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Using enantioselective dispersive liquid–liquid microextraction for the microseparation of *trans*-cyclohexane-1,2-diamine enantiomers

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ABSTRACT

A new chiral separation system effective for the enantioselective extraction of racemic *trans*-cyclohexane-1,2-diamine is presented. Enantioselective dispersive liquid–liquid microextraction has been used for the chiral microseparation of *trans*-cyclohexane-1,2-diamine, with a chiral azophenolic crown ether being identified as a versatile chiral selector. The influence of various process conditions on the extraction performance was studied experimentally. It was found that the operational selectivity in one extraction step is mainly related to the type and volume of the solvents, chiral selector concentration, extraction time, temperature of sample solution, and pH. At optimum conditions (300 μL of diethyl ether as the extraction solvent 1 mL of methanol as the disperser solvent, with 5 mmol L^{-1} chiral selector concentration, pH of the sample equal to 4.5, 30 min extraction time and a temperature of 10 °C), the distribution ratio of (*R*,*R*)- and (*S*,*S*)-*trans*-cyclohexane-1,2-diamine was 18.3 and 1.8, respectively, while the enantioselectivity value of 10.2 was found at the optimum condition.

1. Introduction

Enantiomerically pure substances play an important role in the pharmaceutical and fine chemical industry, as raw materials, intermediates and end products. The importance of chirality with respect to biological activity has been clearly recognized.[1] An important property of biological systems is their ability to differentiate between enantiomers. The interactions of enantiomers with organisms are diastereomeric.[2] Therefore, the enantiomers of drugs, pesticides, or waste compounds are recognized by the organism as different molecules that elicit different physiological responses. This interest can be attributed largely to a heightened awareness that enantiomers of a racemic drug may have different pharmacological activities, as well as different pharmacokinetic and pharmacodynamic effects.[3] Chirality is a major concern in the modern pharmaceutical industry. In the modern pharmaceutical industry, especially in anti-cancer drugs, sometimes only one enantiomer has medicinal properties while the other enantiomer does not, i.e., one enantiomer is useful for the body while the other one is harmful or has no effect on.[4,5] Therefore, separation of a racemic mixture is desirable, and hence there is a great need to develop the technology for analysis and separation of racemic drugs. Current methods of enantiomeric analysis include such non-chromatographic techniques such as polarimetry,[6] nuclear magnetic resonance,[7–9] isotopic dilution,[10,11] calorimetry,[12] and enzyme techniques.[13] The disadvantages of these techniques are the need for pure samples, and no separation of enantiomers is involved. Quantification, which does not require pure samples, and separation of enantiomers can be done simultaneously by either gas chromatography (GC)[13–15] or high performance liquid chromatography (HPLC).[16,17] Current chiral HPLC methods are either direct, utilizing chiral stationary phases[18] and chiral additives in the mobile phase,[19] or indirect, which involves derivatization of samples.[20] Chiral separation is often required as the final product purification step after asymmetric synthesis. The two main industrial-scale separation methods each have serious drawbacks. Diastereomeric crystallization is inflexible and involves solid phase handling,[21] while simulated moving bed chromatography[22] is very expensive and produces diluted product streams. Therefore, there is a need for a flexible cost-effective separation method suitable for commercial production scale. Extraction methods are good methods to achieve this target. Chiral selectors play a very important role in the separation efficiency of all enantiomer separation techniques.[23] In the extraction methods, the enantioselective chiral selector is generally present in the organic solvent because chiral recognition tends to be more selective in a hydrophobic environment.[24] The enantiomer that forms a complex most strongly with the chiral selector is recovered from the extract and the other enantiomer ends up in the raffinate. In

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enantioselective liquid–liquid extraction, an enantiopure host is used as a chiral selector to bind enantiospecifically and reversibly with a racemic substrate.[25] If the host is confined to one phase in a biphasic system, enantiomeric separation of the substrate can take place between the two phases in a single step. If the separation is imperfect, a fractional extraction series is required.[26] Dispersive liquid–liquid microextraction is a mode of liquid–liquid extraction on a smaller scale.[27] Dispersive liquid–liquid microextraction employs a mixture of an extracting solvent and water miscible polar disperser solvent. In dispersive liquid–liquid microextraction, after a rapid injection of an appropriate mixture, containing the extracting and disperser solvents, into the aqueous sample, a cloudy state is formed. The contact area between the extracting solvent and the sample solution is very large. Thus the extraction equilibrium is achieved rapidly. After centrifugation, the extracted phase settles at the bottom or top of the conical test tube.[28,29] If we use a disperser solvent in the extraction process, extracting the solvent can capture the target analyte in less time and higher recovery. Therefore, we can use a chiral selector in dispersive liquid–liquid microextraction for the extraction and separation of enantiomers. In this method, the chiral selector is mixed with the extracting and disperser solvents and then this mixture is injected into the sample solution. Herein we have used enantioselective dispersive liquid–liquid extraction with an azophenolic crown ether (Fig. 1) as the chiral selector for the microseparation of *trans*-cyclohexane-1,2-diamine enantiomers. *trans*-Cyclohexane-1,2-diamine is required for oxaliplatin (anti-cancer drug) synthesis,[30] where only the (*R*,*R*)-isomer is effective, meaning that separation of the two isomers before the synthesis is necessary. Parameters affecting this process, such as selection type and volume of extracting and disperser solvents, chiral selector concentration, pH of sample solution, temperature and extraction time were optimized.

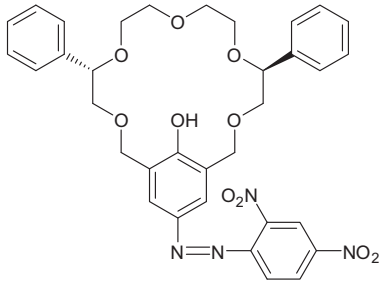

Figure 1. Azophenolic crown ether, the chiral selector used in this study.

2. Experimental

2.1. General

trans-Cyclohexane-1,2-diamine and *m*-toluoyl chloride were purchased from Sigma–Aldrich (Steinheim, Germany). HPLC grade (methanol, isopropyl alcohol, hexane, acetonitrile and acetone), sodium hydroxide, hydrochloric acid and chloride were obtained from Merck (Darmstadt, Germany). Xylene, diethyl ether, dodecane, toluene, octanol and cyclohexane were obtained from Aldrich (Milwaukee, WI, USA). The azophenolic crown ether was synthesised by Syncom BV (Groningen, The Netherlands).[31] The water used was double distilled deionized. A stock solution of *trans*-cyclohexane-1,2-diamine (10.0 g/L) was prepared in methanol and stored in the dark at 4 °C. Working standard solutions were diluted with deionized double distilled water at concentration of 1.0 mg/L whenever needed.

2.2. Instrumentation and operating condition

The resolved diamine was derivatized as the *m*-toluoyl bisamide based on the work of Walsh.[32] Derivatization allows detection of the enantiomers as they are eluted from the column by UV–vis spectroscopy (λ = 254 nm). The derivatization of the racemic and resolved diamine as the bisamides with *m*-toluoyl chloride is easily accomplished under basic conditions. Chromatographic measurements were carried out using a HPLC system equipped with a series 10-LC pump, UV detector model LC-95 set at 254 nm and model 7725i manual injector with a 20 μL sample loop (Perkin–Elmer, Norwalk, CT, USA). Column used was Pirkle L-Leucine (250 × 4.6 mm) and mobile phase was 90:10 hexane/isopropyl alcohol at a flow rate of 1 mL/min. typical chromatogram of *trans*-cyclohexane-1,2-diamine racemic mixture has shown in Fig. 2. The pH of the solutions was measured by a 3030 Jenway pH meter (Leeds, UK).

2.3. Enantioselective dispersive liquid–liquid microextraction procedure

A 10 mL sample solution containing 1.0 mg/L of *trans*-cyclohexane-1,2-diamine was placed in a centrifuge tube with narrow neck (~4 mm i.d.), which was specially designed for ease of removing the supernatant phase. A mixture of 1 mL disperser solvent and 300 μL extracting solvent with 2 mmol L^{-1} of chiral selector was rapidly injected into the sample solution using a 5.0 mL syringe, and mixed by vortex mixer at 500 rpm stirring rate for 20 min, so that a cloudy solution was formed. The cloudy solution was centrifuged for 5 min at 3500 rpm, and the extraction product (supernatant phase) was collected in the neck of the tube. Finally, this supernatant phase was derivatized and injected into the HPLC. All of the experiments were carried out in triplicate and the average of the result was reported.

2.4. Calculation of distribution ratio and selectivity

The extent of extraction is characterized by the distribution ratios D_R and D_S for each enantiomer[33,34]:

$$D_R = \frac{[R]org,\ allforms}{[R]aq,\ allforms} \quad (1)$$

$$D_S = \frac{[s]org,\ allforms}{[s]aq,\ allforms} \quad (2)$$

The concentrations of the enantiomers in the organic phase were determined from HPLC peak area while the amount of residue of *trans*-cyclohexane-1,2-diamine in the initial phase were found by subtracting the initial amount from the amount of *trans*-cyclohexane-1,2-diamine in the organic phase. The operational selectivity α_{op} is defined by the ratio of these distribution ratios. Its upper limit is the intrinsic selectivity α_{int} which is the ratio of the complexation constants:

$$\alpha_{op} = \frac{D_R}{D_S} \quad \text{Assuming } D_R > D_S \quad (3)$$

$$\alpha_{int} = \frac{K_R}{K_S} \quad (4)$$

K_R and K_S are complexation constants for the complexation between the crown ether and *trans*-cyclohexane-1,2-diamine enantiomers. As a result, even if one of the two enantiomers is much less valuable than the other one, the enantiomeric purity of both enantiomers should be high in their appropriate exit streams, to ensure a good yield of the desired enantiomer. From calculations based on the Kremser equation,[35] it is clear that the operational selectivity

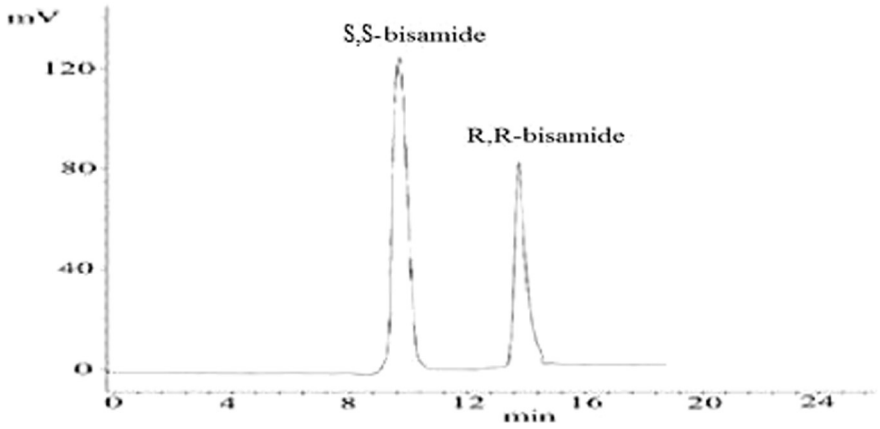

Figure 2. Typical HPLC chromatogram for *trans*-cyclohexane-1,2-diamine enantiomers, mobile phase: 90:10 hexane/isopropyl alcohol; flow rate: 1.0 mL/min; column: Pirkle L-Leucine (250 × 4.6 mm); λ = 254 nm.

in one stage mainly determines the number of stages required for a certain product purity: the larger the selectivity, the lower the number of stages required. The capacity of extraction (expressed as distribution ratio) influences the required solvent-to-feed ratio and wash-to-feed ratio, and thus the productivity. This effect is reflected in the extraction factors. In a fractional extractor, the extraction factors in the wash section are defined by (for enantiomer '*R*' and '*S*'):

$$E_R = D_R \frac{S}{W} \quad \text{and} \quad E_S = D_S \frac{S}{W} \tag{5}$$

For the extraction and separation processes, we have to find the best selectivity between the two enantiomers. It can be said that one enantiomer has to stay in the initial phase while the other enantiomer has to move to the extraction phase with a chiral selector. Based on these statements we can evaluate the selectivity or separation factor between the two enantiomers to determine the optimum conditions.

3. Results and discussion

In order to achieve the highest selectivity between the two enantiomers, the chiral selector experimental parameters, which influence the extraction and selectivity of the enantioselective dispersive liquid–liquid extraction procedure, including the extracting and disperser solvents as well as their volume, pH value of the sample solution, chiral selector concentration, temperature and extraction time were optimized using a one variable-at-a-time optimization method.

3.1. Selection of extracting and disperser solvents

In order to obtain a good selectivity for the enantioselective dispersive liquid–liquid extraction of *trans*-cyclohexane-1,2-diamine, selection of an appropriate solvent is a major factor for the enantioselective dispersive liquid–liquid extraction process. Herein we used solvents lighter than water. The extracting solvent has to meet two properties: it must have less density than water and have low solubility in water. Hence, xylene, *n*-hexane, diethyl ether, dodecane, toluene, octanol and cyclohexane were tested for this purpose. In order to choose which disperser solvent should be used in the enantioselective dispersive liquid–liquid extraction, the miscibility of it in the organic phase (extracting solvent) and the aqueous phase (sample solution) were key factors. Acetonitrile, acetone and methanol were examined as the disperser solvent in the extraction of *trans*-cyclohexane-1,2-diamine. To obtain a high selectivity, all combinations using 250 μL of extraction solvents (2 mmol L^{-1} chiral selector) with 400 μL of dispersive solvents were examined. The results in Table 1 indicate that diethyl ether as the extraction solvent and methanol as the disperser solvent had the best selectivity of approximately 7. Therefore, this diethyl ether-methanol combination was selected for subsequent experiments.

3.2. Effect of extracting solvent volume on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and selectivity

In order to examine the effect of the extraction solvent volume, experiments involving different volumes of diethyl ether were used for the microseparation of *trans*-cyclohexane-1,2-diamine enantiomers from standard sample solutions with 400 μL of methanol as the disperser solvent. When the extraction solvent volumes were lower than 50 μL, no supernatant organic phase was collected on the top of aqueous phase. Table 2 shows the distribution ratio of the enantiomers and the selectivity factor with the extraction solvent volume. The results in Table 2 show that

Table 1
The effect of different types of extraction and disperser solvents on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and selectivity. Extraction conditions: aqueous sample volume, 10 mL (1.0 mg/L of *trans*-cyclohexane-1,2-diamine); extracting solvent volume, 300 μL with 2 mmol L^{-1} of chiral selector; disperser solvent volume, 1 mL

Solvents	Without dispersive solvent			Methanol			Acetone			Acetonitrile		
	D_R	D_S	α	D_R	D_S	α	D_R	D_S	α	D_R	D_S	α
Xylene	4.3	1.7	2.5	8.2	2.1	3.9	7.4	2.3	3.2	7.3	1.9	3.8
n-Hexane	5.3	1.5	3.5	8.6	2.4	3.5	8.1	2.1	3.9	9.5	2.1	4.5
Diethyl ether	6.5	1.9	3.4	**13.2**	**1.8**	**7.3**	11.2	1.9	5.9	11.1	2.2	5.0
Dodecane	5.1	1.5	3.4	9.2	2.3	4.0	9.1	1.8	5.1	10.5	2.5	4.2
Toluene	5.2	1.8	2.8	10.6	2.5	4.2	9.8	1.9	5.1	10.4	3.6	2.9
Octanol	6.5	1.7	3.8	12.1	2.8	4.3	9.6	1.9	5.0	11.2	6.6	1.7
Cyclohexane	4.7	1.4	3.4	9.3	2.0	6.6	7.2	2.0	3.6	8.0	2.2	3.6

Table 2
The effect of the extraction solvent (diethyl ether) volume on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity. Extraction conditions: as Table 1. With different volume of extraction solvent

Extraction solvent volume (μL)	50	100	200	250	300	350	400
D_R	9.2	10.8	12.4	13.2	**15.0**	15.2	15.9
D_S	1.4	1.6	1.7	1.8	**2.0**	3.1	4.5
α	6.6	6.8	7.2	7.3	**7.5**	4.9	3.5

300 μL of the extraction solvent produced the highest selectivity factor. Thus this volume was selected as the optimum volume for the extraction solvent.

3.3. Effect of disperser solvent volume on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and selectivity

In order to determine the best volume of the disperser solvent, the extractions were carried out by changing the volume of methanol in the range of 100–2000 μL. The results in Table 3, show that when increasing the volume up to 1 mL methanol, the selectivity increased and decreased after this volume. The lower selectivity at volume of methanol less than 1 mL can be attributed to the fact that the cloudy state was not well formed and the extracting solvent (diethyl ether) could not be well dispersed among aqueous solution in the form of very little droplets. Hence for the following experiments, 1 mL of methanol was used as the optimal disperser solvent volume.

3.4. Effect of the chiral selector concentration on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and selectivity

The type and concentration of the chiral selectors are very important factors in this method. The choice of a good chiral selector can give high selectivity factor. An azophenolic crown was used in the chiral separation of amine compounds.[36] The influence of the crown ether concentration on the extraction performance has been studied in diethyl ether for *trans*-cyclohexane-1,2-diamine enantiomers. In Table 4, the observed distribution ratios D and the operational selectivity are given for *trans*-cyclohexane-1,2-diamine microseparation. It can be seen that a very good operational selectivity is achieved for the *trans*-cyclohexane-1,2-diamine enantiomers with a relatively low chiral selector concentration (~5 mmol L^{-1}). For two enantiomers, the distribution ratios increased when increasing the chiral selector concentration, but the slope of the distribution ratio increasing for (*S*,*S*)-*trans*-cyclohexane-1,2-diamine was more than the other enantiomer, and so the selectivity decreases at high chiral selector concentration. This concentration was chosen for the optimum concentration.

3.5. Effect of sample pH on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and selectivity

In enantioselective dispersive liquid–liquid extraction, the pH of the sample solution is a key factor for the extraction of acidic and basic compounds. In order to obtain high selectivity for acidic compounds, the sample solution should be acidified to deionize the analyte and consequently increase their transfer from the sample solution into the organic phase. Thus, the pH of the sample solution should be adjusted to make neutral the molecular forms of the analytes prior to the microextraction step, as the pH of the sample might effect the complexation constant between the chiral selector and the two enantiomers. For this purpose, the effect of the pH of the sample solution on the selectivity of *trans*-cyclohexane-1,2-diamine was investigated in the range of 3–8. Values of pH higher than 8.0 were not examined, because *trans*-cyclohexane-1,2-diamine can be hydrolyzed at basic pH. Table 5 shows that the highest selectivity was obtained with pH = 4.5 and so subsequent experiments were performed at this pH. This observation can be explained by the fact that in higher or lower pH, the *trans*-cyclohexane-1,2-diamine molecules are protonated or not and so the complexation constant of the enantiomers changes.

3.6. Effect of extraction time on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity

In enantioselective dispersive liquid–liquid extraction, the extraction time is defined as the gap time between the injection of the mixture of the disperser and extraction solvent with a chiral selector into the aqueous sample and the start of the centrifugation. Herein, the effect of the extraction time was examined from 1 to 120 min. The obtained results showed that the selectivity varied greatly depending on the extraction time. This observation can be explained by the fact that the rate at which the complex forms between the chiral selector and *trans*-cyclohexane-1,2-diamine enantiomers is slow. The results in Table 6 show increasing the

Table 3
Effect of disperser solvent (methanol) volume on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity. Extraction conditions: extracting solvent (diethyl ether) volume, 300 μL. Other conditions as Table 2

Disperser solvent volume (μL)	100	400	900	**1000**	1100	2000
D_R	10.2	14.4	15.2	**16.1**	16.2	16.9
D_S	1.5	1.7	1.8	**1.9**	2.4	2.8
α	6.8	8.4	8.4	**8.5**	6.7	6.0

Table 4
Effect of chiral selector concentration on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity. Extraction conditions: disperser solvent (methanol) volume, 1 mL. Other conditions as Table 3

Chiral selector concentration (mmol L^{-1})	1	4	**5**	6	10
D_R	8.1	15.5	**16.9**	17.0	17.5
D_S	1.5	1.6	**1.7**	4.6	6.1
α	5.4	9.7	**9.9**	3.6	2.8

Table 5
Effect of pH of sample solution on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity. Extraction conditions: chiral selector concentration, 5 mmol L^{-1}. Other conditions as Table 4

pH of sample solution	3	3.5	4	**4.5**	5.0	7.0	8.0
D_R	10.2	12.5	14.4	**17.0**	15.5	12.2	7.0
D_S	1.2	1.4	1.5	**1.7**	1.7	1.3	1.0
α	8.5	8.9	9.6	**10.0**	9.1	9.3	7.0

Table 6
Effect of Extraction time on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity. Extraction conditions: pH of sample solution, 4.5. Other conditions as Table 5

Extraction time (min)	1	10	20	**30**	40	60	120
D_R	8.2	10.8	15.2	**18.2**	18.1	18.1	18.3
D_S	1.5	1.6	1.8	**1.8**	1.9	2.4	2.8
α	5.5	6.7	8.6	**10.1**	9.5	7.5	6.5

Table 7
Effect of Temperature on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity. Extraction conditions: Extraction time, 30 min. Other conditions as Table 6

Temperature (°C)	5	8	**10**	15	25	37
D_R	10.2	14.8	**18.3**	15.2	14.2	11.2
D_S	1.2	1.5	**1.8**	1.8	1.8	1.7
α	8.5	9.8	**10.2**	8.4	7.8	6.6

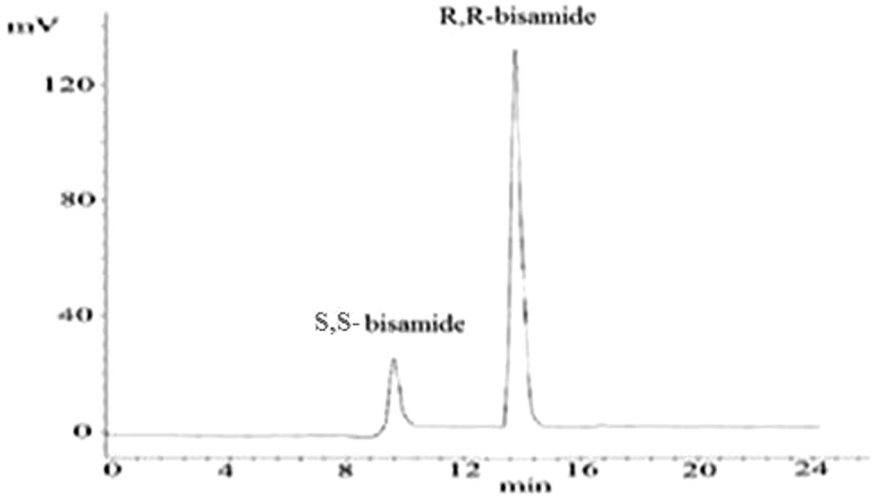

Figure 3. HPLC chromatogram for extraction phase at optimum conditions.

time up to 30 min increased the selectivity factor, after which the selectivity factor decreased. Hence 30 min was chosen for the extraction time.

3.7. Effect of temperature on the distribution ratios of the *trans*-cyclohexane-1,2-diamine enantiomers and the selectivity

Some researchers have reported that the temperature of the sample solution has a beneficial effect on the selectivity in chiral extraction procedures.[15,16] It is well known that increasing the temperature of aqueous sample can improve the selectivity. As can be seen in Table 1, the temperature has a strong influence on the absolute values of the selectivity. Over the temperature range 5–37 °C, the selectivity increased with increasing temperature up to 10 °C while after this temperature, the selectivity strongly decreased. Optimal selectivity was achieved at lower temperatures because at high temperatures, the chiral selector was demolished or the complexation between the enantiomers and the chiral selector did not occur. However, below T = 10 °C, extraction became too difficult due to an increase in the viscosity of the solution and a decrease in chiral selector solubility (Table 7). Therefore, in this case, T = 10 °C was considered to be the optimal temperature for enantioselective dispersive liquid–liquid extraction.

4. Conclusions

The extraction and microseparation of *trans*-cyclohexane-1,2-diamine enantiomers in an aqueous sample solution have been performed using enantioselective dispersive liquid–liquid extraction and HPLC. The results show that enantioselective dispersive liquid–liquid extraction is an effective method for the microseparation of *trans*-cyclohexane-1,2-diamine enantiomers. The azophenolic crown ether is a versatile and highly enantioselective compound for chiral microseparation

trans-cyclohexane-1,2-diamine by enantioselective dispersive liquid–liquid extraction. The chromatogram of the extraction phase at optimum conditions is shown in Fig. 3. The process capacity is good due to the sufficiently high distribution ratios. This method is very inexpensive and can be used in the laboratory. We have used a micro extraction method for the chiral separation. Herein our aim was to find an extraction method for *trans*-cyclohexane-1,2-diamine chiral separation on a semi industrial scale, but we carried out our experiments on a micro scale to save solvent and materials.

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