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Enantioseparation of chiral mandelic acid derivatives by supercritical fluid chromatography

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Abstract

Mandelic acid and its derivatives are important chiral analogs which are widely used in the pharmaceutical synthetic industry. The present study investigated the enantiomeric separation of six mandelic acids (mandelic acid, 2-chloromandelic acid, 3-chloromandelic acid, 4-chloromandelic acid, 4-bromomandelic acid, 4-methoxymandelic acid) on the Chiralpak AD-3 column by supercritical fluid chromatography. The influences of volume fraction of trifluoroacetic acid, type and percentage of modifier, column temperature, and backpressure on the separation efficiency were investigated. And the enantiomer elution order was determined. The results show that, for a given modifier, the retention factor, the separation factor, and the separation resolution decreased gradually with increasing the volume ratio of the modifier. At the same volume ratio of modifier, the retention factor of the mandelic acid and its derivatives increased in the order of methanol, ethanol, and isopropanol, except 3-chloromandelic acid. The separation factor and the separation resolution decreased with the increase of column temperature (below the temperature limit). The backpressure affected the enantioseparation process: As the backpressure increased, a corresponding decrease in retention factor was observed. Under the same chiral column conditions, the SFC method exhibited faster and more efficient separation with better enantioselectivity than the HPLC method.

KEYWORDS

chiral, enantioseparation, mandelic acid, mobile phase additive, supercritical fluid chromatography

1 | INTRODUCTION

As an important structural unit of many natural products and drug molecules, chiral carboxylic acids play a crucial role in biochemistry and life sciences.[1] Mandelic acid (MA), known as α-hydroxyphenylacetic acid, is a kind of chiral carboxylic acid. Mandelic acid and its derivatives are important chiral analogs which are widely used in the pharmaceutical synthetic industry. They are important raw materials for the synthesis of cephalosporins antibiotics, vasodilator drug loop mandelic acid, and urinary tract disinfectant urotropine.[2] Mandelic acid has a chiral center resulting from the chirality of the carbon directly attached to the benzene ring, and thus, it usually exists in the enantiomeric forms of (R)-MA and (S)-MA. As for the mandelic acid compounds, they can be obtained by replacing the hydrogen of benzene ring in mandelic acid by different groups, and therefore also show chirality. It should be noted that the enantiomers of mandelic acid and the mandelic acid compounds are different in activity, toxicity, and environmental degradability. For example, the R-enantiomer of 2-chloridic acid can be

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used to synthesize a new, safe, and efficient antiplatelet aggregation drug clopidogrel,[3] while the S-enantiomer is inactive. The α-cyclohexylmandelic acid is an important chiral drug precursor which is used to synthesize a variety of chiral drugs with biological activity and good effect, such as oxybutynin; its S-enantiomer is better than that of racemate with fewer side effects.[4]

At present, the ways of obtaining single enantiomers of MA and its derivatives are mainly asymmetric synthesis, chiral separation, and enantioselective liquid-liquid extraction.[5] For instance, in the study of Tan,[6] poly (MAH-β-CD-co-NIPAAm), being synthesized by the copolymerization of *N*-isopropylazylamide (NIPAAm) with the maleic anhydride (MAH) modified β-cyclodextrin (β-CD), was used to separate the enantiomer of MA. The maximum separation factor (α) of MA reached 1.27 under the optimum conditions. As a new method of enantiomeric separation, chromatography attracts much interest and is studied widely. The use of high-performance liquid chromatography (HPLC) to analyze MA and its derivatives has been reported many times. High-performance liquid chromatography separates efficiently the chiral organic compounds with macromolecules, strong polarity, poor thermal stability, and high boiling point.[7] Su[8] achieved completed enantioseparation of α-cyclohexylmandelic acid and methyl α-cyclohexylmandelate on an achiral column (ODS3) with HP-β-CD as mobile phase additive by HPLC. The gas chromatographic separation of MA and its derivatives has also been reported. Shi et al[9] found that the separation of enantiomers of methyl mandelate was satisfactory by means of gas chromatography using β-cyclodextrin (CyclodexB, 30 m × 0.25 mm × 0.25 μm). Comparing supercritical fluid chromatography (SFC) with HPLC, first, CO_2 is more easily available and less expensive as the mobile phase of SFC; second, only a small amount of polar modifier is added, which has less impact on the environment and operators[10]; in addition, SFC can expand the range of chromatographic analysis by using a variety of detectors, such as UV detector,[11] electron capture detector, and so forth.[12] This leads to the complementarity in the separation of different target compounds.[13] Supercritical fluid chromatography takes the advantages of short analysis time, fast column equilibration, and simple mobile phase system.[14]

Currently, there are not many reports on the separation of MA and its derivatives by SFC. Medvedovici et al[15] comparatively studied polysaccharide chiral stationary phases (CSPs), Chiralcel OD, Chiralpak AD, and the macrocyclic antibiotic CSPs for the separation of different types of racemic compounds, including MA, by packed column subcritical fluid chromatography. Mangelings[16] evaluated three immobilized chiral polysaccharide-based stationary phases (Chiralpak IA, IB, and IC) in SFC with a set of pharmaceutical racemates, including MA. However, the separate study of the enantiomeric separation of MA and its derivatives on SFC has not been reported.

It must be noted that enantioselective separations in SFC frequently employ temperature below the critical temperature of CO_2 ($T < T_c$), although the main experimental conditions were under its supercritical state. Furthermore, the addition of an organic modifier may lead to subcritical fluid conditions. In other words, SFC sometime was run as subcritical fluid chromatography in fact because of the lower temperature and the addition of organic modifier. However, usually subcritical or supercritical state was not distinguished so strictly in enantioselective separations by SFC or other application of supercritical fluid technique due to the following reasons: (1) The critical temperature and pressure of mixture were difficult to determine, (2) the properties of fluid near critical point changed continuously, and (3) the fluid retains many of the desirable properties of supercritical fluids in spite of the little lower of temperature or the addition of modifier.[17] The term SFC will be utilized throughout this paper although some experiment was carried out at subcritical conditions.

In this work, the enantiomeric separation of six MAs (Figure 1) was studied on the Chiralpak AD-3 column by SFC using supercritical CO_2 as mobile phase. The impacts of volume fraction of trifluoroacetic acid, type and ratio of modifier, column temperature, and backpressure on the separation efficiency were investigated. The effect of enantiomeric separation by SFC was compared with that of traditional HPLC. Besides, the enantiomeric elution order on the Chiralpak AD-3 column was established by analyzing nonracemic samples enriched by the R-enantiomers.

2 | MATERIALS AND METHODS

2.1 | Materials

The organic solvents with HPLC grade-methanol, ethanol, and isopropanol were used as modifier. They were produced by Tianjin Shield Specialty Chemical Ltd. Co (Tianjin, China). CO_2 was of dry-ice grade and purchased from Jingong Specialty Gas Co. Ltd. (Hangzhou, China). The additive trifluoroacetic acid (TFA) was of HPLC grade and purchased from Aladdin Company. The six MAs and their (R)-MA are the original drugs with the purity of above 97.0%. The racemic sample was dissolved in isopropanol to prepare a solution with a mass concentration of about 1000 mg/L for the chromatographic analysis.

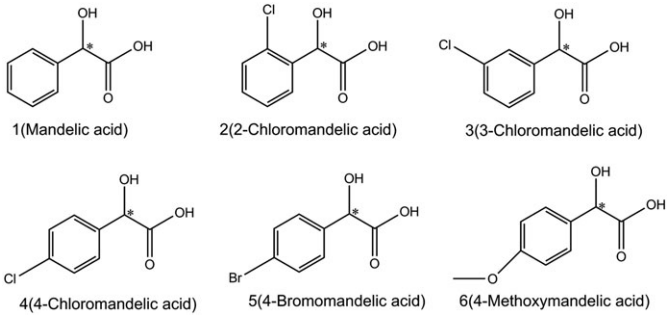

FIGURE 1 Chemical structures of 6 MA compounds in the present study (*denoted chiral center)

2.2 | Instrumentation

The SFC used herein was the Thar SD-ASFC-2 model from Thar Technologies (Pittsburgh, PA, USA) equipped with a Gilson UV/VIS-151 detector (Middleton, WI, USA) and a Rheodyne 7410 injector with a 20 μL loop volume (Cotati, CA, USA). The system was controlled by the software of Thar Instruments Superchrom.

2.3 | Chromatographic conditions

The CSP of the Chiralpak AD-3 column (250 mm × 4.6 mm i.d., 5 μm) is made from an amylose tris (3,5-dimethylphenylcarbamate); the skeleton of the polymeric selector consists of D-glucose units linked by an α-(1,4)-D-glucose linkage, and it is regularly arranged to form a left-handed helix conformation.[18] The 3,5-dimethylphenyl carbamate side chains surround the helical grooves and delimit the chiral environments. Naturally and chemically modified amylose have highly rigid and well-hydrophobic cavity which can serve as a molecular acceptor to recognize a variety of organics, inorganics, biomolecule-forming host guests as well as supramolecular complexes.[19,20] Figure 2 shows the molecular chemical structure of CSP which was purchased from Daicel Chiral Technologies (Shanghai) Co. Ltd. The mobile phase was supercritical CO_2. Different kinds and volume fractions of alcohol modifiers (methanol, ethanol, isopropanol) were added into the mobile phase in order to explore their influences on the chiral separation and to optimize the mobile phase composition.

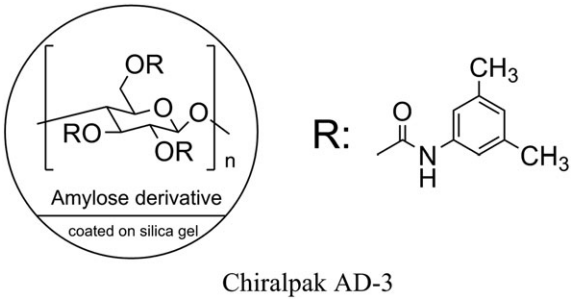

FIGURE 2 Structural formulas of CSPs

The column temperature and the column pressure were changed from 29°C to 41°C and from 13 to 17 MPa respectively. Finally, the optimal separation conditions of all target compounds were determined. According to a large number of preliminary experiments,[21,22] the detection wavelength was set at 220 nm, the flow rate was 2 mL/minute, and injection volume was 10 μL.

The retention factor of *i*th compounds (k_i), the separation factor (α), and the resolution (R_s) are calculated as Equations (1), (2), and (3) respectively.

$$k_i = \frac{t_i - t_0}{t_0} \tag{1}$$

$$\alpha = \frac{k_{i+1}}{k_i} \tag{2}$$

$$R_s = 1.18 \times \frac{t_{i+1} - t}{w_i + w_{i+1}} \tag{3}$$

Here, t_0 is the hold-up time which is the retention time of a nonadsorbing component, t_i is the retention time of the component, and w_i is the peak width at half height of the component.

3 | RESULTS AND DISCUSSION

By exploring different influencing factors, the optimum conditions for the separation of MA compounds could be determined and the related mechanisms could be recognized as well, which might provide an insight for the separation of other enantiomers by SFC.

3.1 | Effect of additive and modifier

3.1.1 | Effect of TFA on the enantioselectivity

Since the MA and its derivatives are rather strong carboxylic acids, they can adsorb onto the silica gel in CSP. This results in the peak type trailing tail, and the data of the peak type fitting are then not accurate. An essential

function of basic as well as acidic additives is to suppress the ionization of strong basic and acidic groups, in order to achieve elution, enantioseparation, and a satisfying peak shape. A small amount of TFA can be added to the mobile phase. As acidic additive, trifluoroacetic acid improves enantioselectivity by enhancing hydrogen bonding interactions between the carboxyl groups of the compounds with carbonyl groups of the carbamate functions of the polysaccharide-based selectors. Addition of TFA to the mobile phase reduces the pH and the ionization of acidic compounds. Thus, the peak trailing can be significantly reduced, the peak broadening can be overcome, the retention time of acidic drugs can be shortened, and selectivity improved drug molecules.

Four analytes were selected to explore the effect of volume fraction of TFA on chiral separation, and the results are shown in Table 1. With the increase of the volume fraction of TFA, the resolution (R_s) presented an increasing trend in general while the retention factors (k_1 and k_2) and the separation factor (α) did not fluctuate significantly. Noting that when the TFA concentration was high, the baseline would become unstable (Figure 3) and the chiral stationary phase might be damaged. Thus, 0.1% of TFA was determined herein.

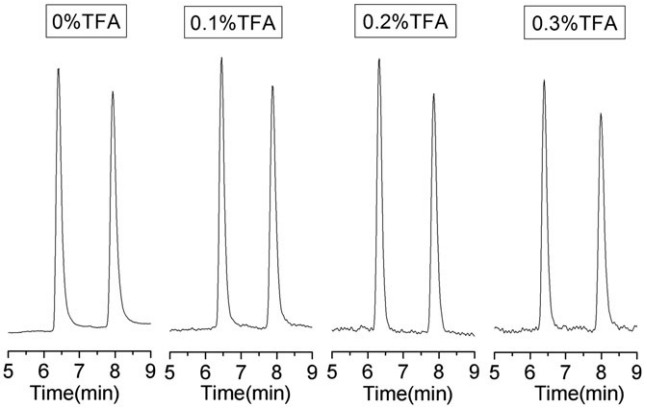

FIGURE 3 Effects of TFA on the base line fluctuation

3.1.2 | Effect of the modifiers on the enantioselectivity

Mandelic acid and its derivatives are known to be of strong molecular polarity whereas the mobile phase CO_2 is a nonpolar substance. Thereby, the attractive interaction between the two cannot be strong. Obviously, it is insufficient to elute with only CO_2 as mobile phase without adding any polar modifier. Methanol, ethanol, and isopropanol used in this study are all proton-type solvents. They are capable of interacting with the stationary phase through hydrogen bonding, competing with the MA compounds for hydrogen bonding sites, and accelerating the elution rate.

For the same modifier, as can be seen from Figure 4, k_1 gradually decreased with increasing the methanol volume fraction. It shows that increasing the volume fraction of modifier accelerated the elution speed, decreasing the retention time. This tendency was pronounced at the initial stage when the modifier volume fraction increased. Methanol is a protic solvent, which is also a proton-donor and proton acceptor. It can occur hydrogen-bonding interaction with the compounds and the chiral stationary phase, thereby competing with compounds for hydrogen bonding sites. Increasing the volume fraction of methanol enhances this competition, reduces the interaction between the compound and CSP, and shortens the retention time.

Figure 5 shows the effect of methanol volume fraction on α. On the whole, the separation factor decreased with the increase of the volume fraction of the modifier. This may be attributed to the fact that the variation degree of k_1 and k_2 changes with the gradual increase of polar component percentage in the mobile phase. The α value is the ratio of two adjacent peak retention factors. Therefore, the change of k value leads to the change of the α value. In essence, the α value indicates the thermodynamic properties of chiral compounds in the distribution equilibrium between the two phases. It reveals the difference

TABLE 1 Effect of TFA on the enantioselectivity of MA and its derivatives

Compd.	TFA content (%)	k_1	k_2	α	R_s	Compd.	TFA content (%)	k_1	k_2	α	R_s
MA	0%	3.28	4.30	1.31	6.08	3-ChloroMA	0%	3.64	4.20	1.15	2.99
	0.1%	3.31	4.26	1.29	5.96		0.1%	3.64	4.18	1.15	3.10
	0.2%	3.22	4.24	1.32	6.70		0.2%	3.60	4.16	1.15	3.21
	0.3%	3.27	4.34	1.33	6.69		0.3%	3.63	4.20	1.16	3.45
2-ChloroMA	0%	4.65	5.20	1.12	2.68	4-Methoxymandelic acid	0%	4.37	4.57	1.05	0.91
	0.1%	4.64	5.16	1.11	2.71		0.1%	4.24	4.50	1.06	1.32
	0.2%	4.55	5.08	1.12	2.84		0.2%	4.36	4.57	1.05	1.15
	0.3%	4.62	5.19	1.12	3.11		0.3%	4.36	4.56	1.05	1.08

Chromatographic condition: Chiralpak AD-3 with the modifier of 12% isopropanol at 35°C column temperature and 15 MPa backpressure.

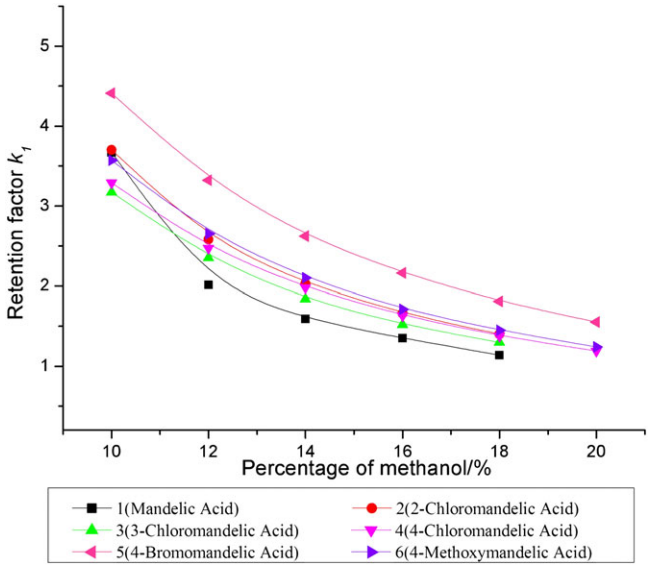

FIGURE 4 Effect of methanol percentage on the retention factor k_1

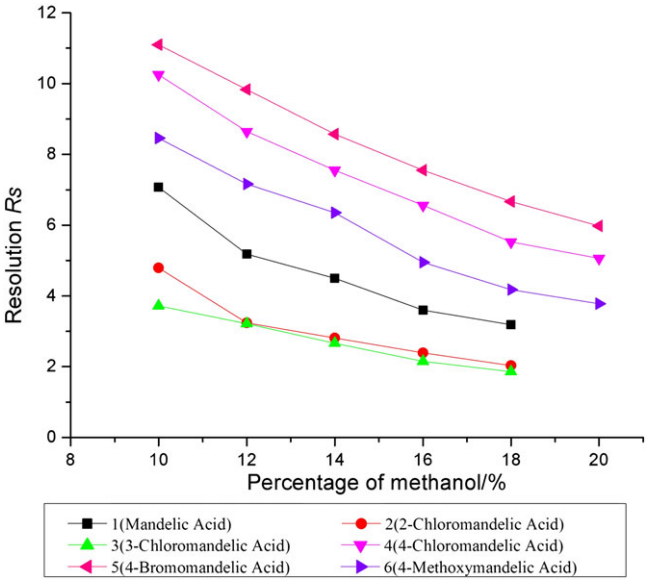

FIGURE 6 Effect of methanol percentage on the resolution R_s

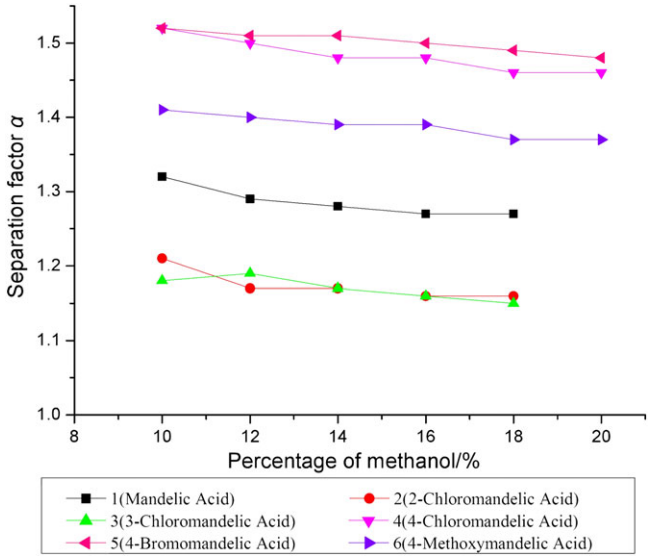

FIGURE 5 Effect of methanol percentage on the separation factor α

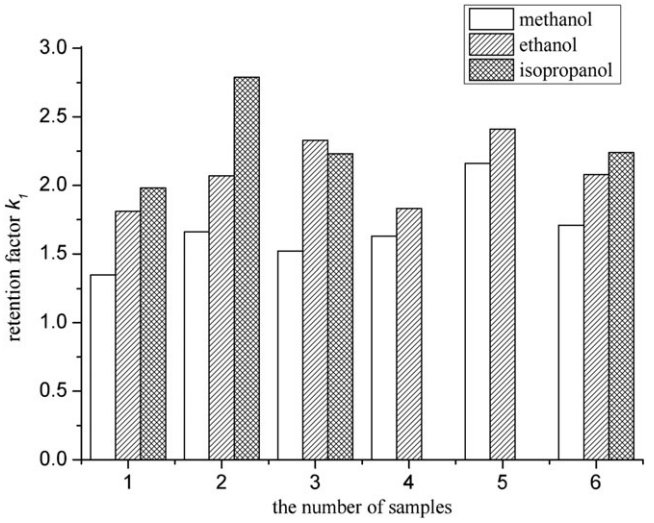

FIGURE 7 Effect of the modifiers on the retention factor k_1

in the interaction force between molecules. That is to say, with the increase of modifier volume fraction, the difference in thermodynamic properties and enantioselectivity of the separated enantiomers turned to be smaller when the distribution equilibrium was achieved.

As can be seen from Figure 6, the resolution (R_s) decreased gradually with the increase of methanol volume fraction since larger volumes of modifier led to the stronger interaction between methanol and the chiral stationary phase. The competition for the adsorption sites on the chiral stationary phase became intensified, and therefore, the retention time of the compounds got shortened. Adequate and effective desorption and adsorption could not be achieved between the enantiomer and CSPs, and thus, the separation of the two enantiomers was inefficient and R_s decreased.

Different modifiers have different effects on chiral separation, due to their differences in polar and steric hindrance. In this paper, we investigated the effect of different modifier species (methanol, ethanol, isopropanol) on chiral separation of MA and its five derivatives. The retention factor k_1, the separation factor α, and the resolution R_s were obtained under the modifier addition of 16% (volume fraction) and listed in Figures 7, 8, and 9 respectively. It should be noted that 4-chloromandelic acid and 4-bromomandelic acid cannot be successfully separated under these isopropanol conditions.

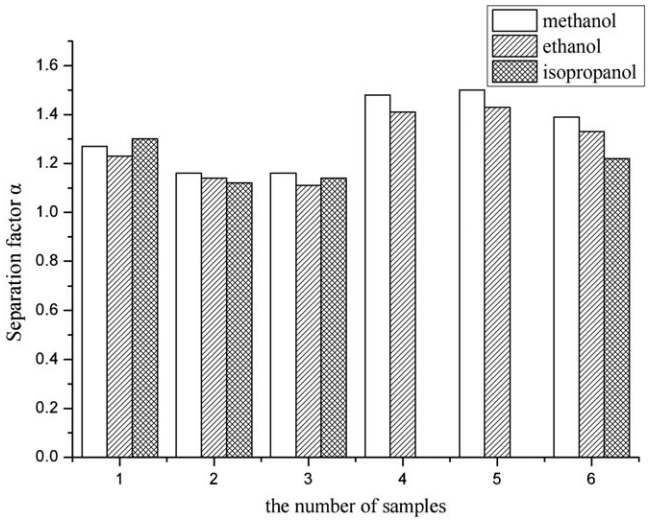

FIGURE 8 Effect of the modifiers on the separation factor α

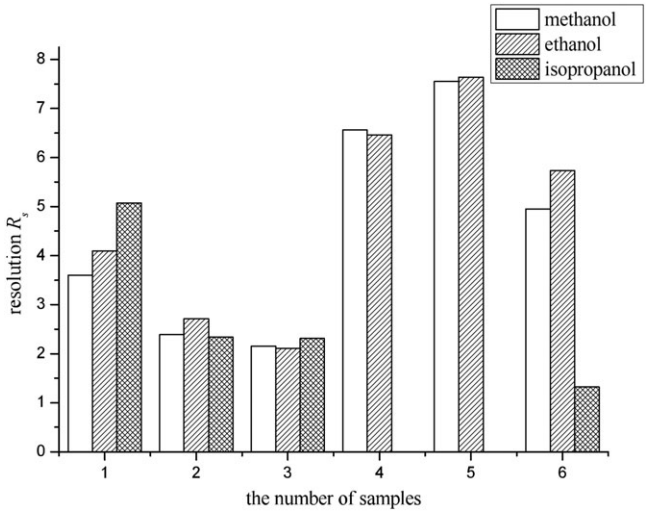

FIGURE 9 Effect of the modifiers on the resolution R_s

As shown in Figure 7, except 3-ChloroMA, the retention factor of MA and its four derivatives increased in the order of methanol, ethanol, and isopropanol for the fixed volume ratio of modifier. The polarity of the three modifiers increased in the order of isopropanol, ethanol, and methanol. The hydrogen bonding strength of the three modifiers to CSP obeyed the same sequence. The chain of isopropanol is more branched than that of methanol and ethanol. Therefore, the hydrogen bonding of isopropanol is much weaker, and the elution rate is much slower. In addition, the molecular volume of the modifier was in the order of isopropanol, ethanol, and methanol. The steric hindrance of the modifier with large molecular volume was larger, and therefore, the attractive interaction between the solvent and the chiral column decreased and the elution ability of the mobile phase reduced. This caused the increase of retention time of enantiomers on the chiral stationary phase.

As shown in Figures 8 and 9, the variation of α and R_s with the modifier has no obvious regularity. This may be because the chiral recognition mechanisms of modifiers used herein to the selected drugs are complex, being affected not only by the polarity of modifier and the molecular volume factors but also associating with the structures of MA compounds. Methanol and ethanol always performed better in the elution of MA and its derivatives than isopropanol. The change of retention factor is basically in accordance with that of the polarity of modifier and the space resistance. In conclusion, methanol usually provided the largest success rate and specificity, which agreed well with the report of Syame Khater.[23]

4 | EFFECT OF THE COLUMN TEMPERATURE

The temperature of chiral chromatographic column importantly influences the separation process of chiral compounds by SFC, changing retention factor, separation factor, and resolution. Given that the volume fractions of modifier were distinct under the preferable separation conditions, thus the separation conditions for this part of experiments were set to be consistent (0.1% TFA, methanol as modifier, 15 MPa). The column temperature ranged from 29°C to 41°C (every 3°C for a gradient), and the impact of column temperature on the separation of MA and its five derivatives was then investigated. The results of enantiomeric separation chromatography at different chiral column temperatures were summarized in Table 2. It can be seen that with the increase of temperature, the retention factor k_1 and k_2 fluctuated while the separation factor α and the resolution R_s decreased. Changing the separating temperature at constant pressure may lead to significant changes of the fluid density. On increasing temperature, a decrease in fluid density, and thus an increase in retention, is usually observed. However, higher temperature can also cause desorption of both $scCO_2$ and modifier from the stationary phase that leads to a decrease in retention.[24] Temperature and density are the main factors affecting viscosity. Both the intermolecular collisions and collisions in the process of molecular free translation can all cause momentum transfer. The combined effects of momentum transfer caused by these two collisions can reflect fluid viscosity. Temperature and density affect the momentum transfer mode, which changes the viscosity of the fluid. Typically, the viscosity of the liquid decreases with increasing temperature; supercritical fluid under high-density conditions, the viscosity decreases with increasing temperature; At low-density conditions, the result is opposite. The temperature of 35°C was appropriate in this

TABLE 2 Separation results of MA and its five derivatives at different column temperatures

Number	MA and its derivatives	Modifier	Column temperature (°C)	k_1	k_2	α	R_s
1	MA	14% Methanol	29	1.08	1.50	1.40	3.15
			32	1.91	2.59	1.35	3.74
			35	1.68	2.20	1.31	2.94
			38	1.65	2.15	1.30	2.82
			41	1.57	1.99	1.26	2.32
2	2-ChloroMA	14% Methanol	29	2.10	2.83	1.35	3.87
			32	2.86	3.69	1.29	3.98
			35	2.61	3.24	1.25	2.76
			38	2.63	3.26	1.24	3.03
			41	2.58	3.09	1.20	2.38
3	3-ChloroMA	10% Methanol	29	3.03	3.67	1.21	3.84
			32	3.00	3.63	1.21	3.86
			35	3.39	4.05	1.20	3.79
			38	3.10	3.70	1.19	2.73
			41	3.00	3.58	1.19	3.58
4	4-ChloroMA	20% Methanol	29	1.18	1.81	1.53	3.55
			32	1.13	1.72	1.52	3.59
			35	1.11	1.68	1.51	3.62
			38	1.06	1.58	1.49	3.60
			41	1.04	1.53	1.48	3.52
5	4-BromoMA	20% Methanol	29	1.38	2.64	1.91	7.59
			32	1.52	2.81	1.85	7.44
			35	1.46	2.61	1.79	6.38
			38	1.46	2.56	1.75	5.88
			41	1.34	2.32	1.72	5.28
6	4-MethoxyMA	20% Methanol	29	1.26	2.05	1.62	5.44
			32	1.37	2.16	1.58	5.03
			35	1.32	2.05	1.55	4.26
			38	1.24	1.90	1.54	4.23
			41	1.25	1.88	1.50	3.72

T = 35°C, 0.1% TFA, on Chiralpak AD-3.

experiment since CO_2 would not reach supercritical state at lower temperatures and the chiral separation process could be hindered at higher temperatures. He[25] reported the enantioseparation of triticonazole by SFC. With the increase of temperature, retention factors, selectivity, column efficiency, and resolution kept stable. It meant that the enantioseparation of triticonazole might be temperature-insensitive under the studied experimental conditions. The separation performance may also be related to the chemicals themselves.

To further investigate the effect of column temperature, the thermodynamic behavior of enantiomeric separation was studied.[26,27] The effect of temperature on the chiral separation process was interpreted according to the van't Hoff equation.

$$\ln k_i = \frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln\phi = -\frac{\Delta H}{RT} + \Delta S^*$$

$$-\Delta\Delta G^\circ = RT\ln\alpha = \ln\left(\frac{k_{i+1}}{k_i}\right) = -\Delta\Delta H^\circ + T\Delta\Delta S^\circ$$

$$\ln\alpha = -\frac{\Delta\Delta H^\circ}{RT} + \frac{\Delta\Delta S^\circ}{R}$$

Here, k_i is the retention factor, α is the separation factor, R is the universal gas constant, T is the absolute temperature, and Φ is phase ratio. ΔH and ΔS are the free enthalpy and entropy of the interaction between each enantiomer and the chiral stationary phase respectively. $\Delta\Delta G°$ is the Gibbs free energy, while $\Delta\Delta H°$ and $\Delta\Delta S°$ are the differences of the free enthalpy and free entropy between two enantiomers respectively. When the $-\Delta\Delta H°/RT$ plays a dominant role or $\Delta\Delta G°$ is governed by the enthalpy, the separation process of enantiomers is called "enthalpy driven." In other words, the separation factor decreases as the temperature increases and an

enthalpy driven occurs. In contrast, an entropy-driven separation occurs if $\Delta\Delta S°/R$ governs the value of $\Delta\Delta G°$ and the separation factor increases as the temperature increases. When the peaks were coeluted because of the balance between $-\Delta\Delta H°/RT$ and $\Delta\Delta S°/R$, the isoenantioselective temperrature (T_{iso}) occurs. T_{iso} can be calculated through the following equation:

$$T_{iso} = \frac{\Delta\Delta H^{\circ}}{\Delta\Delta S^{\circ}}$$

The results of fitting $\ln\alpha - 1/T$ plots were analyzed using the van't Hoff formula to calculate the thermodynamic parameters $\Delta\Delta H°$, $\Delta\Delta S°$, and T_{iso} for chiral separation of enantiomers, which are listed in Table 3.

TABLE 3 The thermodynamic parameters of MA and its five derivatives

Number	MA and its derivatives	Fitting equation	$\Delta\Delta H$ (kJ/mol)	$\Delta\Delta S$ (J/mol/K)	T_{iso} (K)
1	MA	$y = 766.3x - 2.209$	−6.37	−18.37	346.9
2	2-ChloroMA	$y = 892.6x - 2.665$	−7.42	−22.16	334.9
3	3-ChloroMA	$y = 133.9x - 0.2529$	−1.11	−2.10	529.5
4	4-ChloroMA	$y = 298.5x - 0.554$	−2.48	−4.61	538.8
5	4-BromoMA	$y = 842.3x - 2.145$	−7.00	−17.83	392.7
6	4-MethoxyMA	$y = 606.7x - 1.528$	−5.04	−12.70	397.1

TABLE 4 Separation results of MA and its five derivatives under different backpressure

Number	MA and its derivatives	Modifier	Pressure (MPa)	k_1	k_2	α	R_s
1	MA	14% Methanol	13	1.90	2.46	1.29	6.04
			14	1.75	2.23	1.28	5.35
			15	1.59	2.11	1.32	5.17
			16	1.47	2.02	1.38	5.13
			17	1.38	1.96	1.42	5.10
2	2-ChloroMA	14% Methanol	13	2.31	2.71	1.18	3.35
			14	2.17	2.53	1.16	2.95
			15	2.03	2.37	1.17	2.81
			16	1.91	2.24	1.17	2.73
			17	1.78	2.10	1.18	2.68
3	3-ChloroMA	10% Methanol	13	3.54	4.21	1.19	4.36
			14	3.33	3.94	1.18	4.03
			15	3.17	3.74	1.18	3.72
			16	3.04	3.55	1.17	3.36
			17	2.86	3.35	1.17	3.23
4	4-ChloroMA	20% Methanol	13	1.58	2.20	1.39	5.66
			14	1.38	1.95	1.41	5.26
			15	1.19	1.74	1.46	5.06
			16	1.03	1.53	1.48	4.69
			17	0.90	1.27	1.40	3.08
5	4-BromoMA	20% Methanol	13	1.88	2.67	1.42	6.29
			14	1.71	2.48	1.45	6.12
			15	1.55	2.31	1.48	5.98
			16	1.44	2.18	1.51	5.89
			17	1.37	2.11	1.54	5.63
6	4-MethoxyMA	20% Methanol	13	1.41	1.98	1.40	4.64
			14	1.31	1.83	1.40	4.28
			15	1.24	1.70	1.37	3.78
			16	1.18	1.62	1.37	3.63
			17	1.17	1.54	1.32	3.07

T = 35°C, 0.1% TFA, on Chiralpak AD-3.

The highest temperature (41 °C) in this study was much lower than T_{iso} of the enantiomers of the MA and its five derivatives. The chiral recognition process was mainly enthalpy driven, and the separation factor α decreased with temperature increasing. This was consistent with the fact that the separation factor of enantiomers of chiral drugs varied with temperatures in this study.

5 | EFFECT OF BACKPRESSURE

The density of mobile phase is proportional to pressure which may influence the component distribution coefficient. For the chiral resolution by supercritical CO_2 chromatography, the pressure range is narrow, basically between 15 and 20 MPa. Under the optimum separation condition, the effect of backpressure on MA and its five derivatives was investigated. Herein, the backpressure of the SFC system ranged from 13 to 17 MPa and a gradient was set to be 1 MPa.

It can be seen from Table 4 that, when the backpressure increased, the retention factors of MA compounds k_1 decreased gradually, and the trend turned to be obvious at low backpressures. It indicates that the increase of backpressure accelerated the speed of elution and reduced the retention time. This could be interpreted by the fact that the increase of backpressure led to the increase of the overall density of the mixed mobile phase as well as the enhancement of the solvation ability. The variation of retention factors is similar to the report of Tarafder.[28] With the increase of the system backpressure, the separation factor α remained within a certain range whereas the resolution R_s decreased. Considering the factors, including peak speed, separation degree, and instrument maintenance, herein, 15 MPa was chosen for the chiral separation of MA and its derivatives.

6 | OPTIMUM SEPARATION RESULTS

The resolution results under different conditions show that all the MA and its five derivatives achieved the baseline separation within 7 minutes ($R_s > 1.5$). The SFC separation method should be optimized based on the principle of relatively fast separation time and high tolerance of instruments and CSP chiral column. The racemic standards and the pure R-enantiomers were tested separately (one chromatogram of 4-bromo mandelic acid shown in Figure 10), and qualitative analysis was performed according to the retention times of the two enantiomers to determine the enantiomer

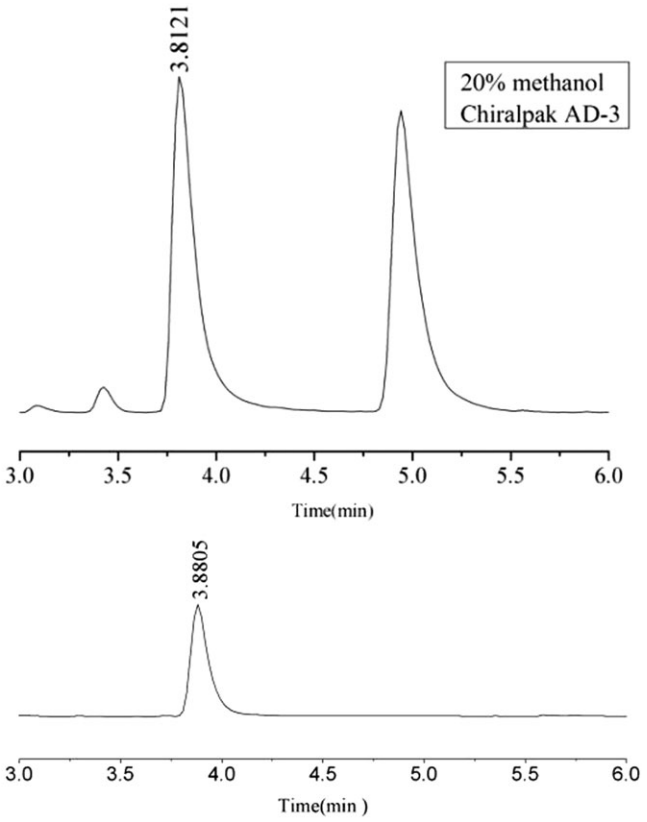

FIGURE 10 The chromatograms of rac-4-bromomandelic acid and R-4-bromomandelic acid in standard solutions

elution order, then mark on the chromatogram under the optimum conditions (Figure 11). The results in this study on the MA and its five derivatives were compared with those reported in literature.[29] Under the same chiral column conditions, SFC resolved all the studied compounds and the HPLC only succeed for two-thirds of the compounds. The SFC method exhibited faster and more efficient separation with better enantioselectivity than the HPLC method (Table 5). As a separating technique,[30] SFC has a cost advantage over normal phase HPLC in terms of (1) the cost of CO_2 versus the cost of normal phase solvents (hexane, heptane, isooctane, ect), (2) the significant cost reduction in handling waste, (3) the improved efficiency of isolating the active pharmaceutical ingredient from the mobile phase after collection, (4) the ease of using SFC as a normal phase system, and (5) SFC arise from the reduced viscosities and increased diffusivities of supercritical fluids when compared to liquids. The reduced viscosity decreases the pressure drop across the column. Increased diffusivity shifts the optimum linear velocity to higher values so that higher flow rates can be used without compromising efficiency.

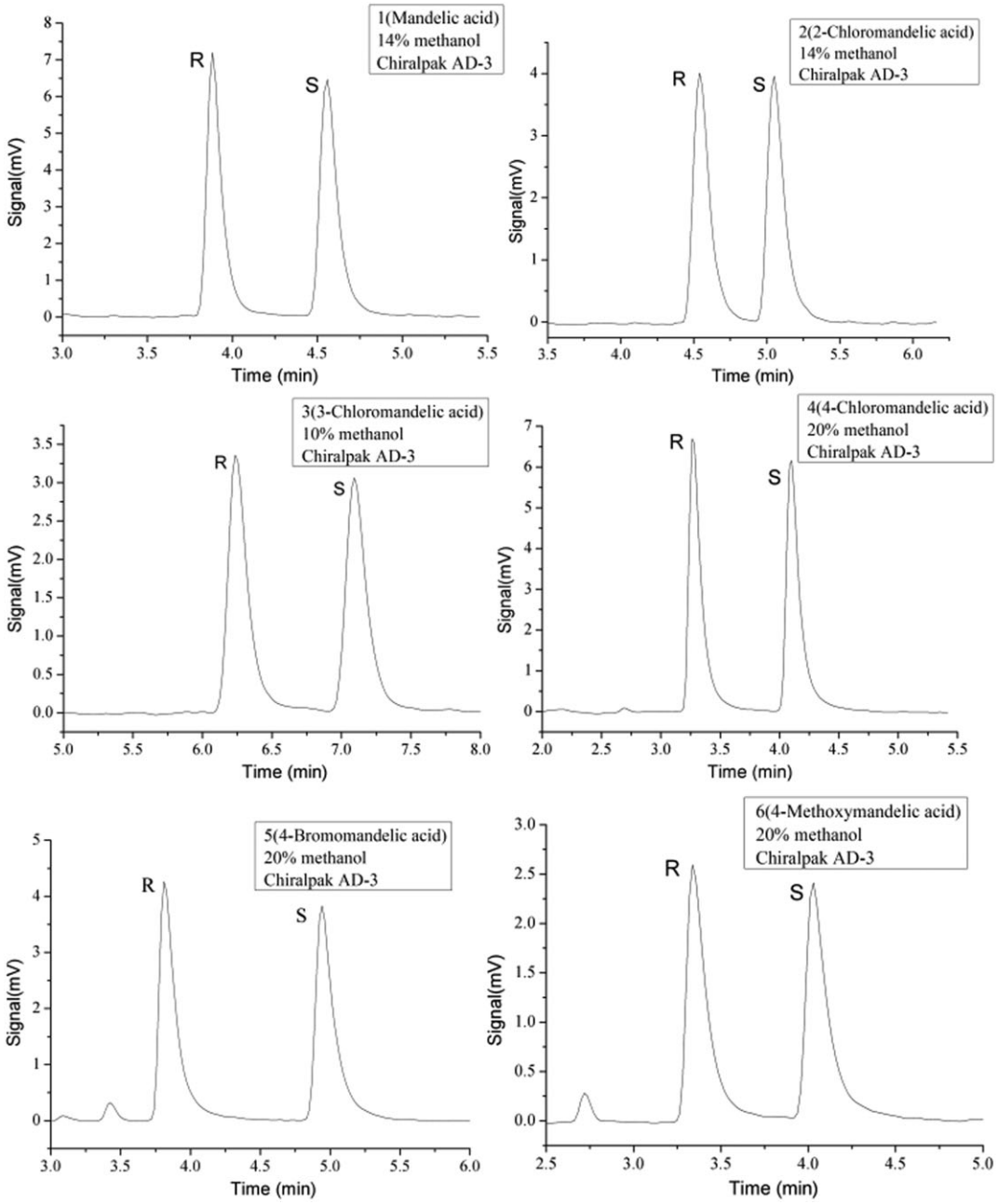

FIGURE 11 SFC chromatograms of MA and its 5 derivatives under optimal separation condition P = 15 MPa, 0.1% TFA, T = 35°C

TABLE 5 Comparison for the enantioseparation of 6 MAs by SFC and HPLC

Number	MA and its derivatives	t_2		α		R_s	
		SFC	HPLC	SFC	HPLC	SFC	HPLC
1	MA	4.6	12.2	1.28	1.20	4.50	2.86
2	2-ChloroMA	5.1	12.9	1.17	1.10	2.81	1.60
3	3-ChloroMA	7.1	19.0	1.18	1.12	3.72	1.83
4	4-ChloroMA	4.1	10.1	1.46	1.00	5.06	0
5	4-BromoMA	5.0	11.0	1.48	1.00	5.98	0
6	4-MethoxyMA	4.0	46.0	1.37	1.10	3.78	2.07

Chiral columns are Chiralpak AD-3.

7 | CONCLUSION

This study explored the enantiomeric separation of MA and its five derivatives on the Chiralpak AD-3 column by SFC using supercritical CO_2 and various organic modifiers. The effects of volume fraction of TFA, type and percentage of mobile phase, column temperature, and backpressure on the separation efficiency were investigated. The results can be summarized as follows:

1 TFA could effectively solve the problem of peak tailing. Modifier species and volume fraction greatly affected the enantioseparation process. Increasing the volume fraction of modifier facilitated the elution of the components and thus decreased the retention time. The tendency was pronounced when the volume fraction began to increase. As the volume fraction increased, both of α and total R_s of MA and its five derivatives showed decreasing trend. When the volume fraction was fixed to be 16%, k_1 of MA and its derivative increased following the order of modifiers: isopropanol > ethanol > methanol. The values of α and R_s did not change significantly with the type of modifier.
2 The retention factors k_1 and k_2 fluctuated with temperatures. The separation factor and resolution decreased when the column temperature increased. According to the van't Hoff equation, the chiral separation process of all MA and its five derivatives was enthalpy driven. The temperature of 35°C was appropriate since CO_2 would not reach supercritical state at lower temperatures and the chiral separation process could be hindered at higher temperatures.
3 The backpressure could affect the enantioseparation process. As the backpressure increased, a corresponding decrease in retention factor was observed. The separation factor did not suffer great fluctuation.
4 The desired enantioseparation efficiencies of six mandelic acids on the AD-3 column were achieved by adding 0.1% TFA and methanol as modifier within 7 minutes at the column temperature of 35°C and the backpressure of 15 MPa. In addition, the enantiomer elution order was determined by comparing the chromatograms of MA enantiomers and the pure R-enantiomers.

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