in each figure, which represent the borderline between one and two phases have the same shape but there is a parallel displacement between the two meaning that higher polymer concentrations are needed to obtain two phases with the cashew-nut tree gum which can be correlated with its lower molecular mass ($\approx 110\,000$) [25] compared with dextran 500 ($\approx 215\,000$) and dextran T500 ($\approx 507\,000$). The carbohydrate polymer is enriched in the denser bottom phases while PEG is found in the upper
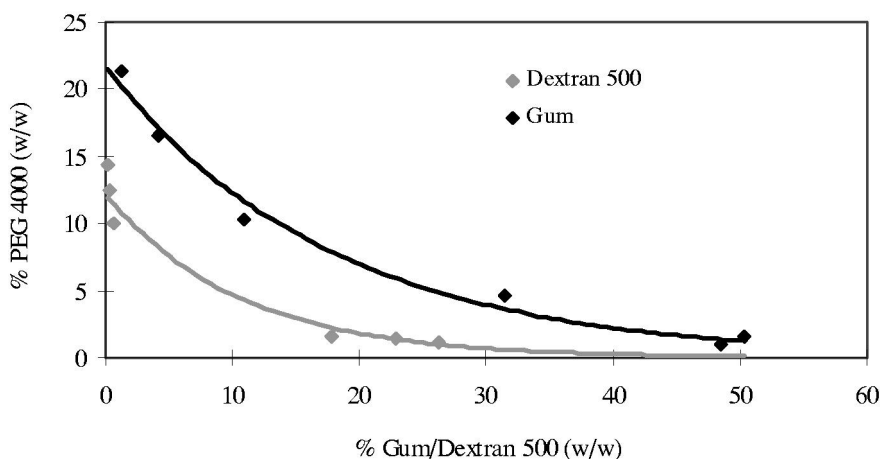
Fig. 1. Phase diagrams showing the binodials for the systems PEG 4000–cashew-nut tree gum and PEG 4000–dextran 500, both at 25±2°C. The data for the system with dextran are taken from Zaslasvsky [29].

phases [25]. The polymer compositions of the tested systems are shown in Table 1.

3.2. Effect of pH on binodials

The influence of the pH on the binodials of the PEG–cashew-nut tree gum systems is shown in Figs. 3 and 4. It can be observed that there is no a significant displacement of the binodials with the pH for both PEG 4000 and 8000.

The influence of pH on the phase diagram seems to be dependent on the kind of system [1]. For PEG–salt systems, the variation of pH has a more pronounced effect on the phases formation [30,31]. This is probably due to an increase of the $[HPO_4^{2-}]/[H_2PO_4^-]$ ratio, which promotes the shift of the phase diagram to lower polymer and salt concentrations. It is well known that the small multivalent anions such as HPO_4^{2-}, used in conjunction with PEG, are more effective in inducing phase formation than mono-
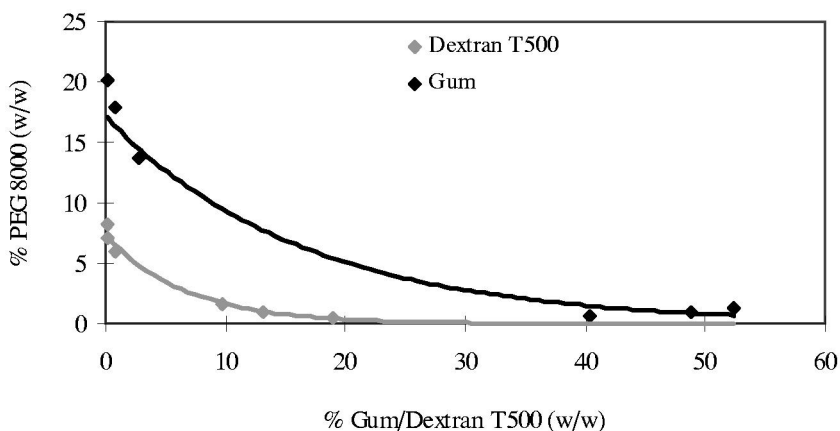

Fig. 2. Phase diagrams showing the binodials for the systems PEG 8000–cashew-nut tree gum and PEG 8000–dextran T500, both at 25±2°C. The data for the system with dextran are taken from Zaslasvsky [29].
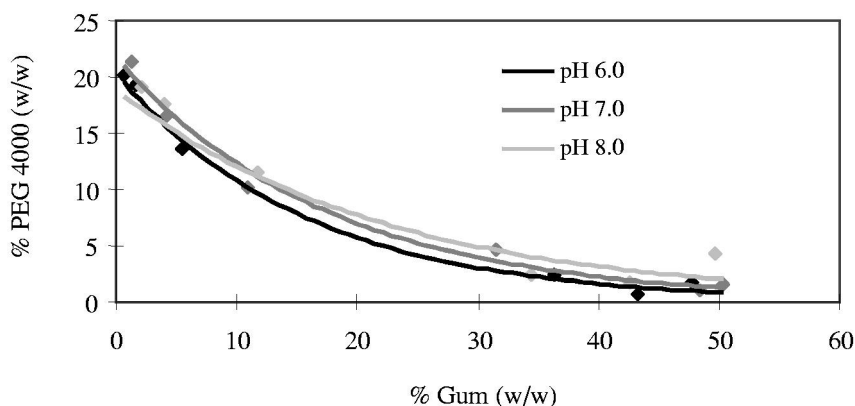
Fig. 3. Effect of pH on the binodial of the PEG 4000–cashew-nut tree gum systems at $25\pm2°C$.

valent anions [32] owing to the conflicting interaction between ether oxygens of PEG and small ions of high charge density [33].

3.3. Effect of temperature on binodials

The influence of temperature on the phase diagram is very different from system to system and depends on the kind of phase-forming polymer used. The effect of temperature on binodials was studied for 25 and 40°C and is shown in Figs. 5 and 6. From the figures it is evident that the binodials remains

practically unaltered when temperature is changed. Diamond and Hsu [34] described that a methylcellulose–dextran system was not affected by the temperature. On the other hand, decreasing the temperature of the PEG–dextran system will lead to lower polymer concentrations required for phase separation [1]. Venâncio et al. [35] and Forciniti et al. [36], observed that for higher temperatures, higher polymer concentrations were present in the upper phase for PEG–hydroxypropyl starch and PEG–dextran systems, respectively.
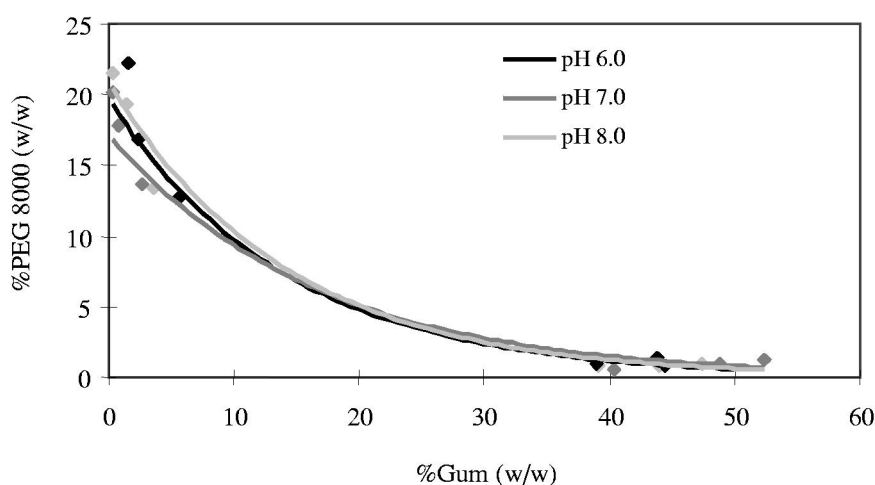
Since the phase diagram was unchanged at 25 and

Fig. 4. Effect of pH on the binodial of the PEG 8000–cashew-nut tree gum systems at $25\pm2°C$.
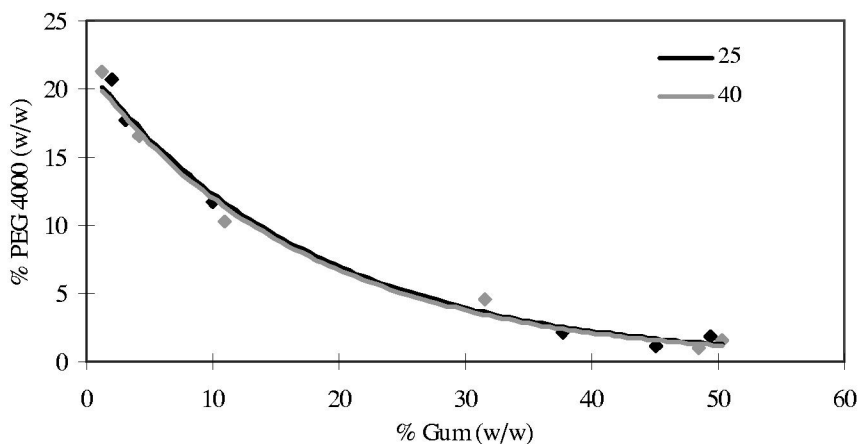
Fig. 5. Effect of temperature on the binodial of the PEG 4000–cashew-nut tree gum systems at pH 7.0.

40°C, the PEG–cashew-nut tree gum system can be used in this temperature range without significant modifications. Consequently, it will be necessary to add more polymer to the formation of aqueous two-phase systems.

3.4. Rheological properties (densities and viscosities)

The densities and viscosities of the PEG–cashew-nut tree gum systems at room temperature (25 ± 2°C)

are shown in Table 2. Because of the high content of water the densities of the phases, as expected, are close to 1 g/ml. The increase in tie-line length increased the viscosities of the phases due to the increase in system concentration. Analysing the variation of viscosities with pH, an increase of viscosity was observed proportionally to this parameter in most samples. The alteration of viscosity of the cashew-nut tree gum rich-phase can be explained by the variation of charge density in the cashew gum molecule. The reduction of the pH promotes a lower
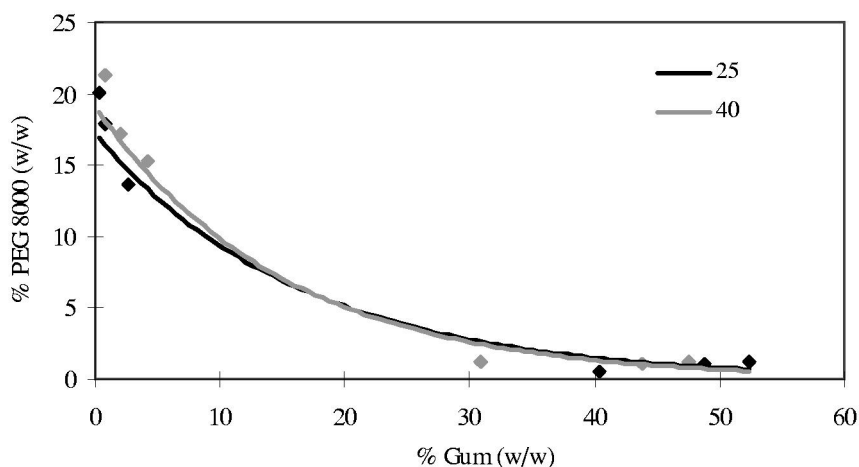

Fig. 6. Effect of temperature on the binodial of the PEG 8000–cashew-nut tree gum systems at pH 7.0.

Table 2
Rheological properties of the PEG 4000– and 8000–cashew-nut tree gum systems at $25\pm2°C$, pH values of 6.0, 7.0 and 8.0

System (tie−line)	pH	Upper phase		Lower phase	
		Density (g/ml)	Viscosity (mPa s)	Density (g/ml)	Viscosity (mPa s)
9% PEG 4000–18% gum	6.0	1.05	177.0	1.13	995.2
11% PEG 4000–20% gum		1.05	181.3	1.20	1002.3
13% PEG 4000–22% gum		1.04	192.5	1.25	1010.5
9% PEG 8000–16% gum		1.04	235.0	1.14	1235.7
11% PEG 8000–18% gum		1.03	286.0	1.21	1377.2
13% PEG 8000–20% gum		1.03	290.0	1.28	1412.0
9% PEG 4000–18% gum	7.0	1.03	182.5	1.20	1111.5
11% PEG 4000–20% gum		1.04	195.2	1.21	1215.7
13% PEG 4000–22% gum		1.01	201.6	1.31	1302.5
9% PEG 8000–16% gum		1.03	242.0	1.16	1287.2
11% PEG 8000–18% gum		1.00	275.2	1.29	1421.7
13% PEG 8000–20% gum		0.99	277.0	1.40	1477.8
9% PEG 4000–18% gum	8.0	1.04	185.3	1.16	1322.4
11% PEG 4000–20% gum		1.04	200.0	1.22	1377.9
13% PEG 4000–22% gum		1.00	187.3	1.30	1412.3
9% PEG 8000–16% gum		1.02	237.2	1.24	1287.3
11% PEG 8000–18% gum		1.02	271.0	1.21	1400.7
13% PEG 8000–20% gum		0.99	271.0	1.36	1511.7

charge density, i.e., repulsion between RCOO$^-$ groups of glucuronic acid [25], permitting chains refolding thus decreasing the intrinsic viscosity.

The phases viscosities of the PEG–cashew-nut tree gum systems are higher than other polymer–polymer systems as the PEG–dextran [37] and the PEG–maltodextrin [21] systems, while are similar to the Klucel L-pluronic P105 [38] and PEG–hydroxy-propyl starch [35] systems.

The rheological properties of the PEG–cashew-nut tree gum systems at $40\pm2°C$ are shown in Table 3. As expected, an increase of temperature decreased the viscosities of the systems.

3.5. Volume ratio and phase separation times

It is clear that only systems that offer a reasonable separation time can be considered for normal gravitational operation. The volume ratios (V_r) and phase separation times are given in Table 4. It can be seen

Table 3
Rheological properties of the PEG 4000– and 8000–cashew-nut tree gum systems at $40\pm2°C$, pH 7.0

System (tie−line)	pH	Upper phase		Lower phase	
		Density (g/ml)	Viscosity (mPa s)	Density (g/ml)	Viscosity (mPa s)
9% PEG 4000–18% gum	7.0	1.08	1.58	1.18	357.20
11% PEG 4000–20% gum		1.06	1.58	1.27	611.10
13% PEG 4000–22% gum		1.06	2.56	1.21	626.36
9% PEG 8000–16% gum		1.06	3.22	1.07	468.00
11% PEG 8000–18% gum		1.05	3.43	1.17	810.00
13% PEG 8000–20% gum		1.05	5.50	1.24	869.06

Table 4
Volume ratio and phase separation times of the PEG–cashew-nut tree gum systems

System (tie-line)	pH	Temperature (°C)	Volume ratio	Phase separation times (s)
9% PEG 4000–18% gum	6.0	25±2	2.1	45
11% PEG 4000–20% gum			2.0	90
13% PEG 4000–22% gum			1.9	180
9% PEG 8000–16% gum			2.6	100
11% PEG 8000–18% gum			2.1	140
13% PEG 8000–20% gum			2.0	360
9% PEG 4000–18% gum	7.0	25±2	3.3	45
11% PEG 4000–20% gum			2.1	100
13% PEG 4000–22% gum			1.9	165
9% PEG 8000–16% gum			3.1	110
11% PEG 8000–18% gum			2.3	180
13% PEG 8000–20% gum			2.1	330
9% PEG 4000–18% gum	8.0	25±2	3.7	50
11% PEG 4000–20% gum			2.0	100
13% PEG 4000–22% gum			2.0	120
9% PEG 8000–16% gum			3.7	110
11% PEG 8000–18% gum			2.6	155
13% PEG 8000–20% gum			2.1	320
9% PEG 4000–18% gum	7.0	40±2	2.1	40
11% PEG 4000–20% gum			1.8	40
13% PEG 4000–22% gum			1.8	105
9% PEG 8000–16% gum			2.9	60
11% PEG 8000–18% gum			2.2	160
13% PEG 8000–20% gum			2.0	220

that the volume ratio has a tendency to decrease with the increase in tie-line length and the decrease of pH. The same behaviour was observed for the PEG–arabinogalactan systems [24]. The increase of temperature, as discussed earlier, reduced the phases viscosities and consequently the phase separation times.

The time of phase separation in PEG–cashew-nut tree gum systems was very short (between 45–360 s), while the salt–polyethylene glycol and dextran–polyethylene glycol systems have settling time of 5–30 min and the dextran–ficoll or dextran–methyl-cellulose systems have settling time of 1–6 h [1]. The large-scale application of an aqueous biphasic system is related to the high requirement of chemical reagents. On the other hand, the reduced operation time required for phases separation is the major factor in obtaining the low operational cost in these systems [39]. Thus, the fast phase separation of the

PEG–cashew-nut tree gum system seems to be very effective in reducing costs.

4. Conclusions

Phase diagrams of the PEG–cashew-nut tree gum were described for different PEG molecular mass, temperatures and pH.

It was observed that increasing polymer mass from 4000 to 8000 led to lower polymer concentrations required for phase separation. The carbohydrate polymer is enriched in the denser bottom phase while PEG is found in the upper phase. There was no significant displacement of the system binodials with the pH and temperature.

The increase in tie-line length and pH increased the phase viscosities and the increase of the tempera-

ture decreased the viscosities of the systems. The volume ratio has a tendency to decrease with the increase in tie-line length and the decrease of pH. The time of phase separation in PEG–cashew-nut tree gum systems was very short, but increased with the tie-line length due to the high phases viscosities. Temperature increase reduced the phase viscosities and consequently the phase separation times.

For industrial purposes, polymer–salt systems are preferential to the polymer–polymer systems [16,40,3] due to the lower viscosity, lower cost of chemicals and shorter phase separation time [40,41]. On the other hand, the results obtained in this work show some attractive properties of the PEG–cashew-nut tree gum once this is a low-cost polymer–polymer system [25], have phase-forming chemicals biodegradables and non-toxic and exhibits a fast phase separation. These characteristics make the PEG–cashew-nut tree gum system very attractive as a separation technique.

Acknowledgements

References

[1] P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, 3rd ed., Wiley, New York, 1986.

[2] S. Bamberger, G.V.F. Seaman, J.A. Brown, D.E. Brooks, J. Colloid Interface Sci. 99 (1984) 187.

[3] M.-R. Kula, K.H. Kroner, H. Hustedt, Adv. Biochem. Eng. 24 (1982) 73.

[4] B.Y. Zaslavsky, L.M. Miheeva, G.Z. Gasanova, A.U. Mahmudov, J. Chromatogr. 403 (1987) 123.

[5] S. Bamberger, D.E. Brooks, K.A. Sharp, J.M. Van Alstine, J.J. Webber, Partitioning in Aqueous Two-Phase System, Academic Press, London, 1985.

[6] K. Kohler, B. Nisson, A. Veide, Bioseparation 3 (1993) 241.

[7] R. King, Polymer Application For Biotechnology: Macromolecular Separation and Identification, Prentice-Hall, Englewood Cliffs, NJ, 1992.

[8] J.G. Huddleston, A. Lyddiatt, Downstream Processing of Natural Products, Wiley, New York, 1996.

[9] Y. Guan, T.E. Treffry, T.H. Lilley, Bioseparation 4 (1994) 89.

[10] S.L. Mistry, J.A. Asenjo, C.A. Zaror, Bioseparation 3 (1993) 343.

[11] D.E. Brooks, R. Norris-Jones, Methods in Enzymology, Vol. 228, Academic Press, San Diego, CA, 1994.

[12] P.-Å. Albertsson, Adv. Protein Chem. 24 (1970) 309.

[13] P.-Å. Albertsson, B. Andersson, C. Larsson, H.E. Åkerlund, Methods Biochem. Anal. 28 (1982) 115.

[14] H. Walter, Methods of Cell Separation, Vol. 1, Plenum Press, New York, 1977.

[15] H. Walter, E.J. Krob, D.E. Brooks, Biochemistry 15 (1974) 2959.

[16] D.E. Brooks, K.A. Sharp, D. Fisher, Partitioning in Aqueous Two-Phase Systems. Theory, Methods, Uses, And Applications To Biotechnology, Academic Press, Orlando, FL, 1985.

[17] G. Johansson, Mol. Cell. Biochem. 4 (1974) 169.

[18] J. Vernau, M.R. Kula, Biotechnol. Appl. Biochem. 12 (1990) 397.

[19] K.H. Kroner, H. Hustedt, M.R. Kula, Process Biochem. 24 (1984) 170.

[20] K.H. Kroner, H. Hustedt, M.R. Kula, Biotechnol. Bioeng. 24 (1982) 1015.

[21] D.C. Szlag, K.A. Giuliano, Biotechnol. Techn. 2 (1988) 277.

[22] T.G.I. Ling, H. Nilsson, B. Mattiasson, Carbohydr. Polym. 11 (1989) 43.

[23] S. Sturesson, F. Tjerneld, G. Johansson, Appl. Biochem. Biotechnol. 26 (1990) 281.

[24] T.J. Christian, M. Manley-Harris, G.N. Richards, Carbohydr. Polym. 35 (1998) 7.

[25] L.A. Sarubbo, L.A. Oliveira, A.L.F. Porto, H.S. Duarte, A.M.A. Carneiro-Leão, J.L. Lima-Filho, G.M. Campos-Takaki, E.B. Tambourgi, J. Chromatogr. B 743 (2000) 79.

[26] J.F. Rodrigues, R.C.M. Paula, S.M.O. Costa, Polímeros: Ciência Tecnol. 1 (1993) 31.

[27] B. Skoog, J. Blood Transfus. Immunopathol. 37 (1979) 345.

[28] G.L. Miller, Anal. Chem. 31 (1959) 426.

[29] B.Y. Zaslavsky, Aqueous Two-Phase Partitioning – Physical Chemistry and Bioanalytical Applications, Marcel Dekker, New York, 1995.

[30] M. Videira, M.R. Aires-Barros, J. Chromatogr. A 668 (1994) 273.

[31] T.T. Franco, Use of Modified Proteins in Aqueous Two-Phase Systems. Effect of Surface Hydrophobicity and Charge, University of Reading, Faculty of Agriculture and Food, Department of Food Science and Technology, Reading, 1992.

[32] K.P. Ananthapadmanabhan, E.D. Goddard, J. Colloid Interface Sci. 113 (1986) 294.

[33] J. Huddleston, A. Veide, K. Kohler, J. Flanagan, S.-O. Enfors, A. Lyddiatt, Trends Biotechnol. 9 (1991) 383.

[34] A.D. Diamond, J.T. Hsu, Adv. Biochem. Eng. 47 (1992) 89.

[35] A.A.D. Venâncio, J.A. Teixeira, M. Mota, Biotechnol. Prog. 9 (1993) 635.

[36] D. Forciniti, C.K. Hall, M.R. Kula, Bioseparation 2 (1991) 115.

[37] F. Tjerneld, S. Berner, A.E. Cajarville, G. Johansson, Enzyme Microb. Technol. 8 (1986) 417.

[38] D.R. Skuse, R. Norris-Jones, M. Yalpani, D.E.S. Brooks, Enzyme Microb. Technol. 14 (1992) 785.

[39] J.M.S. Cabral, M.R. Aires-Barros, in: Liquid–Liquid Extraction of Biomolecules Using Aqueous Two-Phase Systems – Recovery Process For Biological Materials, Wiley, New York, 1993, p. 273.

[40] A. Kaul, R.M. Pereira, J.A. Asenjo, J.C. Merchuk, Biotechnol. Bioeng. 48 (1995) 246.

[41] H. Walter, G. Johansson, Methods in Enzymology, Vol. 32, Academic Press, London, 1994.