DOI: 10.1002/cjoc.201100224

Extraction of Phenylalanine Enantiomers by Aqueous Two Phase Systems Containing Combinatorial Chiral Selector

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In order to obtain a better enantioselectivity of phenylalanine enantiomers and establish the optimal chiral extraction conditions, the distribution behavior was investigated in aqueous two-phase systems which were composed of polyethylene glycol and ammonium sulfate containing combinatorial chiral selector: *β*-cyclodextrin and HP-*β*-cyclodextrin. The influence of the molar concentration ratio of combinatorial chiral selectors, the total molar concentration of combinatorial chiral selectors, pH value, buffer type and its concentration were thoroughly studied, respectively. The results show that the enantioselectivity reaches 1.53 under the optimal chiral extraction conditions. This extraction is a potential economical and effective way for chiral resolution.

Keywords aqueous two phase extraction, combinatorial chiral selectors, chiral resolution, enantioselectivity, phenylalanine enantiomers

Introduction

An aqueous two-phase system (ATPS) is a new separation technique and shows unique advantage of separation and purification in biology substance, natural production, antibiotics, *etc*.[1] These systems are formed when aqueous solutions of two mutually incompatible components are separated into two phases of different densities under the force of gravity. It has been reported that aqueous two phase extraction shows significant advantages including less process time, small interfacial tension, non-residual solvents, innocuity, facile operation conditions, and easy polymer recycling.[2-8] Sellergren and Ekberg proposed the use of aqueous two-phase system by a few counter-current extractions for semi-preparative chiral separation at 1988.[9] However, it does not achieve any important result from then on in the field of chiral separation.

Combinatorial methods have more and more important effect on screening medication lead compound and a widely application in chemistry research. Vries *et al*.[10] first proposed the application of combinatorial methods in chemistry resolution, which is a significant breakthrough in resolution methodology. They took the combination of diaryl formyl derivant to separate chiral amine by crystallization. The results showed that the combination improved resolution efficiency greatly, accelerated crystallization and increased enantioselectivity. The combinatorial resolution was also applied in resolution of racemic terbutaline and mandelic acid.[11,12]

Based on the previous results, the distribution behavior of phenylalanine enantiomers (*D*,*L*-Phe, Figure 1a) was investigated in ATPS composed of polyethylene glycol (PEG) and ammonium sulfate [$(NH_4)_2SO_4$] containing combinatorial chiral selectors: *β*-cyclodextrin (*β*-CD, Figure 1b) and hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD, Figure 1c). The influence of the molar concentration ratio of combinatorial chiral selectors, the total molar concentration of combinatorial chiral selectors, pH value, buffer type and its concentration were studied, respectively. This study provides an environmental friendly, effective and economical chiral separation method and is important for the development of aqueous two phase extraction (ATPE) technique.

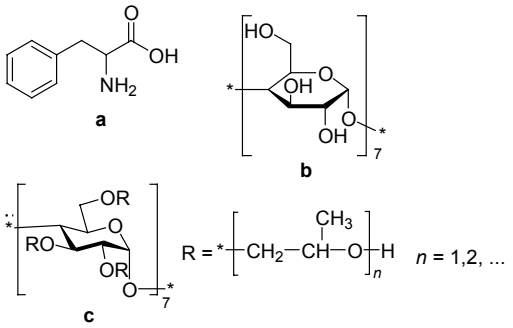

Figure 1 Molecular structures of (a) *D*,*L*-Phe, (b) *β*-CD and (c) HP-*β*-CD.

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Received August 24, 2011; accepted October 20, 2011; published online March 7, 2012.

Experimental

Chemicals

Racemic Phe was purchased from Alfa Aesar, a Johnson Matthey Company (American). β-CD and HP-β-CD were purchased from Chemical Reagent Sinopharm Group Co. Ltd (Shanghai, China). $(NH_4)_2SO_4$ was obtained from the third chemical factory (Jiaozuo, China). PEG with molecular weight 2000 was supplied by Chemical Reagent Sinopharm Group Co. Ltd (Shanghai, China). Perchloric acid was purchased from Guangfu Institute of Fine Chemicals (Tianjin, China). Disodium hydrogen phosphate was purchased from Xin'an Science and Technology Co. Ltd (Jiaozuo, China). Acetic acid and phosphoric acid were purchased from Chemical Reagent Factory of Hunan Normal University (Changsha, China). All the chemicals are of analytical-reagent grade.

Quantification

The quantification of Phe enantiomers in the bottom phase was performed by the liquid chromatograph (Shimadzu, Japan) with a Crownpak CR(+) column (150 mm×4.0 mm i.d.) (Daicel, Japan) and coupled with an SPD-10 UV/VIS spectrophotometer detector (Shimadzu, Japan) and an AT-130 temperature controller (Autoscience, Tianjin, China).

The mobile phase for Phe enantiomers was perchloric acid aqueous solution with pH 2.0 at a flow of 0.8 mL/min. The UV spectrometer was operated at 200 nm, while the column temperature was set at 25 ℃. The retention time of (*D*)-enantiomer is less than that of (*L*)-enantiomer (Figure 2).

ATPE

For each extraction experiment, an ATPS was prepared by mixing 5 mL of a given concentration of chiral selectors aqueous solution with Phe enantiomers and 20 mL of a given quantity PEG2000 solution and $(NH_4)_2SO_4$. The pH value was adjusted with 5 mL of buffer solution and measured by a pH meter. The contents were mixed thoroughly using a magnetic stirrer at the rotation speed of 800 r/min. Subsequently the mixture was separated for about 2 h in a thermostat water bath with fixed temperature. The phase separation was completed by putting the contents in a vibrating centrifuge at the speed of 3000—3500 r/min, operated for about 6—8 min. After clear separation of the two phases, the volumes of the top and bottom phase were noted. The concentration of *D*,*L*-Phe enantiomer in the bottom phase was analyzed by HPLC. The concentration of *D*,*L*-Phe enantiomer in the top phase was calculated by subtractive method. Each experiment was repeated twice at least and the standard deviation between two should be less than ±2%.

Theory of ATPE

Distribution coefficient (K) and enantioselectivity

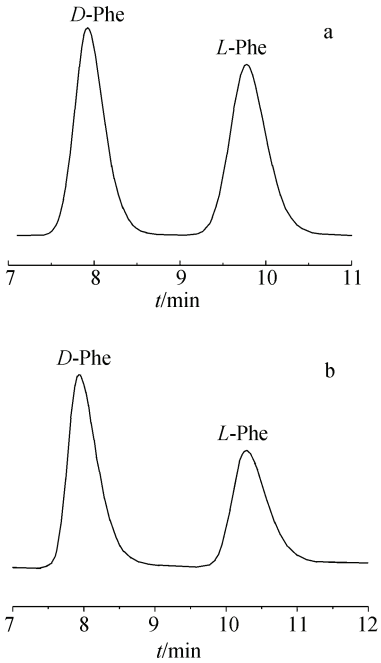

Figure 2 (a) Chromatogram of *D*,*L*-Phe in the lower phase. Initial aqueous concentration of *D*,*L*-Phe was 400 mmol/L, pH_{eq} 5.50; (b) separation of Phe enantiomers after extraction with combinatorial chiral selectors β-CD and HP-β-CD.

which is expressed in term of separation factor (α) and enantiomeric excess (*ee*), are important parameters to estimate aqueous two-phase chiral-extraction performance of extractant. They can be calculated by the following formulas:

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

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

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

Top phase:

$$ee = \frac{C_{t,D} \mid C_{t,L}}{C_{t,D} + C_{t,L}} \times 100\% \quad (4)$$

Bottom phase:

$$ee = \frac{C_{b,L} \mid C_{b,D}}{C_{b,L} + C_{b,D}} \times 100\% \quad (5)$$

among which $C_{t,D}$ and $C_{b,D}$ represent the concentration of *D*-Phe enantiomer in the top phase and bottom phase, respectively. Similarly, $C_{t,L}$ and $C_{b,L}$ represent the concentrations of *L*-Phe enantiomer, respectively. K_D and K_L represent the distribution coefficient of *D*-Phe enantiomer and *L*-Phe enantiomer, respectively.

In the process of ATPE, chiral extraction is carried out by the formation of two diastereomeric inclusion complexes between chiral selector and *D*,*L*-enantiomer due to potential molecular interactions, hydrogen bond,

polarization, induction, or electrostatics existing. These two diastereomeric inclusion complexes, with different physical and chemical properties, have different interaction with PEG of the top phase, which leads to different distribution behavior of Phe enantiomer in ATPS.

Results and Discussion

Influence of different chiral selectors

The composition of ATPE system should be a parameter under control. The molecular weight of PEG and mass fraction of $(NH_4)_2SO_4$ were decided after thoroughly literature survey. Molyneux *et al.*[13] supported that small molecular weight PEG can be dissolved in the water while high molecular weight PEG is less hydrosoluble. Thus, some researchers adopted small molecular weight PEG, such as PEG400 PEG425 PEG600 and PEG725, to separate amino enantiomers in aqueous phase.[14,15] Other researcher groups suggested to use high molecular weight PEG, from PEG3350, PEG6000 to PEG 8000, to separate hydrophobic amino acids.[16,17] However the viscosity of ATPE system increases dramatically by increasing the molecular weight or concentration of PEG.[18] Pedro *et al.*[17] proposed that the ATPE systems with a mass fraction of PEG between 10% to 50% showed better separation in the PEG/salt system. Moreover, Gao *et al.*[19] proved that, the mass fractions of PEG and ammonium sulfate should be 10%—30% and less than 20%, respectively. In our work, PEG2000 was selected with a mass fraction of 30% whilst the quality fraction of $(NH_4)_2SO_4$ is 20%. Larger molecular weight PEG may increase the viscosity of ATPE system and reduce the separation efficiency.

The concentration of Phe was 3.00 mmol/L. the concentration of chiral selector was 0.01 mol/L (in combinatorial selectors, C(*β*-CD)∶C(HP-*β*-CD)=1∶1). The pH of Na_2HPO_4-H_3PO_4 buffer solution was 5.5 and the temperature was 25 ℃. The influence of different chiral selectors on the distribution behavior of racemic Phe was studied. As shown in Table 1, ATPS shows extractive effect but no resolution ability without chiral selector. Moreover, the parameters of *α* and *ee* were improved using combinatorial selectors composed of *β*-CD and HP-*β*-CD instead of single selector such as *β*-CD or HP-*β*-CD, though *K* was not noticeably improved. The distribution characteristics of Phe were similar to distribution trend reported previous study,[20] which shows combinatorial chiral selectors own better separation effect.

Table 1 Relationship between chiral selectors and resolution results

	K_L	K_D	α	*ee*/% (lower phase)	*ee*/% (higher phase)
None	1.98	1.92	1.03	0.02	0.05
β-CD	3.04	2.10	1.45	7.08	10.40
HP-*β*-CD	3.49	2.37	1.47	7.62	11.03
β-CD and HP-*β*-CD as combinatorial selector	3.25	2.15	1.53	9.81	12.83

Influence of different molar concentration ratio of combinatorial chiral selectors

The effect of various molar concentration ratios of combinatorial chiral selectors for the distribution behavior of racemic Phe was investigated under the same conditions as above except that the concentration ratio of *β*-CD and HP-*β*-CD was variational though the total concentration was 0.01 mol/L fixedly. Table 2 shows that, *α* and *ee* reached maximum when the molar concentration ratio of *β*-CD to HP-*β*-CD was 2∶3. The possible reason is that different ratio of *β*-CD to HP-*β*-CD has different chiral recognition ability and enantioselectivity separation efficiency for racemic Phe. HP-*β*-CD has better chiral recognition ability while *β*-CD has higher separation efficiency. Therefore, the optimal concentration ratio of *β*-CD to HP-*β*-CD is 2∶3 in combinatorial chiral selectors.

Table 2 The influence of ratio of combinatorial chiral selectors on *K*, *α* and *ee*

$C_{(\beta\text{-CD})}$∶$C_{(\text{HP-}\beta\text{-CD})}$	K_L	K_D	α	*ee*/% (lower phase)	*ee*/% (higher phase)
4∶1	3.12	2.17	1.44	7.11	10.44
3∶2	3.30	2.21	1.49	8.65	11.59
1∶1	3.25	2.15	1.51	9.81	12.83
2∶3	3.30	2.33	1.53	10.27	13.31
1∶4	3.44	2.36	1.46	7.86	11.23

Influence of different total concentration of combinatorial chiral selectors

The effect of various total concentrations of combinatorial chiral selectors for the distribution behavior of racemic Phe was investigated under the same conditions as the section of Influence of different chiral selectors, except that the molar concentration ratio of *β*-CD to HP-*β*-CD is 2∶3 in combinatorial chiral selectors. As shown in Figure 3, *α* and *ee* rapidly increase with the increasing total concentration of combinatorial chiral selectors within the range of 5—10 mmol/L, and then the increases slow down, finally they get stable when the concentration exceeds 10 mmol/L. This conversion of changing rate permits concentration variation in a wide range with a stable resolution. Hence, the experimental results reappear easily. On the contrary, the use of single chiral selector usually has an optimal concentration. The slight change of concentration nearby the optimal concentration could cause noticeable variation, which causes a difficult control of separation conditions and has difficulty in repeating results. Stephen reported a model for the separation of pairs of enantiomeric molecules in capillary electrophoresis.[21] Once the con-

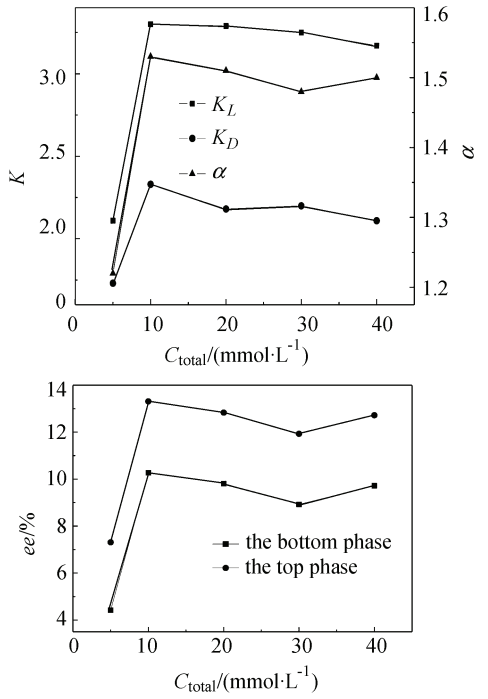

Figure 3 Influence of the total molar concentration of combinatorial chiral selector on the distribution behavior of racemic phenylalanine.

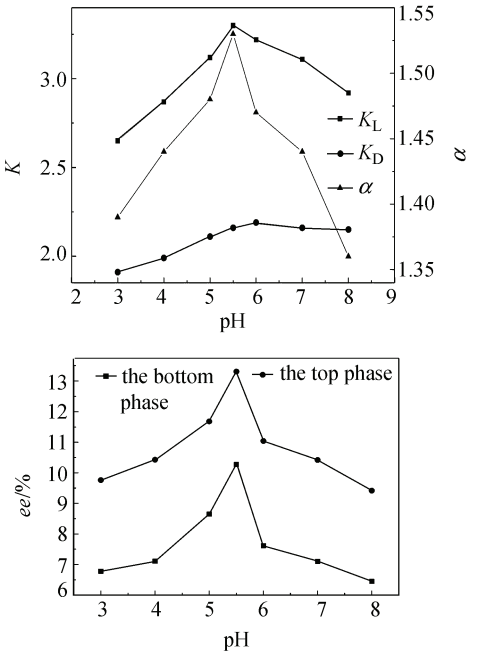

Figure 4 Influence of the pH values on the distribution behavior of racemic phenylalanine.

centration of chiral selector is lower or higher than the optimum concentration, the separation rate decreased dramatically with the decreasing of mobility difference. The mobility difference decreased over 50% when the concentration of chiral selector was 20 mmol higher than optimum condition. That is the reason why the popularization and application of the single chiral selector have been restricted.

Influence of pH

The effect of pH of Na_2HPO_4-H_3PO_4 buffer for the distribution behavior of racemic Phe was investigated under the same conditions as the section of Influence of different chiral selectors, except that the molar concentration ratio of β-CD and HP-β-CD is 2∶3 in combinatorial chiral selectors. Figure 4 shows that the pH has obvious effect on the distribution behavior of racemic Phe. The items of α and *ee* achieve the maximum at the same time when pH is 5.5. The possible reason is that Phe reaches its isoelectric point when pH is 5.5 in this case, Phe exists in form of molecule. Its ionization process increases in other pH, which leads to ionic Phe more than molecular Phe. However, HP-β-CD has little recognition effects for ionic Phe. Therefore, K, α and *ee* decrease when the pH is higher or lower than the isoelectric point. The extraction system of pH 5.5 has been selected in this experiment.

Influence of different kinds and concentration of buffer solution

The effect of type and concentration of buffer solution for the distributive action of racemic Phe was investigated under the same conditions as the section of Influence of different chiral selectors, except that the molar concentration ratio of β-CD to HP-β-CD is 2∶3 in combinatorial chiral selectors. The buffer solution in this study contains H_3PO_4-Na_2HPO_4, citric acid-sodium citriate (Cit-NaCit), HAc-NaAc, H_3PO_4-$Na_2B_4O_7$, Cit-TEOA and Cit-Tris. Table 3 indicates that, the separation effect of H_3PO_4-Na_2HPO_4 buffer solution is the best in all the experimental buffer solution. The possible reason is that TEOA competes for the cavity of CD with object. The competition could reduce the affinity between the object and the cavity of CD and weaken the action existing in sample molecule and chiral selector. The other four buffer solutions showed less enantioselectivity ability based on the experimental data (Table 3). The reasons for less recognition ability could be explored in the further study.

Table 3 Influence of different buffer on the distribution behavior of racemic phenylalanine

Item	H_3PO_4/Na_2HPO_4	Cit/NaCit	HAc/NaAc	Cit/TEOA	Cit/Tris
K_L	3.30	2.56	3.12	2.12	1.86
K_D	2.16	1.80	2.11	1.70	1.58
α	1.53	1.42	1.48	1.25	1.18
ee/%(lower phase)	10.27	7.01	8.65	4.92	4.02
ee/%(higher phase)	13.31	10.32	11.68	7.81	6.98

Figure 5 indicates that the appropriate concentration of buffer solution is 0.02 mol/L in the experiment. The concentration of buffer solution has the similar influence on separation effect. The mutual restraint between

ions reinforces with the increase of buffer solution concentration. It minishes the chance that anion and cation combine into molecule from electrolyte dissociation, subsequently decreases the concentration of weak electrolyte molecule and increases ionic concentration as well as degree of dissociation. Thus, the separation effect was weakened.

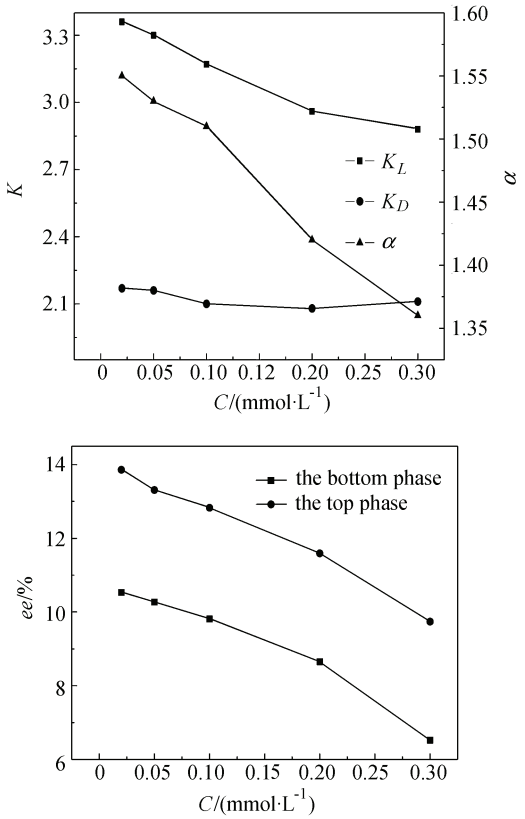

Figure 5 Influence of phosphate buffer concentration on the distribution behavior of racemic phenylalanine.

Conclusions

The chiral separation behavior of racemic Phe was investigated in aqueous two-phase systems composed of polyethylene glycol and ammonium sulfate containing combinatorial chiral selector: *β*-CD and HP-*β*-CD. The experimental results show that a better enantioseparation efficiency can be obtained using combinatorial chiral selectors. The optimized concentration ratio of *β*-CD to HP-*β*-CD is 2∶3 in combinatorial chiral selectors. The total concentration of combinatorial chiral selectors is 0.01 mol/L and pH of Na_2HPO_4-H_3PO_4 buffer solution is 5.5. The research indicates that repeatability of experiment result is better and experiment conditions can be more easily using combinatorial chiral selectors instead of single chiral selector. At the same time, separation efficiency has also been improved. Therefore, we conclude the method we proposed is an economical and effective way for chiral resolution.

Acknowledgements

The authors would like to thank National Natural Science Foundation of China (Nos. 20805058 and 201176262) and Provincial Natural Science Foundation of Hunan (No. 09JJ3026) for the financial supports of this work.

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