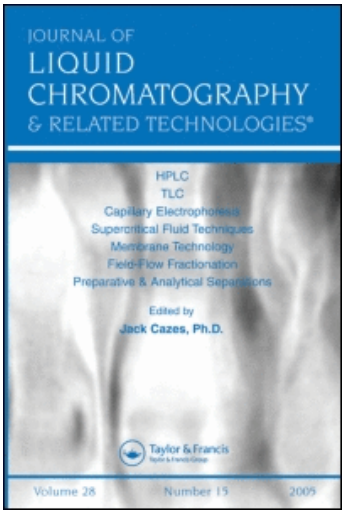

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Mohamed M. Hefnawy[a]
[a] Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

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Optimization of the Chiral Resolution of Metyrosine by Capillary Electrophoresis and/or Micellar Electrokinetic Capillary Chromatography

Mohamed M. Hefnawy
Department of Pharmaceutical Chemistry, College of Pharmacy,
King Saud University, Riyadh, Saudi Arabia

Abstract: The chiral resolution of metyrosine was developed by capillary electrophoresis (CE) (method I) and by micellar electrokinetic capillary chromatography (MEKC) (method II) using a fused-silica capillary (72 cm × 50 μm ID). The background electrolyte (BGE) for method I was Tris-phosphate buffer (pH 2.5, 50 mM)—methanol (95 : 5, v/v) containing 20 mM β-cyclodextrin (CD), and for method II was acetate buffer (pH 2.5, 50 mM) containing 5 mM sodium taurocholate (NaTC). The separation was achieved at 20 kV applied voltage and 30°C for both investigations. The detection wavelength was 220 nm. The values of R_s for methods I and II were 1.5 and 2.1, respectively. The electrophoretic conditions were optimized varying the pH and the ionic strength of the BGE, concentration of the chiral selector, applied voltage, temperature, and percent of organic modifier. The chiral recognition mechanisms between the analyte and chiral selectors are discussed.

Keywords: Capillary electrophoresis, metyrosine, chiral resolution, β-cyclodextrin, sodium taurocholate

INTRODUCTION

Capillary electrophoresis (CE), a versatile technique of high speed, high efficiency, low consumption of run buffer, and small sample size, is a major

Address correspondence to Mohamed M. Hefnawy, Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia. E-mail: mhefnawy2003@yahoo.com

trend in analytical science, and numerous publications have appeared on the chiral resolution of a variety of racemic drugs and pharmaceuticals in recent years.[1–4] The most common technique for enantiomeric separation is to add a chiral selector to the run buffer. Neutral and charged cyclodextrins (CDs) are the most commonly used chiral selectors, due to low cost, commercial availability, and UV transparency.[5–7] Native CDs are cyclic, chiral oligosaccharides with hydrophobic cavity and hydroxyl groups on the rim. The intermolecular forces including hydrophobic, hydrogen bonding, dipole–dipole, and van der Waals interactions are present in the formation of inclusion complexes between CDs and chiral compounds.[8]

Another technique for enantiomeric separations is micellar electrokinetic capillary chromatography (MEKC).[9,10] The method is based on micellar solubilization and electrophoretic migration of the micelle and solutes. Separations occur due to the differential distribution of solute between the micelle and the surrounding aqueous phase and the differential migration of the two phases. In MEKC, the types of surfactant and buffer modifications are important in manipulating the selectivity of the method.[11,12] Changes in micellar composition can produce micelles of different sizes, aggregation numbers, and geometrics, leading to different interactions or solubilization. The use of bile salts in the background electrolyte (BGE) of MEKC has been described as an effective method to separate enantiomers,[13,14] as well as hydrophobic uncharged molecules[15,16] and ionic compounds.[17,18] Anionic surfactant systems have been preferred in MEKC since the resultant micelles migrate electrophoretically opposite to the electroosmotic flow, and do not interact with the negatively charged walls of the fused silica capillary columns.

In aqueous solution, amphiphilic bile salts form micelles when their concentration exceeds the critical micelle concentration (CMC).[19] Trihydroxy bile salts generally have higher CMC than dihydroxy bile salts. At physiological pH, the CMC of most bile salts varies between 2 and 5 mM.[14] The bile salts commonly used in MEKC are sodium cholate, sodium taurocholate (NaTC), sodium deoxycholate, and sodium taurodeoxycholate.

Metyrosine is clinically administered as a racemate for the control of hypertension in-patients with phaeochromocytoma. It may be administered as a pre-operative medication for those patients for whom surgery is contra-indicated.[20] The L-enantiomer is biologically active while the D-enantiomer is inactive.[20] Therefore, the US Food Drug Administration and other health regulatory agencies issued certain guidelines regarding the racemic drugs.[21–23] One prior report on the optical resolution of metyrosine was published by Saito et al.[24] Hefnawy and Stewart[25] separated metyrosine enantiomers by HPLC as methyl esters derivatized with 2,3,4,6-tetra-*o*-acetyl-β-D-glucopyranosyl isothiocyanate. Metyrosine has also been determined in serum by HPLC, in the presence of its major metabolite α-methyldopa, using solid phase extraction and fluorescence detection.[26]

It has also been determined in biological fluids and tissues by GC-MS[27] and fluorometry.[28] Metyrosine in dosage forms has been determined either polarographically through treatment with nitrous acid[29] or colorimetrically via its reaction with 4-amino antipyrine in the presence of an alkaline oxidizing agent.[30] The USP 23 recommended a non-aqueous titration method with potentiometric detection of the end point for the evaluation of the bulk drug substance.[31] It is very important to observe that the above-cited reports[24,25] of the chiral resolution of metyrosine by HPLC suffer certain drawbacks and complications. The use of chiral pre-column derivatization makes the methodology complex and expensive. Therefore, attempts are made to develop simple methods for the chiral resolution of metyrosine using underivatized β-CD and NaTC and the results are presented herein.

EXPERIMENTAL

Chemicals

Metyrosine (4-hydroxy-α-methylphenylalanine) was obtained from Aldrich Chemical Co., (Milwaukee, WI, USA). Phosphoric acid, methanol, acetic acid, and ammonium acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Tris (hydroxymethyl amino methane) and NaTC were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Heptakis- (2,6-di-*o*-methyl)-β-cyclodextrin (DM-β-CD) and heptakis (2,3,6-tri-*o*-methyl)-β-cyclodextrin (TM-β-CD) were also obtained from Sigma. Native-β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (OH-β-CD), and sulfated-β-cyclodextrin (S-β-CD) were gifts from American Maize Products (hammond, IN, USA). Methyl-β-cyclodextrin (M-β-CD, degree of substitution 12.7) was kindly supplied by Cerestar USA, Inc. (Hammond, IN, USA). All solutions were filtered through a 0.2 μm nylon filter (Acrodisc13, Gleman Science, Ann Arbor, ML, USA).

Chromatography

The CE experiments were performed using an ABl model 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a UV detector. An uncoated fused slica capillary (L = 72 cm, 1 = 50 cm, 50 μm ID, Polymicro Technologies, Pheonix, AZ, USA) was used for the analysis. A 0.5 cm detection window was created by stripping the polymide coating of the capillary. The sample was injected on the anodic end and detected on the cathodic side of the capillary. The electropherograms were recorded on a HP 3395 integrator (Hewlett Parkard, Avondale, PA, USA).

Sample Preparation

A stock solution of DL-metyrosine was prepared in water to give a concentration of 100 μg/mL. The solution was stored at 4°C and was stable for 1 week. Ammonium acetate solution (50 mM) was prepared by dissolving 3.85 g of ammonium acetate in 1 L of double distilled deionized water and the pH adjusted to 2.5 with acetic acid. Tris-phosphate solution (50 mM) was prepared by dissolving 6.05 g of Tris (hydroxymethyl aminomethane) in 1 L of double distilled, deionized water and adjusted to pH 2.5 with phosphoric acid. The run buffer solution was prepared by weighing the appropriate amount of β-cyclodextrin or NaTC into a 5 mL volumetric flask and the Tris-phosphate or ammonium acetate buffer solution added to volume.

Capillary Electrophoretic Procedure

The BGE consisted of either 50 mM Tris phosphate buffer pH 2.5-methanol (95 : 5 v/v) or 50 mM ammonium acetate pH 2.5, countering various concentrations of β-cyclodextrin or NaTC, respectively. The analytes were monitored at 220 nm. New capillaries were conditioned by rinsing with 1 M sodium hydroxide, water, and BGE for 15 min each. The sample was introduced using a vacuum injection for 20 sec prior to each analysis, and the capillary was washed with 0.1 M sodium hydroxide for 2 min and then with BGE.

RESULTS AND DISCUSSION

The resolution factor (R_s) and retention time for the resolved enantiomers of metyrosine by methods I and II were calculated. The values of R_s were 1.5 and 2.1 for methods I and II, respectively. Typical electropherograms of the resolved enantiomers of metyrosine by both methods are given in Figure 1(a) and (b). The retention times for first and second enantiomers of metyrosine were 34.2 and 35.1 min for method I and 30.9 and 32.4 min for method II, respectively. The retention time may be considered as an indirect way to avoid the interference due to impurities in the biological samples; as most of the biological impurities elute before 30 min of time. The retention times of the enantiomers have been reduced by using high voltage and concentration of BGE, but the reproducibility was poorer. A variation in the electrophoretic parameters was carried out to obtain the best resolution by both methods. To optimize the electrophoretic conditions, variations in pH and the ionic strength of the BGE, concentration of chiral selector, methanol, and applied voltage were carried out. As a result of extensive experimentation, the optimized chromatographic conditions for methods I and II were developed and reported herein.

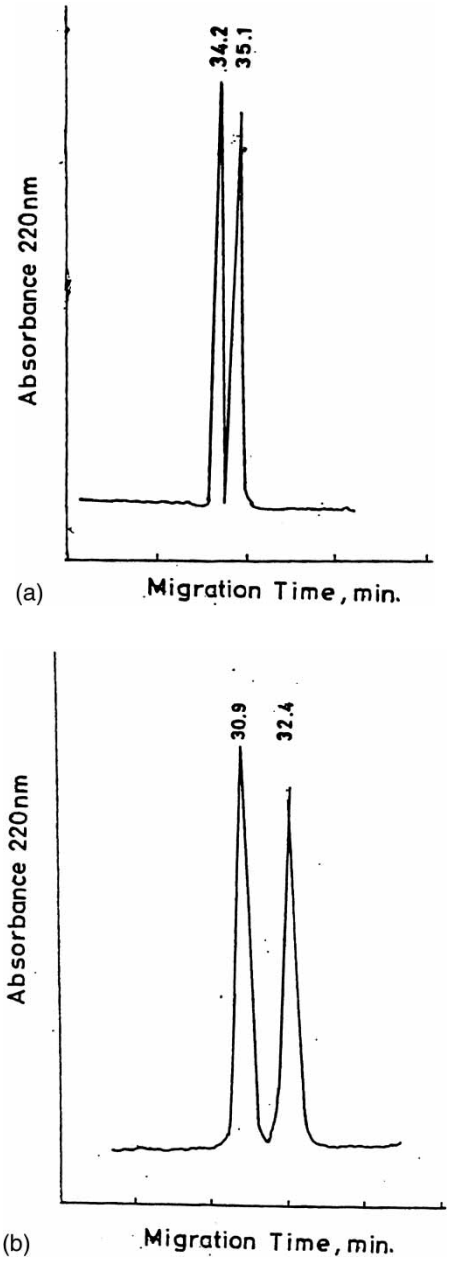

Figure 1. (a) Electropherogram of metyrosine enantiomers, BGE, Tris-phosphate buffer (pH 2.5, 50 mM)–methanol (95 : 5 v/v) containing 20 mM β-cyclodextrin. (b) Electropherograms of metyrosine enantiomers, BGE, acetate buffer (pH 2.5, 50 mM) containing 5 mM sodium taurocholate.

Method I

Effect of pH of the BGE

To achieve the best resolution, a variation in the pH was carried out from 2.5 to 6.5. The results of the pH variation are shown in Table 1, which clearly

Table 1. Effect of various parameters on resolution of metyrosine enantiomers using β-cyclodextrin as the chiral selector

		Migration time (min)	
Parameters	R_s	P_1[a]	P_2[b]
Chiral selector concentration (mM)			
5	—	17.2	—
10	0.8	25.5	25.8
15	1.1	32.3	32.8
20	1.5	34.0	34.6
25	1.1	38.9	39.6
30	1.0	45.8	46.3
Buffer concentration (mM)			
10	1.1	19.4	19.8
30	1.2	26.6	27.1
50	1.5	34.2	34.8
60	1.3	39.3	39.8
70	1.3	44.8	45.3
pH			
2.5	1.5	33.9	34.6
4.5	1.0	25.6	26.1
6.5	—	19.7	—
Voltage (kV)			
15	1.5	44.8	45.6
20	1.4	34.1	34.8
25	1.1	21.9	22.4
30	0.8	12.2	12.4
Temperature (°C)			
30	1.5	33.6	34.3
40	1.1	24.5	24.9
50	—	20.7	—
Organic modifier (% v/v)			
5	1.5	34.6	35.1
10	1.1	36.1	36.6
20	—	41.5	—

[a]Peak of enantiomer I.
[b]Peak of enantiomer II.

indicates pH 2.5 as the optimum. It may be observed from this table that the R_s values first increase and then start to decrease. The effect of pH on the chiral resolution of metyrosine enantiomers may be attributed due to the different stabilities of the diastereomeric complexes formed between metyrosine enantiomers and β-CD at different pH. At pH 2.5, the analytes were fully ionized and the electroosmotic flow was negligible, which resulted in increased interaction of the β-CD-analyte. Above pH 6.5, there was less interaction of the analyte with the β-CD, resulting in a loss of resolution due to the increased electroosmotic flow.

Effect of the Ionic Strength of BGE

The ionic concentration of the buffer was varied from 10 to 70 mM and the values of R_s obtained are plotted in Table 1. The values of R_s increase up to 1.5 and then start to decrease. A solution of 50 mM concentration of Tris-phosphate buffer was found suitable and, hence, used throughout all the experiments. Generally, the value of conductivity increases by increasing the ionic strength of the BGE. Therefore, the variation in the enantioselectivity at different buffer concentrations may be due to the different conductivities of the buffer.

Effect of the Type of CD

The metyrosine racemate was run in Tris-phosphate buffer pH 2.5 in the absence of a chiral additive. Owing to the protonation of the analyte, the enantiomer moved towards the cathode as expected, and no enantiomeric separation was obtained. Enantiomeric resolution of metyrosine was studied using Tris-phosphate buffer containing different types of CD, such as β-CD, hydroxypropyl-β-CD, sulfo-β-CD, methyl-β-CD, di-methyl-β-CD, and tri-methylβ-CD (Table 2). Complete baseline resolution of metyrosine enantiomers was obtained with β-CD. Under the experimental conditions, the electroosmotic flow was relatively low compared with the effective mobility of the cationic sample and the β-CD probably acted as a quasi-stationary phase.

Effect of the Concentration of β-CD

The effect of the concentrations of β-CD on the enantioselectivity of metyrosine is shown in Table 1, which shows 20 mM as the optimum concentration. The values of R_s increase from 10 to 20 mM concentrations, but become almost constant at higher concentrations of β-CD. It may be concluded that 20 mM concentration of β-CD is suitable for the formation of a stable complex with metyrosine enantiomers. The poor resolution of metyrosine at higher concentrations of β-CD may be due to the change in the composition

Table 2. Resolution of metyrosine enantiomers using various CD derivatives

		Migration time (min)	
Chiral selector	R_s	P_1[a]	P_2[b]
β-CD[c]	1.5	34.1	34.6
OH-β-CD[c]	—	25.3	—
S-β-CD[c]	—	23.7	—
M-β-CD[c]	—	30.9	—
DM-β-CD[c]	—	23.5	—
TM-β-CD[c]	—	12.5	—

[a]Peak of enantiomer I.
[b]Peak of enantiomer II.
[c]Electrophoretic conditions: Tris-phosphate buffer (pH 2.5, 50 mM)–methanol (95 : 5 v/v) containing 20 mM CD derivatives as the chiral BGE additive; applied voltage, 20 kV; temperature, 30°C; detection UV at 220 nm.

of the BGE, as the high concentration of β-CD may decrease the conductivity of the BGE.

Effect of the Applied Voltage

The values of the applied voltage were from 15 to 30 kV and the resulting R_s values are plotted in Table 1. The values of R_s decrease by increasing the applied voltage. Of course, the values of R_s are greater at 15 kV voltage but the migration times of the enantiomers were high, leading to increased interaction of the β-CD-analyte. Due to electrodispersion, band broadening was seen at higher migration times. Therefore, 20 kV applied voltage was selected as the optimum. Again, the poor resolution of metyrosine enantiomers at higher values of applied potential is due to the higher values of conductivity and migration velocities of BGE.

Effect of the Temperature

The migration time decreased as the temperature increased, which was attributed to decreased viscosity of the BGE at higher temperature and decreased binding constants (Table 1). The resolution of metyrosine enantiomers, as expected, decreased due to the decrease in the binding constants for the β-CD–analyte complexes.

Effect of the Concentration of Organic Modifier

The influence of organic solvent in the run buffer was investigated. Methanol was used as the organic modifier. Increased amounts of organic modifier caused a general increase in the migration times of metyrosine enantiomers. This might be attributed to two factors: methanol decreased the electrical conductivity, thereby decreasing the current, and methanol decreased the amount of electroosmotic flow modifier adsorbed onto the inner wall of the fused-silica capillary. It was also observed that with the addition of methanol in the range 5–20% v/v, the migration times of the enantiomers increased from 5–20% and resolution of the enantiomers decreased (Table 1). The enantioselectivity increased from 2% to 5% concentrations of methanol and became almost constant at further higher concentrations. Therefore, 5% methanol was selected as the optimum. The resolution of metyrosine enantiomers decreasing with an increase in the percentage of organic modifier may be due to a decrease in the binding constants, resulting from the competition of the organic solvent molecules with analyte for the β-CD cavity.

Method II

Effect of the Type of Bile Salt

The presence of surfactants in chiral selector containing buffers enhance the resolution of stereoisomers and, in many situations, the chiral resolution cannot be achieved without the presence of an ionic micellar system. One of the most striking properties of micellar systems relevant to their use in chemical and chiral separation is their ability to solubilize compounds. Solubilization starts at the CMC and increase in proportion to micellar concentration.[19] When an ionic surfactant is added to BGE at concentration above its CMC, surfactant monomers tend to aggregate, forming micelles. The surface of the micelles acquires a charge, which gives them an electrophoretic mobility.[32] Bile salts were initially introduced by Terabe et al.[13] as an additive in run buffers for chiral separations. In our study, the chiral separation of metyrosine enantiomers by MEKC was investigated with four different bile salts, namely, sodium cholate, sodium deoxycholated, NaTC, and sodium taurodeoxycholate. No separation was obtained using deoxycholate or taurodeoxycholate, and only partial separation of metyrosine enantiomers was observed when sodium cholate (5 mM) was the chiral selector. Complete baseline resolution of metyrosine enantiomers was obtained with NaTC. As a result, it can be concluded that both hydroxyl groups on the cholic acid part of the NaTC, along with the taurine group, have an effect on enantiomeric resolution of metyrosine enantiomers.

Effect of the Concentration of NaTC

The effect of the concentrations of NaTC on the enantioselectivity of metyrosine is shown in Table 3, which shows 5 mM as the optimum concentration. The values of R_s increase from 2 to 5 mM concentrations, and then start to decrease. The metyrosine enantiomers were not resolved at low NaTC concentrations (1 mM). There was retention of the analytes showing that micelles were present, but the resolution was limited by poor peak efficiency due to

Table 3. Effect of various parameters on resolution of metyrosine enantiomers using NaTC as the chiral selector

		Migration time (min)	
Parameters	R_s	P_1[a]	P_2[b]
Chiral selector concentration (mM)			
1	—	15.6	—
2	0.8	19.1	19.4
3	1.4	23.3	23.8
4	1.7	26.2	26.9
5	2.1	30.9	31.8
6	1.1	41.4	41.9
7	0.7	53.2	53.6
Buffer concentration (mM)			
10	0.9	18.6	18.9
20	1.2	21.3	21.7
30	1.6	25.4	26.1
50	2.0	30.1	30.9
pH			
2.5	2.1	30.8	31.7
4.5	1.1	21.3	21.7
6.5	—	19.5	—
8.5	—	17.5	—
Voltage (kV)			
15	1.4	41.3	41.9
20	2.1	30.9	31.8
25	1.5	22.6	23.0
30	0.6	16.8	17.1
Temperature (°C)			
30	2.0	30.5	31.4
40	1.3	21.9	22.3
50	—	19.4	—

[a]Peak of enantiomer I.
[b]Peak of enantiomer II.

the effects of micelle polydispersity.[33] When concentrations of NaTC increased from 2 to 5 mM, reasonable high efficiency and resolution of metyrosine enantiomers was obtained. This indicated that, under the conditions employed, the bile salt micelles may be nearly monodisperse or the rate of micelle-monomer exchange is rapid on the electrophoretic time scale.[33] Otherwise, with higher concentrations (>6 mM) the resolution of metyrosine enantiomers decreased. Increased surfactant concentration produced large electrophoretic currents, which led to significant band broadening due to thermal gradients created by joules heating within the capillary.[34]

Effect of pH of the BGE

The effects of pH on the resolution of metyrosine enantiomers revealed that maximum resolution was observed at pH 2.5 where the electroosmotic flow was negligible. The results of pH variation (2.5–8.5) are shown in Table 3. It may be observed that the R_s values first increase and then start to decrease. As the pH increased above 5.5, there was a complete loss of resolution of metyrosine enantiomers with a concurrent decrease in migration time.

Effect of the Ionic Strength of BGE

The buffer type and concentration affect not only the electroosmotic flow but also efficiency and resolution. The effect of the type and buffer concentration was investigated from 10 to 50 mM of acetate, phosphate, and borate buffer. It was found that the resolution of metyrosine enantiomers was improved by using acetate buffer. The values of R_s increase up to 2.0 and then start to decrease as shown in Table 3. A solution of 50 mM concentration acetate buffer was found suitable and, hence, used throughout all the experiments.

Effect of the Applied Voltage

The effect of applied voltage on separation and resolution of metyrosine enantiomers was investigated in the acetate buffer. Higher voltage led to diminished resolution due to the decreased interaction of the micelle–analyte on the capillary column. The migration time was higher at lower voltages leading to increased interaction of the micelle–analyte. The values of R_s decrease by increasing the applied voltage over 20 kV (Table 3). Therefore, 20 kV applied voltage was selected as the optimum.

Effect of the Temperature

Higher temperature led to decreased migration times and comparatively less resolution due to increased kinetics of the micelle–analyte interaction and a decrease in the viscosity of the run buffer (Table 3).

In addition to these parameters, we also tried to optimize the chiral resolution varying the amount of metyrosine sample loading. The amount of loading was varied from 5 to 60 sec as the sample loading time. It was observed that the resolution was good with 5–15 sec as the loading time but detection was poor. At 20 sec as the loading time, both the resolution and the detection were optimum. However, the detection was very good at 30 sec or higher sampling time but the resolution was poor. Therefore, 20 sec was selected as the best sample loading time throughout all the experiments. The effect of different concentrations of metyrosine was studied using 20 sec as the loading time. The solutions of metyrosine prepared were 2.0, 5.0, 10.0, 15.0, and 20.0 μg/mL separately. It was observed that the peaks were very small with 1.0 μg/mL concentration while the resolution became poor when using 15.0 and 20.0 μg/mL concentrations. Therefore, 10.0 μg/mL was selected as the best concentration for the chiral resolution of metyrosine.

CONCLUSIONS

The present study reports a successful chiral resolution of metyrosine enantiomers by two methods, CE and MEKC. It may be concluded from this study that the chiral resolution of metyrosine enantiomers depends on many of the operational parameters of CE. The value of the lower limit of detection was 1.0 μg/mL for both methods. To ascertain reproducibility, the standard deviation was found to be ± 0.2 and ± 0.3 for methods I and II, respectively, which indicates the good reproducibility of the developed methods. Briefly, the developed CE and MEKC methods are sensitive and reproducible for the chiral resolution of metyrosine enantiomers.

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