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Enantiomer separation of flavour and fragrance compounds by liquid chromatography using novel urea-covalent bonded methylated β-cyclodextrins on silica

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Abstract

A novel methylated β-cyclodextrin chiral stationary phase (CSP-ME), which was chemically immobilised onto porous silica via multiple urea-linkages was synthesised. The CSP-ME chiral stationary phase depicted good enantiomer separation abilities for some well-known flavour as well as fragrance compounds using high-performance liquid chromatography under reverse phase conditions. The optimum resolution for α-ionone, 3-methyl-α-ionone, flavanone, 5-methoxyflavanone, 6-methoxyflavanone, 7-methoxyflavanone, hesperetin, naringenin and taxifolin was achieved using a mobile phase composition consisting of 1 wt.% triethylammonium acetate buffer (pH 4.68)–methanol. The effects of pH of triethylammonium acetate buffer and the methanol–acetonitrile content of the mobile phase composition on their retention time and resolution were examined to optimise the separation conditions.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Flavanones; Ionones; β-Cyclodextrin

1. Introduction

Chiral compounds from natural origins usually exist as one predominant enantiomer. Through the inspection of enantiomeric ratios, one can characterise the regional differences between oils. Although sometimes as a result of processing, the presence of racemic pairs most often indicates adulteration or unnatural origin. For many years, chiral capillary gas chromatography has proven to be a convenient method for characterizing essential oils and for differentiating natural flavours from those of synthetic origin [1,2]. Currently, the enantioseparation of many fragrance compounds was commonly performed using gas chromatography and there are very few literature reports on high-performance liquid chromatography separation [3–8].

Cyclodextrins and their derivatives are widely used in gas chromatography, capillary electrophoresis and high-performance liquid chromatography for the separation of chiral compounds [9,10]. The hydroxyl groups of the cyclodextrins can be modified and such derivatizations can have great impact on the selectivities towards racemates [11,12]. Moreover,

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the methods for chemical immobilization of derivatised cyclodextrin onto polymeric support have been extensively studied since 1983 [13] to investigate the effects of different covalent linkers on chiral separation. Accordingly, our research is focused primarily on chemical immobilization of functionalised β-cyclodextrin CSP [14,15], heptakis(6-azido-6-deoxy-2,3-di-*O*-methyl)-β-cyclodextrin onto the surface of aminated silica gel via Staudinger reaction [16]. The derived CSP-ME possesses crosslinked multiple urea bonds which are highly stable in acidic media as well as under mobile phase compositions with high aqueous content.

In this paper, we describe how to develop and optimise a particular chiral separation for neutral and acidic flavour compounds using the ureido-bonded methylated-β-cyclodextrin based (CSP-ME) chiral stationary phase under reverse phase separation conditions.

2. Experimental

2.1. Synthesis of ureido-bonded methylated β-cyclodextrin (CSP-ME)

The synthesis of ureido-bonded methylated methylated β-cyclodextrin (CSP-ME) was performed according to the schematic route depicted in Scheme 1.

*2.1.1. Synthesis of heptakis(6-iodo-6-deoxy)-β-cyclodextrin, **1***

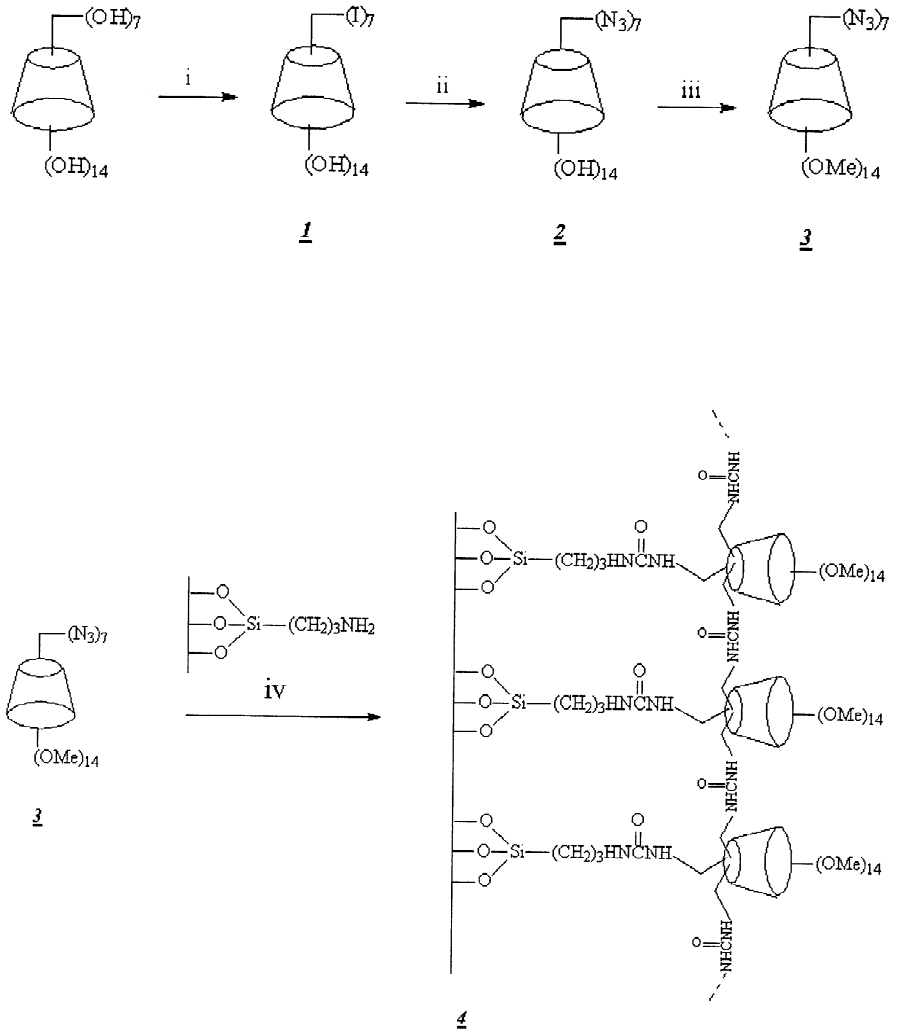
Iodine (10.1 g) and triphenylphosphine (10.5 g) were dissolved in 40 ml dry DMF, after which 2.1 g of dry β-CD (heated at 120 °C, 8 h under high vacuum) was added and stirred overnight at 90 °C. The excess DMF (20 ml) was removed under vacuum and the reaction mixture was adjusted with 3 *M* NaOMe until pH around 9–10 using an external ice-bath. The precipitated brown-red solid was filtered and washed with a large amount of acetone to afford about 3.1 g yellow-brown solid in high yield of 90%. Elemental analysis $(C_6H_9O_4I)_7$: Calculated values: C: 26.46%, H: 3.31%. Determined values: C: 27.11%, H: 2.96%; IR (cm^{-1}): 3366 (O–H str), 2914 (C–H str), 1038 (C–O str); ^{1}H NMR (DMSO, TMS) δ (ppm): 6.2–5.6 (14H), 5.1–4.9 (7H), 4.0–3.5 (42H); melting point: 205.7 °C, [α]: +180° (c: 0.05, DMSO).

*2.1.2. Synthesis of heptakis(6-azido-6-deoxy)-β-cyclodextrin, **2***

Iodinated β-cyclodextrin, **1** (3.2 g) in 50 ml DMF, was stirred with 3.2 g of sodium azide under 95 °C for overnight. DMF was removed at low temperature at about 50–60 °C, and the mixture was poured into water. The solids were filtered followed by washing with a large amount of water to remove sodium azide. Solid (**2**) obtained was dried under vacuum (yield: 89.5%). Elemental analysis $(C_6H_9N_3O_4)_7$: calculated values: C: 38.47%, H: 4.809%, N: 22.44%. Determined values: C: 37.90%, H: 4.850%, N: 22.11%; IR(cm^{-1}): 3367 (O–H str), 2925 (C–H str), 2106 (N_3 str), 1053 (sym C–O–C str); ^{1}H NMR (DMSO, TMS) δ (ppm): 5.9–5.7 (14H); 5.0–4.9 (7H); 4.0–3.5 (42H); melting point: 220.4 °C; [α]: +240° (c: 0.05, DMSO).

*2.1.3. Synthesis of heptakis(6-azido-6-deoxy-2,3-di-o-methyl)-β-cyclodextrin, **3***

Heptakis(6-azido-6-deoxy)-β-cyclodextrin, **2,** (2.12 g, 1.83 mmol) was dissolved in DMF (c.a. 35 ml). Next, 0.646 g (27.00 mmol) of sodium hydride (55–60% in paraffin) was washed with *n*-hexane to remove the paraffin. The dissolved **2** was then added to the sodium hydride, with a vigorous stirring. After the vigorous reaction ceased, methyl iodide (4.00 ml, 28.2 mmol) was added slowly to the reaction mixture and stirred for 12 h. After the concentration and filtering of the reaction mixture, ethyl acetate (200 ml) was added to extract the product. The sodium iodide formed in the reaction mixture was removed by washing with brine (3×50 ml). The organic layer was separated and dried over anhydrous sodium sulfate. The solution was concentrated and purified by flash chromatography over silica gel using ethyl acetate–acetone (1:1) as eluent, yielding heptakis(6-azido-6-deoxy-2,3-di-*O*-methyl)-β-cyclodextrin, **3** (yield: 80.9%). Elemental analysis $(C_8H_{13}N_3O_4)_7$: calculated values: C: 44.65%, H: 6.04%. Determined values: C: 44.64%, H: 5.81%; IR (cm^{-1}): 2923 (C–H str), 2107 (N_3 str), 1055 (sym C–O–C str); ^{1}H NMR ($CDCl_3$, TMS) δ (ppm): 4.90 (H-1), 3.30



Scheme 1. Synthesis scheme for urea-bonded methylated β-cyclodextrin and reaction conditions: (i) iodine, triphenylphosphine, dimethylforamide, 90 °C; (ii) sodium azide, dimethylforamide, 95 °C; (iii) sodium hydride, methyl iodide and (iv) tetrahydrofuran, carbon dioxide and triphenylphosphine.

(H-2), 3.70 (H-3), 3.50 (H-4), 3.55 (H-5), 3.70 (H-6), 3.57 (CH_3-2), 3.67 (CH_3-3).

2.1.4. Preparation of amino-functionalised silica gel

The amino-functionalised silica gel was prepared by refluxing 3-aminopropyltriethoxysilane and silica gel in toluene for 15 h. The aminised silica gel obtained was washed with acetone in soxhlet apparatus for 24 h to remove excessive 3-aminopropyltriethoxysilane completely. Elemental analysis: C, 2.76%; H, 0.12%; N, 1.35%.

*2.1.5. Immobilization of functionalised β-CD onto silica gel (**4**)*

The dry amino-functionalised silica gel (4.50 g) was stirred in anhydrous tetrahydrofuran (15 ml) and carbon dioxide was then bubbled into the mixture. After 5 min, the solution of compound **3** (2.30 g) in 5 ml of tetrahydrofuran was added. About 20 min later, another solution of triphenylphosphine (1.40 g)

in 5 ml of tetrahydrofuran was added. The reaction mixture was stirred overnight with continuous bubbling of carbon dioxide at room temperature. The silica gel was filtered off and extracted with acetone for 24 h. The chemically bonded chiral stationary phase, (CSP-ME) **4** was obtained, after the removal of acetone. Elemental analysis: C, 17.69%; H, 2.95%; N, 2.83%.

2.1.6. Preparation of mobile phase

Triethylammonium acetate buffers were prepared by using 1 wt.% of triethylamine solutions, which were adjusted by adding glacial acetic acid to the desired pH. The mobile phase, consisting of triethylammonium acetate buffer and the appropriate amount of the organic modifier, were freshly prepared, filtered, and degassed. The analytical column 250 mm length×4.6 mm I.D. containing CSP-ME was to allowed to equilibrate in order to obtain good reproducible results.

2.2. Instruments

Nuclear magnetic resonance spectroscopy was carried out on a Bruker ACF300FT-NMR spectrometer. Fourier transform infra-red spectroscopy was performed on Bio-Rad TFS156 instrument with KBr pellets. Elemental analysis was determined on a Perkin-Elmer 2400CHN analyzer. Melting point determination was conducted on BüCHI capillary melting point apparatus.

Evaluation of CSP-ME column was performed using a HPLC system, which comprised a Shimadzu HPLC system SPD-10AV, a Shimadzu UV/VIS detector and a Shimadzu Autosampler. All chromatographic experiments were carried out at room temperature at about 23 °C. The UV absorbance detection was performed at 254 nm, and the flow rate of the mobile phase was kept constant at 0.5 ml/min.

2.3. Chemicals

β-Cyclodextrin and 3-aminopropyl-triethoxysilane were purchased from Fluka. Iodine and triphenylphosphine were purchased from Sigma while sodium azide was obtained from Merck. *N*,*N*-Dimethylformamide (DMF) was distilled over calcium hydride before use. The silica gel used was purchased from Kromasil, 100-5-SIL, (particle size 5 μm, pore size 100 Å, surface area 306 m^2/g). Carbon dioxide (99.9% pure) was purchased from National Oxygen Pte Ltd, Singapore. Organic solvents used to prepare CSP were of analytical grade and used for packing of HPLC column. HPLC grade solvents were used for performing chromatographic separations on the HPLC. All racemic compounds were obtained from Aldrich-Sigma Chemical Company or Sino Chemical Company.

3. Results and discussion

3.1. Influence of mobile phase composition on enantiomeric separation of flavour and fragrance compounds

The enantioseparation of some well-known flavour and fragrance compounds was performed using a novel ureido-bonded methylated β-cyclodextrin (CSP-ME) as the chiral selector. The choice of an appropriate eluent for each type of analyte is the most important part of method development and optimization.

Chromatographic separation was first attempted using methanol/water mobile phase composition, since methanol is commonly regarded as the weakest displacer of analytes from the CD-cavity. The ratio of methanol/water volume ratio composition was varied from 60:40 (condition 1) to 50:50 (condition 2) and 40:60 (condition 3). The separation factor, α and resolution R_s of various analytes under different separation conditions are shown in Table 1. All of the following analytes consisting of alpha-ionone, 3-methyl-alpha-ionone, 5-methoxy-flavanone, 6-methoxy-flavanone, 7-methoxy-flavanone, flavanone and hesperetin were effectively separated using methanol/water mobile phase composition, with α values ranging from 1.08 to 1.88 and R_s values ranging from 0.53 to 3.62. Out of these nine analytes, alpha-ionone, 3-methyl-alpha ionone and 7-methoxy-flavanone are separated with R_s values greater than 1.5.

From the plot of R_s values for alpha-ionone and 3-methyl-alpha ionone versus the mobile phase

Table 1
Chromatographic enantiomeric separation results of alpha-ionone, 3-methyl alpha-ionone, flavanone, 5-methoxyflavanone, 6-methoxyflavanone, 7-methoxyflavanone, naringenin, hesperetin and taxifolin on CSP-ME at constant flow rate of 0.5 ml/min, detection wavelength of 254 nm and at different separation conditions (v/v): condition 1: methanol/water (60/40); condition 2: methanol/water (50/50); condition 3: methanol/water (40/60); condition 4: methanol/water (30/70); condition 5: methanol/buffer pH 5.0 (40/60); condition 6: methanol/buffer pH 5.0 (50/50) and condition 7: methanol/buffer pH 4.68 (30/70)

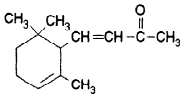
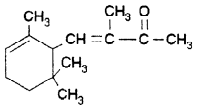
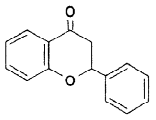
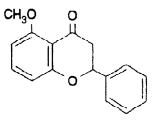
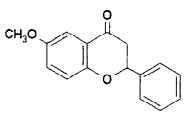
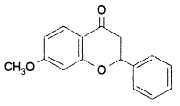
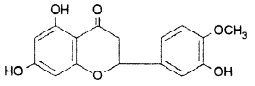
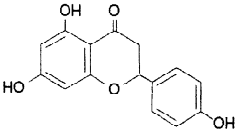
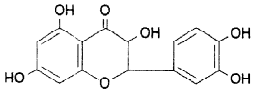
Compounds	Chromatographic separation results						Separation conditions
	k'_1	k'_2	α	R_s	t_1 (min)	t_2 (min)	
Alpha-ionone	0.66	0.94	1.41	1.42	10.01	11.86	1
	1.87	2.75	1.33	2.54	15.34	20.14	2
	3.88	6.11	1.57	3.07	29.55	42.66	3
	8.66	14.11	1.62	3.62	58.13	91.06	4
	3.82	6.00	1.56	3.21	27.52	39.52	5
3-Methyl alpha-ionone	0.77	1.38	1.78	3.14	10.91	14.28	1
	2.00	3.50	1.75	3.37	17.90	27.06	2
	5.44	9.77	1.79	3.54	38.24	65.08	3
	12.67	23.88	1.88	3.61	82.29	148.9	4
	4.75	8.56	1.80	4.06	35.18	58.31	5
Flavanone	2.05	2.22	1.08	0.66	18.24	19.23	1
	4.38	4.77	1.08	0.73	32.31	34.69	2
	10.33	11.44	1.10	1.00	68.12	74.27	3
	21.22	23.44	1.10	0.91	133.1	146.3	4
	8.86	9.73	1.09	0.96	58.57	63.64	5
5-Methoxyflavanone	1.50	1.67	1.11	0.75	15.00	15.90	1
	3.05	3.38	1.10	0.85	24.21	26.28	2
	7.11	8.00	1.13	1.06	48.44	53.86	3
	6.12	6.87	1.12	1.09	41.33	45.76	5
6-Methoxyflavanone	2.11	2.33	1.10	0.72	18.78	20.17	1
	4.61	5.22	1.13	1.00	33.78	37.20	2
	11.55	13.11	1.13	1.03	75.26	84.49	3
	25.00	28.55	1.14	1.01	156.2	177.7	4
	9.37	10.50	1.12	1.12	60.12	66.60	5

Table 1. Continued

Compounds	Chromatographic separation results						Separation conditions
	k_1'	k_2'	α	R_s	t_1 (min)	t_2 (min)	
7-Methoxyflavanone	2.22	2.50	1.12	1.00	19.45	21.31	1
	4.94	5.66	1.14	1.23	35.64	39.94	2
	12.55	14.55	1.16	1.38	81.24	93.02	3
	10.25	11.87	1.15	1.63	65.26	74.30	5
Hesperetin	No separation				13.86	–	1
	2.27	2.55	1.12	0.53	19.82	21.36	2
	4.78	5.44	1.13	1.00	34.80	38.52	3
	9.44	10.88	1.15	1.08	63.19	73.00	4
	3.39	3.84	1.13	1.30	25.56	28.36	5
Naringenin	No separation				16.00	–	1
	No separation				23.91	–	2
	No separation				130.0	–	5
	No separation				60.55	–	6
	8.23	9.11	1.10	0.96			7
Taxifolin	No separation				48.44	–	1
	No separation				61.05	–	2
	Shoulder peak				65.41	67.92	5
	10.50	11.00	1.04	0.53			7

composition it is deduced that increased R_s values can be obtained by decreasing the methanol content. The same phenomena had also been observed for 6-methoxy-flavanone, flavanone and hesperetin.

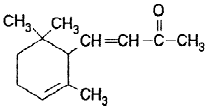
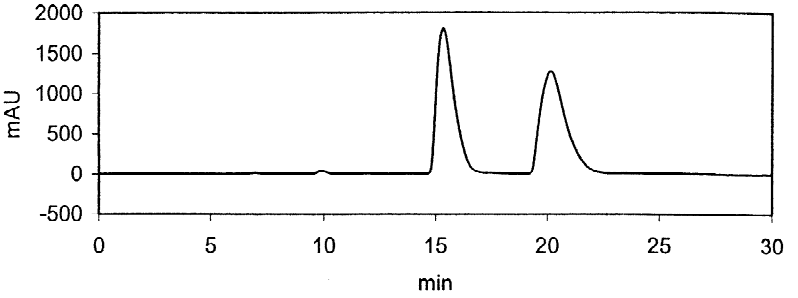
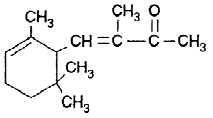
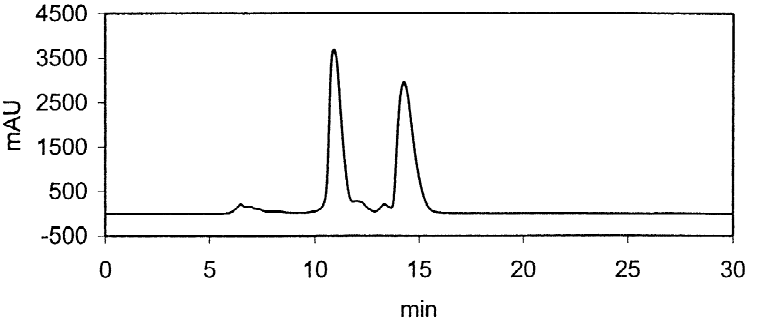
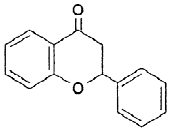
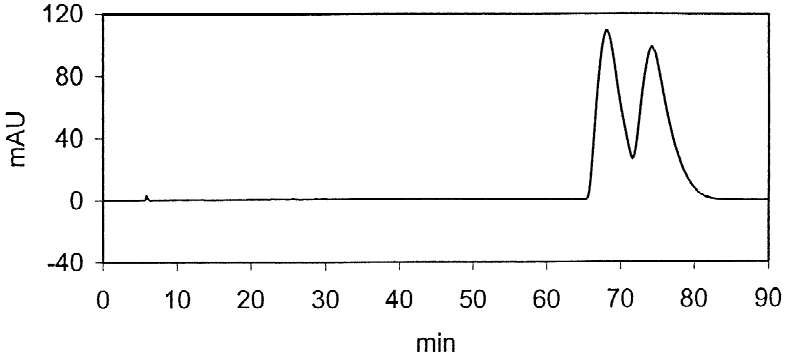
Indeed, when methanol/water mobile phase is used on CSP-ME column, it is observed (see Table 1) that the greater the amount of water in the eluent, the longer is the retention time of the analyte. Thus, for neutral analytes such as alpha-ionone, 3-methyl-alpha ionone, flavanone and 6-methoxy flavanone, a simple mobile phase composition consisting of a simple mixture of water and methanol/acetonitrile is sufficient to obtain a satisfactory separation (see Tables 2 and 3).

Both naringenin and taxifolin showed no signs of separation using water/methanol as mobile phase. The lack of enantioselectivity for naringenin and taxifolin may be due to the presence of acidic hydroxyl groups in these analytes as compared to other neutral analytes like ionone and flavanone.

3.2. Influence of buffer pH on chromatographic separation

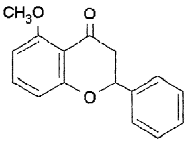
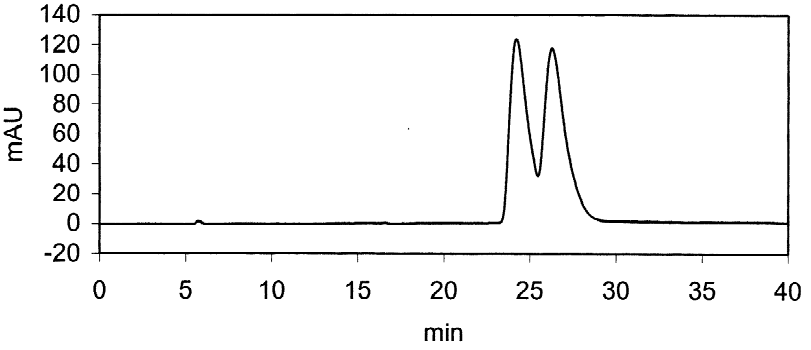
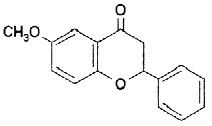
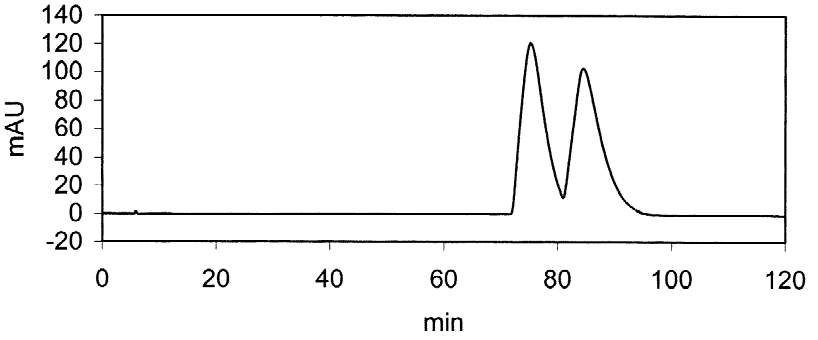
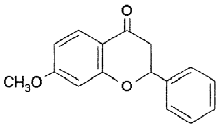
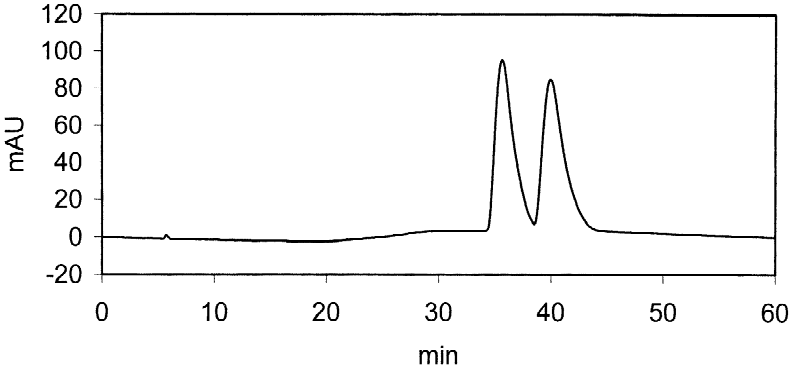
It is also important to classify the analytes according to their chemical properties. Alpha-ionone, 3-methyl-alpha ionone, flavanone and 6-methoxy flavanone are considered as relatively neutral compounds, whereas hesperetin, naringenin and taxifolin are weakly acidic compounds in nature. Since these

Table 2
HPLC Chromatograms of alpha-ionone, 3-methyl alpha-ionone and flavanone on CSP-ME at constant flow rate of 0.5 ml/min, detection wavelength of 254 nm and under different separation conditions

Compounds	Chromatograms achieved under different separation conditions (v/v)
Alpha-ionone	Condition 2: Methanol/water (50/50)
	
3-Methyl alpha-ionone	Condition 1: Methanol/water (60/40)
	
Flavanone	Condition 3: Methanol/water (40/60)
	

analytes have different pK_a values, they are expected to show a different extent of selectivity under buffered separation conditions. When the eluent used consisted of an acidic 1 wt.% triethylammonium acetate (TEAA) buffer solution, there was essentially no significant effect on a given separation.

Table 3
HPLC Chromatograms of 5-methoxyflavanone, 6-methoxyflavanone, 7-methoxyflavanone, on CSP-ME at constant flow rate of 0.5 ml/min, detection wavelength of 254 nm and under different separation conditions

Compounds	Chromatograms achieved under different separation conditions (v/v)
5-Methoxyflavanone	Condition 2: Methanol/water (50/50)
	
6-Methoxyflavanone	Condition 3: Methanol/water (40/60)
	
7-Methoxyflavanone	Condition 2: Methanol/water (50/50)
	

For illustration, the R_s value of 3-methyl-alpha ionone under condition 3: water/methanol: 60/40 was 3.54 and the selectivity factor, α, obtained was 1.79. Upon replacement of water with TEAA buffer using condition 5: buffer (pH 5.0)/methanol: 60/40, both the R_s and α values obtained for 3-methyl-ionone are 4.06 and 1.80 respectively. Also for 6-methoxy flavanone, the R_s and α values obtained under conditions 3 and condition 5 are around 1.0 and 1.1 respectively. Likewise, the other neutral analytes such as alpha ionone and flavanone, also depicted a similar trend. Therefore the enantioselectivities of these neutral analytes were not influenced significantly by the pH value of the buffer, but only depended upon the polarity of the mobile phase.

The acidic analytes such as hesperetin, naringenin and taxifolin, on the other hand, show no separation when water/methanol mobile phase composition is used. This indicates that buffered (pH 4.68)/methanol eluent is necessary for achieving effective separation on CSP-ME column. Therefore, when acidic buffered solutions containing 1% TEAA were used in place of water in the mobile phase composition, naringenin and taxifolin showed partial separation under condition 7 (buffer containing 1% TEAA, pH 4.68/methanol: 70/30). Moreover, by replacing methanol with another organic modifier that has higher solvent strength, such as acetonitrile, the retention time required was reduced. But however, the resolution of test analytes was not improved.

From chromatographic separation results achieved on CSP-ME column, the separation mechanism of analytes for neutral chiral analytes such as alpha-ionone, 3-methyl-alpha ionone, flavanone and 6-methoxy flavanone depended greatly upon chirality of methylated-cyclodextrin and its ability to form inclusion complexes with the chiral analytes. For other acidic analytes such as hesperetin, naringenin and taxifolin, the presence of acidic TEAA buffer increased the retention by the CSP-ME chiral stationary phase.

4. Conclusion

A novel methylated-β-cyclodextrin based (CSP-ME) chiral stationary phase material which was chemically bonded via multiple-ureido linkages was developed. By controlling the mobile phase composition, that is methanol and water/buffer ratio, the CSP-ME stationary phase can provide efficient chiral separation of several flavour and fragrance compounds under reverse phase separation conditions. For neutral chiral compounds, the relative simplified separation conditions such as methanol/water or acetonitrile/water is sufficient for achieving chiral separation. The resolution, R_s of alpha-ionone, 3-methyl-alpha ionone, flavanone and 6-methoxy flavanone may be optimised by decreasing the polarity of the mobile phase. Whereas, for acidic chiral compounds such as hesperetin, naringenin and taxifolin, effective separation may be obtained using methanol/TEAA buffer as mobile phase. Further work on separation abilities of CSP-ME on basic test analytes is currently in progress.

Acknowledgements

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