J. Iran. Chem. Soc., Vol. 8, No. 4, December 2011, pp. 889-896.

JOURNAL OF THE
Iranian
Chemical Society

Enantioselective Extraction of Mandelic Acid Enantiomers by Aqueous Two-Phase Systems of Polyethylene Glycol and Ammonium Sulfate Containing *β*-Cyclodextrin as Chiral Selector

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(Received 16 January 2009, Accepted 8 May 2009)

A new chiral separation technology, aqueous two-phase extraction, was proposed for the separation of racemic mandelic acid. The distribution behavior of mandelic acid enantiomers was investigated in aqueous two-phase systems composed of polyethylene glycol and ammonium sulfate containing *β*-cyclodextrin as chiral selector. The influences of the pH, the mass fraction of polyethylene glycol and ammonium sulfate, the polymerization degree of polyethylene glycol, the initial concentration of *β*-cyclodextrin, mandelic acid enantiomers and extractive temperature on the distribution behavior were studied respectively. The results show that *β*-cyclodextrin is inclined to recognize *L*-enantiomer; under the optimized conditions, the separation factor reaches 2.46 and the enantiomeric excess is 42.13% in the top phase and 40.43% in the bottom phase, respectively. Aqueous two phase chiral-extraction with strong chiral separation ability, plays great role in preparative separation of racemic compounds and is important for the development of aqueous two phase extraction technique.

Keywords: Aqueous two phase extraction, Chiral separation, Mandelic acid enantiomers, *β*-cyclodextrin

INTRODUCTION

With the need of pure isomer of racemic mixture increasing rapidly after the Food and Drug Administration issued guidelines for the marketing of chiral drugs in May 1992 [1], more and more attentions have been paid to chiral resolution technology [2-11]. As a potential large scale production technique, chiral solvent extraction has attracted the attention of many researchers to make great efforts in recent years [10-11]. But there still exist some disadvantages in traditional chiral solvent extraction such as large volume residual organic solvent, especially most of which are toxic, flammable and volatile.

A queous two-phase systems (ATPS) have been known since the early work of Beijerinck in 1896 [12], but was not gained importance until the work of Albertsson in the 1950s [13-14]. These systems are formed when aqueous solutions of two mutually incompatible components separate into two phases of different densities under the force of gravity. This technique allows clarification, concentration, and partial purification to be integrated in one step, and besides providing biocompatibility. Since both phases consist mainly of water (80-90%, w/w), it can be highly selective and easily scaled up. This fact has been widely exploited to separate proteins, amino acids, lipids, nucleic acids, and animal cells without significant interfacial denaturing effects [15-19]. It has been reported that aqueous two phase extraction shows significant advantages including: short phase-separation time, small interfacial tension, non residual solvents, innocuity, easier operation conditions and polymer recycling [15-25].

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Sellergren and Ekberg proposed the use of aqueous two-phase system by a few counter-current extractions for semipreparative chiral separations in 1988 [26]. But it does not gain any important results and focus from then on.

Based on the previous results, the distribution behavior of mandelic acid enantiomers (*D,L*-MA), a major metabolite of styrene widely used as a biological indicator of occupational exposure to styrene, was investigated in aqueous two-phase systems (ATPS) which was composed of polyethylene glycol (PEG) and ammonium sulfate [$(NH_4)_2SO_4$] containing *β*-cyclodextrin (*β*-CD) as chiral selector. The influences of the pH, the mass fraction of PEG and $(NH_4)_2SO_4$, the polymerization degree of PEG, the initial concentration of *β*-CD, *D,L*-MA and extractive temperature on the distribution behavior were studied respectively. This technology provides an environmental friendly, effective and economical chiral separation method and is important for the development of aqueous two phase extraction (ATPE).

EXPERIMENTAL

Chemicals

Racemic MA (purity > 99.0%) and *L*-phenylalanine ($[\alpha]_D^{20} = -34 \pm 1°$) were purchased from Guangfu Institute of Fine Chemicals (Tianjin, China); $CuSO_4.5H_2O$ (purity > 99.0%) was bought from Kemiou Chemical Reagents Co. Ltd. (Tianjin, China); *β*-CD was purchased from Abxing Biological Technology Co. Ltd. (Beijing, China); $(NH_4)_2SO_4$ (purity > 99.0%) was obtained from the third chemical factory (Jiaozuo, China); PEG1500, 2000, 4000, 6000 were supplied by Chemical reagent Sinopharm Group Co. Ltd. (Shanghai, China); All other chemicals are of analytical-reagent grade.

Apparatus

The quantification of MA enantiomer in the bottom phase was performed by HPLC using a UV detector (Shimadzu, Japan) at the UV wavelength of 300 nm. The column was Lichrospher C_{18}, 5 μm particle size of the Packing Material, 150 mm × 4.6 mm I.D. (Hanbon Science & Technology Co. Ltd., China).

Analytical Method

The mobile phase for MA enantiomer was 3 mM $CuSO_4$ and 6 mM *L*-phenylalanine aqueous solution: methanol (90:10) at a flow velocity of 0.4 ml min^{-1}. The retention time of the *D*-enantiomer is less than that of the *L*-enantiomer (Fig. 1).

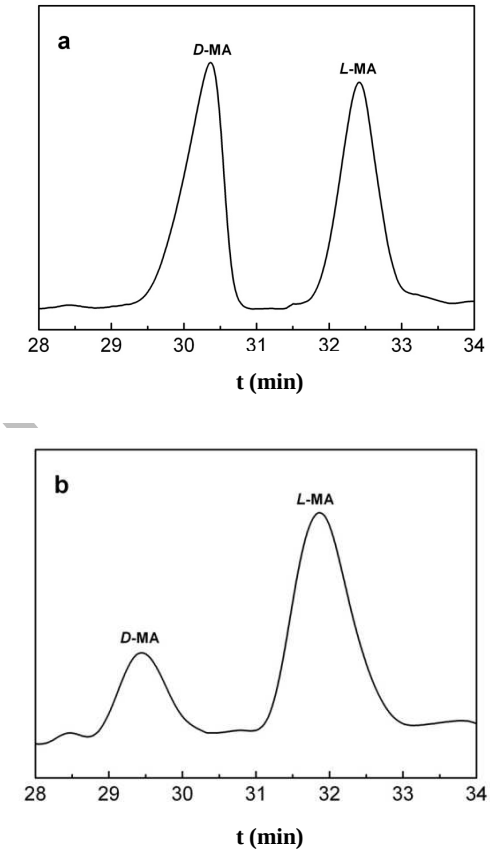


Fig. 1. Chromatogram of *D,L*-MA before and after extraction. a) 0.2 M *D,L*-MA solution; b) *D,L*-MA 0.11 M, the mass fraction of PEG and $(NH_4)_2SO_4$ is 30% and 20%, the polymerization degree of PEG is 2000, pH 1.0 and temperature: 30 °C.

Aqueous Two Phase Extraction (ATPE)

The temperature of all experiments was maintained at 30 °C by a constant temperature water bath apparatus, except when the influence of temperature was investigated.

For each extraction experiment, an ATPS was prepared by mixing 10 ml *β*-CD aqueous solution with MA enantiomers

and 20 ml PEG solution and $(NH_4)_2SO_4$. The pH value, measured by a pH meter, was adjusted by phosphate buffer at a concentration of 0.1 M. The mixing contents were placed in a 50 ml Eppendorftube signed with scale, then were mixed thoroughly using a magnetic stirrer (15 × 6 mm I.D.) at the rotation speed of 800 rpm about 2 h for equilibration. The phase separation is completed by putting the contents in a centrifuge at the speed of 3000~3500 rpm operated about 3~6 min. After clear separation of the two phases, the volumes of the top and bottom phase are noted. The concentration of MA enantiomers in the bottom phase is analyzed by HPLC. The concentration of MA enantiomers in the top phase is calculated by subtractive method.

RESULTS AND DISCUSSION

In the process of ATPE, chiral extraction is carried out by the formation of two diastereomeric inclusion complexes between chiral selector and (*D*) or (*L*)-enantiomer due to potential molecular interactions, hydrogen bond polarization, induction, or electrostatics exists. These two diastereomeric inclusion complexes, with different physical and chemical properties, have different interactions with PEG of the top phase, which leads to different distribution behavior of single enantiomer MA in ATPS.

The distribution coefficient (*K*) and the enantioselectivity, calculated in terms of the separation factor (β) and the enantiomeric excess (*ee*, %), are important parameters to estimate aqueous two phase chiral-extraction performance of extractant, which can be calculated by the following formulas:

$$K_D = C_{t,D} / C_{b,D} \quad (1)$$

$$K_L = C_{t,L} / C_{b,L} \quad (2)$$

$$\beta = K_D / K_L \quad (3)$$

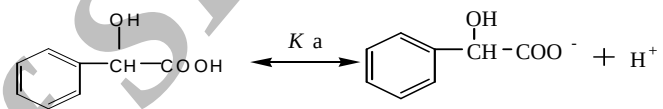
Top phase: $$e.e.\% = \frac{C_{t,D} - C_{t,L}}{C_{t,D} + C_{t,L}} \times 100 \quad (4)$$

Bottom phase: $$e.e.\% = \frac{C_{b,L} - C_{b,D}}{C_{b,L} + C_{b,D}} \times 100 \quad (5)$$

among which $C_{t,D}$ and $C_{b,D}$ are the concentrations of the *D*-MA enantiomer in the top phase and bottom phase, respectively, similarly, $C_{t,L}$ and $C_{b,L}$ the concentrations of the *L*-MA enantiomer, respectively, K_D and K_L the distribution coefficients of the *D*-MA enantiomer and *L*-MA enantiomer, respectively.

Influence of pH

Mandelic acid (*D*- or *L*-MA) has one carboxylic group and an aromatic group. One dissociation equilibrium exists in aqueous solution:



(6)

The dissociation constant can be described by

$$K_a = \frac{[A^-][H^+]}{[A]} \quad (7)$$

Where A and A^- are the unionized and anion of *D*- or *L*-MA, respectively.

Therefore, the influence of pH in ATPE is significant. As shown in Fig. 2, the distribution coefficient of MA enantiomer decreases with the increasing of the pH. Regarding the enantioselectivities of the extraction process, it is obvious that the enantioselctivities decrease when increase the pH value.

The possible reasons for these phenomena might be that the amount of ionic MA increases with the increase of the pH. β-CD mainly has chiral recognition ability and affinity for molecular MA, but not for ionic MA. The majority of ionic MA exists in the bottom phase. The concentration of complexes, formed by β-CD and MA enantiomer, decreases along with the increase of pH in ATPE. As a result, the distribution coefficient, enantioselectivity greatly decrease with the increasing pH. Hence, it should be kept at lower pH in order to obtain a higher resolution efficiency.

Influence of the Mass Fraction of PEG and $(NH4)_2SO_4$

The influences of the mass fraction of PEG and $(NH_4)_2SO_4$

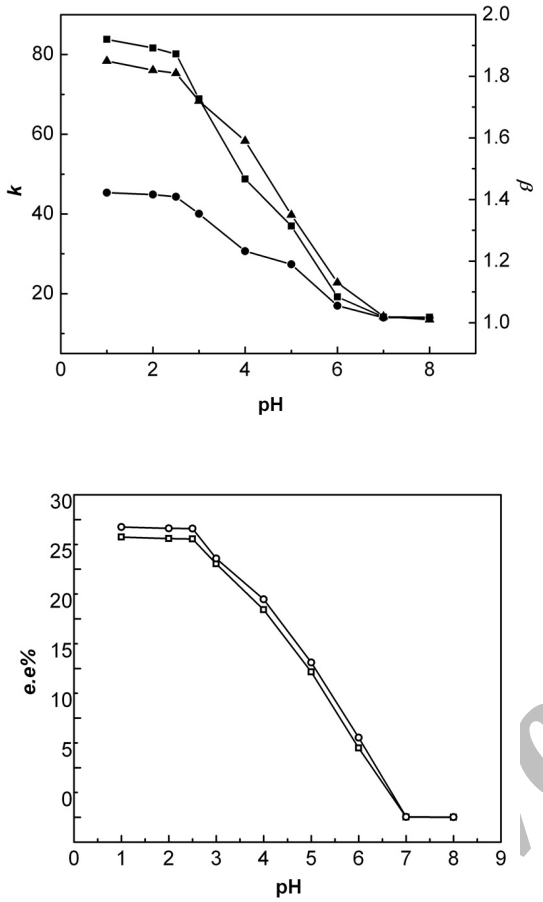


Fig. 2. Influence of the PH values on the distribution behavior of MA enantiomer. Initial concentration: *D*,*L*-MA 0.11 M, *β*-CD 0.004 M, the mass fraction of PEG and $(NH_4)_2SO_4$ is 30% and 20%, the polymerization degree of PEG is 2000, temperature: 30 °C. ((■) K_D, (●) K_L, (▲) b, (□) the bottom phase, (○) the top phase).

are illustrated in Fig. 3. On the one hand, as shown in Fig. 3a, the distribution coefficient, enantioselectivity all increase before the mass fraction of PEG is up to 30%, however, when the mass fraction of PEG increases further, the distribution coefficient and enantioselectivity remain at a moderate extent showing no obvious trend. The possible reasons for these are discussed as follows: as the increase of the mass fraction of PEG, the relative concentration of PEG in the top phase increases, which makes the phase-separation more complete, so the distribution coefficient, enantioselectivity all increase with it. When the mass fraction of PEG reached 30%, the phase-separation has already been completed, so the distribution coefficient, enantioselectivity remain at a moderate extent though further increase the mass fraction of PEG. In addition, it is found during the experiments that the solution viscosity and the time of phase-separation increase with the increasing mass fraction of PEG. The appropriate mass fraction of PEG is therefore about 30% in this experiment system.

On the other hand, as shown in Fig. 3b. The distribution coefficient, enantioselectivity all increase before the mass fraction of $(NH_4)_2SO_4$ is up to 20%, but follow an opposite tendency when the mass fraction of $(NH_4)_2SO_4$ further increases. The results indicate that, water could get into the top phase at the extraction and phase-separation time, but with the increase of the mass fraction of $(NH_4)_2SO_4$, PEG and water will be gradually separated, which leads to the increase of the relative concentration of PEG in the top phase, and makes the phase-separation more complete, so the distribution coefficient, enantioselectivity all increase. But when the mass fraction of $(NH_4)_2SO_4$ reaches 20%, the phase-separation has already been completed, then if increase the mass fraction of $(NH_4)_2SO_4$ in succession, the excessive salt will produces a salt effect, which makes the amount of ionic MA increase and the amount of molecular MA decrease. As the results in section 2.1 show that *β*-CD mainly has chiral recognition ability and affinity for molecular MA, so the distribution coefficient, enantioselectivity continuously decrease when further increase the mass fraction of $(NH_4)_2SO_4$. Hence, the better mass fraction of $(NH_4)_2SO_4$ is 20%.

Influence of the Degree of Polymerization of PEG

PEG is not a single molecular weight compound, but a mixture of the same series. The average molecular weight of PEG is a token of its polymerization degree, the bigger the PEG average molecular weight is, the bigger the polymerization degree of PEG is. The influence of the polymerization degree of PEG is shown in Fig. 4. With the increase of the average molecular weight of PEG, the distribution coefficient, enantioselectivity gradually increase, however, the viscosity of the solution increases with the increase of the average molecular weight of PEG, which not

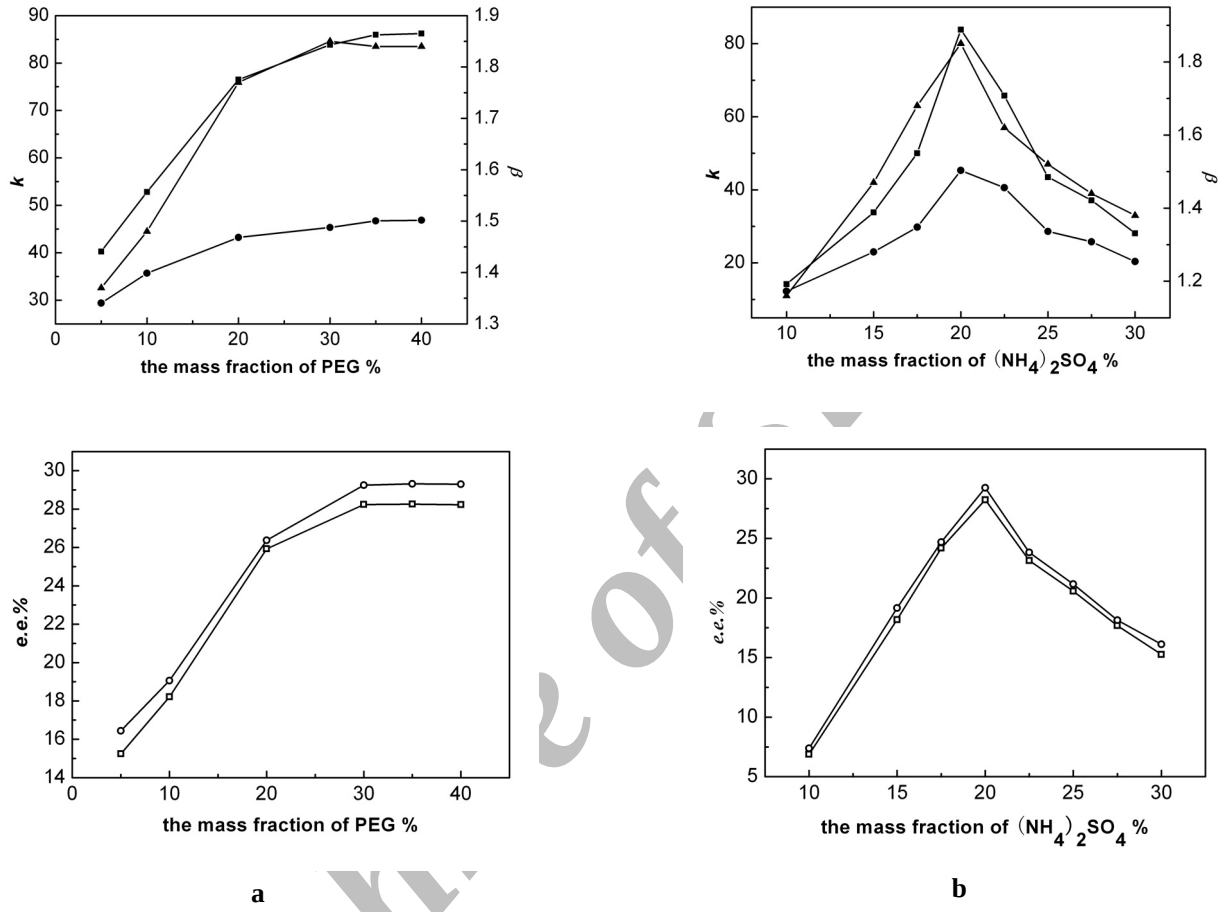
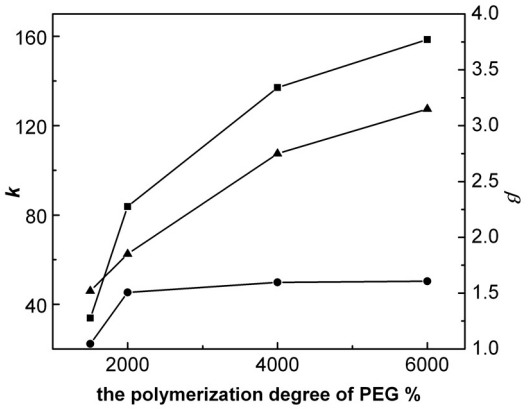


Fig . 3. Influence of the mass fraction of PEG and $(NH_4)_2SO_4$. a) Influence of the mass fraction of PEG on the distribution behavior of MA enantiomer. D,L-MA 0.11 M, β -CD 0.004 M, the mass fraction of $(NH_4)_2SO_4$ is 20%, the polymerization degree of PEG is 2000, pH 1.0 and temperature: 30 °C; b) Influence of the mass fraction of $(NH_4)_2SO_4$ on the distribution behavior of MA enantiomer. *D,L*-MA 0.11 M, *β* -CD 0.004 M, the mass fraction of PEG is 30%, the polymerization degree of PEG is 2000, pH 1.0 and temperature: 30 °C. ((■) K_D, (●) K_L, (▲) b, (□) the bottom phase, (○) the top phase).

only prolongs the time of phase-separation, but also increases the cost and decreases the economic efficiency. So in this experiment we choose PEG2000 as the study object.

Influence of the Initial Concentration of β-CD

There exists three naturally occurring cyclodextrins with different numbers of glucose monomers, namely *α*-, *β*-, and *γ*-cyclodextrins. Among these three cyclodextrins, β-CD has suitable toroid for forming inclusion complexes with various molecules by recognition ability [27]. Therefore, it plays an important role in the process of extraction and has different chiral recognition for *D*-MA and *L*-MA. Changing the concentration of *β*-CD will make great influence on the distribution behavior of MA enantiomer. Figure 5 reflects the influence of the concentration of *β*-CD on the distribution behavior of *D,L*-MA. With an increase of *β*-CD content, the



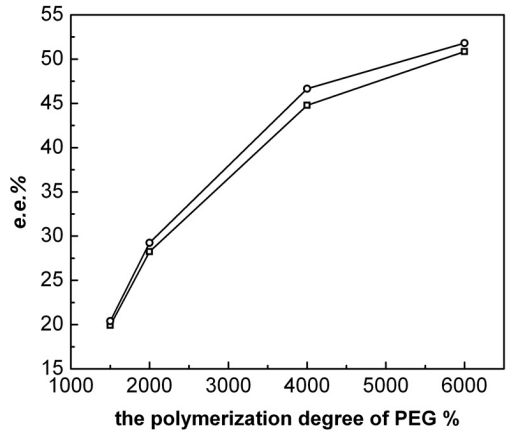
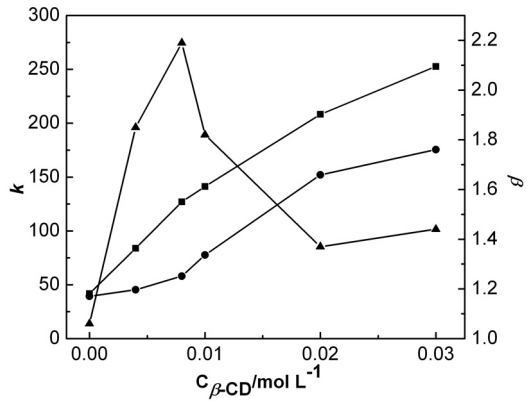


Fig. 4. Influence of the polymerization degree of PEG on the distribution behavior of MA enantiomer. *D,L*-MA 0.11 M, *β* -CD 0.004 M, the mass fraction of PEG and $(NH_4)_2SO_4$ is 30% and 20%, pH 1.0 and temperature: 30 °C ((■) K_D, (●) K_L, (▲) b, (□) the bottom phase, (○) the top phase).



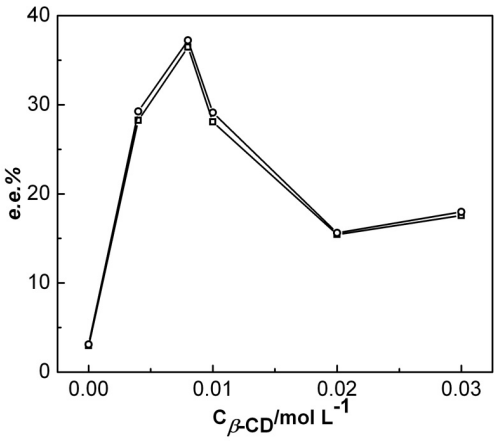


Fig. 5. Influence of the initial concentration of *β* -CD on the distribution behavior of MA enantiomer. *D,L*-MA 0.11 M, the mass fraction of PEG and $(NH_4)_2SO_4$ is 30% and 20%, the polymerization degree of PEG is 2000, pH 1.0 and temperature: 30 °C ((■) K_D, (●) K_L, (▲) b, (□) the bottom phase, (○) the top phase).

distribution coefficient greatly increases. Meanwhile, the enantioselectivity increases before the concentration of *β*-CD is up to 0.008 M. When the concentration of *β*-CD increases further, the distribution coefficient increases continuously, while the enantioselectivity follows an opposite tendency. This may be the results of the cooperation actions of the two diastereomeric complexes between *β*-CD and MA enantiomer and PEG. It is also found that, K_D values are always larger than K_L, namely $\beta > 1$, which indicates that *β*-CD has a stronger recognition ability for *L*-MA than for *D*-MA.

Influence of the Initial Concentration of D,L-MA

The study on the influence of initial concentration of *D,L*-MA on the distribution behavior of MA enantiomer indicated that the distribution coefficient were enhanced upon an increase of the initial concentration of MA enantiomers. However, the value of the enantioselectivity is relatively higher at low concentration. It indicates better

Table 1. The Distribution Coefficients and Selectivity Factor under the Optimum Conditions

	pH	PEG (%)	$(NH_4)_2SO_4$ (%)	$M_{(PEG)}$	$C_{(\beta\text{-CD})}$ (M)	$C_{(D,L\text{-MA})}$ (M)	T (°C)
	1.0	30%	20%	2000	0.008	0.05	30
K_D				66.25			
K_L				26.98			
β				2.46			

enantioseparation efficiency at a low initial concentration.

The Influence of the Temperature

The influence of the temperature on the distribution behavior of *D,L*-MA was studied in the range of 30-70 °C, which suggests better enantioseparation efficiency can be obtained at a low temperature for the distribution coefficient, the enantioselectivity decreases slowly with the increase of the temperature.

Along with the increase of the temperature, the decreased stability of the two diastereomeric complexes formed between *β*-CD and single enantiomer of MA leads to a faster dissociation speed of the complexes, and the interaction between the complexes and PEG has somewhat been weaken, hence, the distribution coefficient, the enantioselectivity all decrease slowly with the increase of the temperature.

After the above experiments, the optimized conditions were obtained and summarized as Table 1.

CONCLUSIONS

ATPE is a new chiral separation technique for separation of MA enantiomers. Due to its strong chiral separation ability, ATPE will play a great role in preparative separation of racemic compounds. This technology may also be very helpful to optimize the extraction systems and realize the large-scale production of enantiomer. In this study, it is found that *β*-CD has stronger recognition ability for *L*-MA than for *D*-MA. The chiral separation ability was changed with the content of the elements composed of the aqueous two phase system. The distribution coefficient, the separation factor and enantiomeric excess are greatly decreased with the rise of pH, and better enantioseparation efficiency can be obtained at low temperature. High enantioseparation efficiency with a maximum separation factor of 2.46 is obtained at the optimal conditions.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of China (20805058), National High Technology Research and Development Program of China (2007AA022006), Hunan Provincial Postdoctoral Special Foundation of China (2009RS3039) and Hunan Provincial Natural Science Foundation of China (09JJ3026).

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