Article

CD-MEKC Method to Analyze Triterpene Acids in Traditional Chinese Medicines

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Um método cromatográfico eletrocinético micelar modificado por ciclodextrina (CD-MEKC) foi desenvolvido para separar e determinar ácido betulínico (BA), ácido oleanólico (OA) e ácido ursólico (UA) usados na medicina tradicional Chinesa (TCMs). Três triterpenos pentacíclicos ácidos foram separados em tampão (pH 9,0) de metanol 6% (v/v), contendo 10 mmol L^{-1} de tetraborato dissódico, 10 mmol L^{-1} de hidrogênio fosfato de sódio, 50 mmol L^{-1} de dodecilsulfato de sódio, 15 mmol L^{-1} de 2-hidroxipropil-β-ciclodextrina em 15 min. Uma boa relação linear entre a área de pico e a concentração foram obtidos nos intervalos de 16,5-330 μg mL^{-1}, 18-360 μg mL^{-1} e 19,5-390 μg mL^{-1} para BA, OA e UA, respectivamente. Comparado com cromatografia líquida de alta eficiência com γ-CD como fase móvel quiral aditiva (CMPA-HPLC), CD-MEKC apresentou melhor eficiência analítica para os três ácidos e o tempo analítico (15 min) foi mais curto do que para CMPA-HPLC (80 min).

A cyclodextrin-modified micellar electrokinetic chromatographic (CD-MEKC) method was developed to separate and determine betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA) in traditional Chinese medicines (TCMs). Three pentacyclic triterpene acids were baseline seperated in the buffer (pH 9.0) of 6% (v/v) methanol containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen phosphate, 50 mmol L^{-1} sodium dodecylsulfate, 15 mmol L^{-1} 2-hydroxypropyl-β-cyclodextrin within 15 min. The good linear relationships between the peak area and concentration were obtained in the ranges of 16.5-330 μg mL^{-1}, 18-360 μg mL^{-1} and 19.5-390 μg mL^{-1} for BA, OA and UA, respectively. Compared with high performance liquid chromatography with γ-CD as chiral mobile phase additive (CMPA-HPLC), CD-MEKC has better analytical efficiency for three acids, and the analytical time (15 min) was shorter than that of CMPA-HPLC (80 min).

Keywords: CD-MEKC, betulinic acid, oleanolic acid, ursolic acid, CMPA-HPLC

Introduction

Triterpenoids, such as betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA), existing in traditional Chinese medicines (TCMs) in the form of free acids or aglycones for triterpenoid saponins,[1] possess important pharmacological properties. BA, a plant-derived pentacyclic lupane-type triterpene, has been shown to exert anti-inflammatory[2] and anti-HIV activities.[3] OA and UA are position isomers (Figure 1). The only difference between them is the position of the methyl group on the ring E. They have been ascertained to have anti-diabetogenic,[4] hepatoprotective,[5] antitumorigenesis[6,7] and antioxidant effects.[8]

The main analytical methods of triterpenoids in TCMs are gas chromatography (GC),[9-11] high-performance liquid chromatography (HPLC),[12] capillary zone electrophoresis (CZE)[13,14] and micellar electrokinetic chromatography (MEKC).[15] However, the resolution of OA and UA was not satisfied by common HPLC or MEKC due to their similar molecular structures. Gas chromatography-mass spectrometry (GC-MS) *via* a time-consuming derivatisation reaction has already been used to identify OA and UA,[16] and liquid chromatography-electrospray/atmospheric pressure chemical mass spectrometry (LC-ESI/APCI-MS) can be employed to identify BA[17] and UA,[18] as the triterpenoids have weak chromophores.

Cyclodextrins (CDs, α-, β-, γ-) are torus-shaped, enzymatically synthesized, non-reducing oligosaccharides
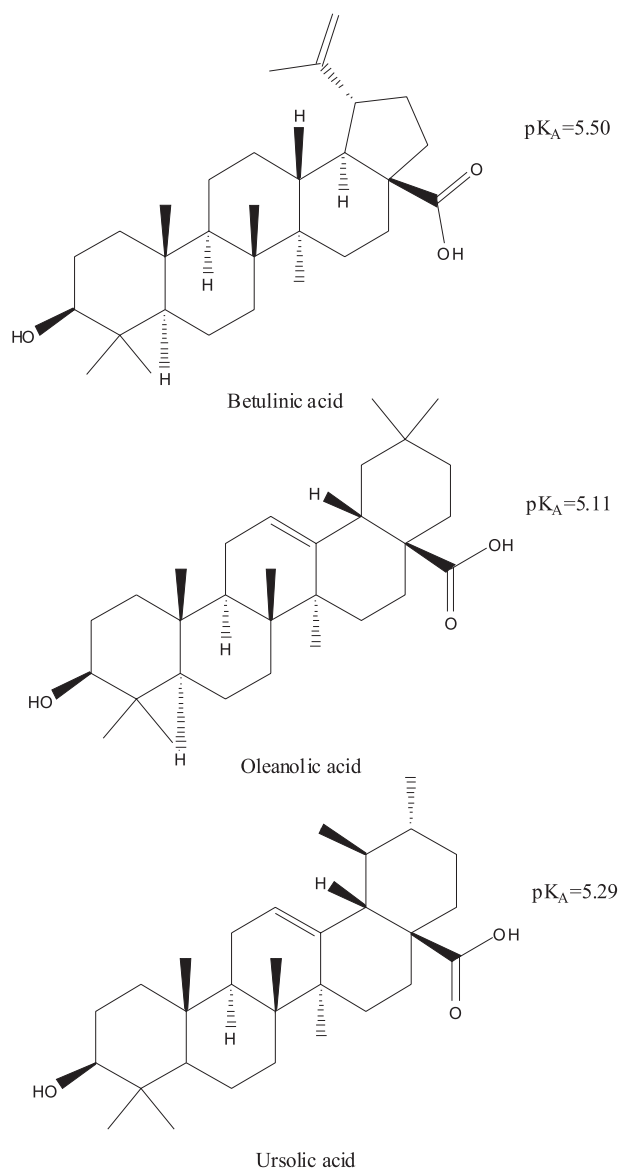
Figure 1. Chemical structures of the three pentacyclic triterpene acids.

consisting of *D*-glucopyranose units bonded through α-1,4-1inkages. The cavities of CDs are relatively hydrophobic while the external faces are hydrophilic. A cyclodextrin-modified MEKC (CD-MEKC) method[19] and HPLC with CDs as chiral mobile phase additive (CMPA-HPLC)[20] greatly expand the analytical applications. The hydrophobic cavities of CDs can form inclusion complexes with the analytes, which improves the separation of components with hydrophobic, isomerous or chiral properties significantly.

In this work, for the first time, a comparative study was carried out, in which a CD-MEKC method was validated for separation and determination of triterpene acids in samples and it was compared with CMPA-HPLC. And the factors affecting the separation of triterpenoids,

including pH value, buffer concentration, organic modifier composition, SDS and CDs concentration in CD-MEKC were investigated.

Experimental

Materials and reagents

BA, OA and UA were provided by the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). *Prunella vulgaris* L., *Diospyros kaki* L. f., and *Crataegus pinnatifida* Bge., were purchased from Hubei, Shanxi and Hebei province of China, respectively. Sodium dodecylsulfate (SDS), sudan III and β-cyclodextrin (β-CD) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), 2-hydroxypropyl-β- cyclodextrin (HP-β-CD), 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD) and γ-cyclodextrin (γ-CD) from Sigma (St. Louis, MO, USA). Methanol was of LC grade (Hanbon Technologies, Jiangsu, China). All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

Apparatus and conditions

The CE analysis was carried out on a CL1030 capillary electrophoresis system (Beijing Cailu Scientific Inc., Beijing, China) equipped with a UV-Vis detector that can perform wavelength scanning from 190 to 740 nm. An uncoated fused-silica capillary (75 μm × 58 cm, Ruipu Chromatogram Equipment Co., Ltd, Hebei, China) with an effective length of 50 cm was used to separate samples, which were injected into the capillary by hydrodynamic flow at a height differential of 10 cm for 10 s. The applied voltage was 20 kV, and the detection wavelength was 210 nm. At the beginning of experiment, the capillary was purged with 0.5 mol L^{-1} NaOH for 5 min, followed by 0.1 mol L^{-1} NaOH for 5 min, deionized water for 5 min and then running buffer for 5 min. Between runs, the capillary was flushed with 0.1 mol L^{-1} NaOH for 2 min followed by deionized water for 2 min and then running buffer 2 min.

HPLC analysis was performed on a Shimadzu LC-2010 apparatus equipped with a Shimadzu SPD-M10A photodiode array detector (Shimadzu Corporation, Kyoto, Japan). Separations were carried out at 30 °C on a Hedera ODS C_{18} column (5 μm, 250 × 4.6 mm, Hanbon Technologies, Jiangsu, China). The optimum wavelength for determination was 210 nm. The flow rate of mobile phase was 1.0 mL min^{-1}.

Preparation of standard and sample solutions

The stock solutions of BA, OA and UA were prepared by dissolving standards (6.6 mg for BA, 7.2 mg for OA and 7.8 mg for UA, respectively) in 10 mL of methanol. Standard solutions of BA, OA and UA at various concentrations were prepared by diluting the stock solutions.

The pulverized samples (2.0 g for *P. vulgaris*, 4.6 g for *Diospyros kaki* L. f. and 1.0 g for *C. pinnatifida*, respectively) were extracted with petroleum ether (50 mL) at 60 °C for 10 min in an ultrasonic bath. After filtration, the residue was extracted with 95% ethanol (50 mL) for 20 min in an ultrasonic bath, and it was repeated twice. The extracts were combined, filtered and concentrated. Finally, the residue was dissolved with 10 mL of running buffer. The solution was then filtered through a 0.45 μm membrane filter before injection.

Results and Discussion

Method optimization

Mobility calculation

The velocity of analytes can not be expressed by the migration time or apparent electrophoretic mobility in the separation systems. The effective electrophoretic mobility (μ_{ef}) described by Fu[22] as follows, avoiding the influence of electroosmotic flow, has been taken as the revised velocity of analytes.[21]

$$\mu_{ef} = \frac{k}{k+1} \mu_{ap(mc)} \qquad (1)$$

where k is the column capacity factor, and $\mu_{ap(mc)}$ is the apparent electrophoretic mobility of micella.

$$k = \frac{t - t_0}{t_0 \left(1 - t / t_{mc}\right)} \qquad (2)$$

$$\mu_{ap(mc)} = \frac{L_{ef} L_t}{V t_{mc}} \qquad (3)$$

where t, t_0 and t_{mc} are the migration time of analyte, electroendosmotic flow marker (methanol in this experiment), and micella (sudan III used for marker) respectively. L_{ef} is the effective length of capillary between injection and detection, L_t is the total length of capillary. V is the applied voltage.

Integration of these equations gives, the calculated equation of μ_{eff} was obtained as follows.

$$\mu_{ef} = \frac{L_{ef} L_t}{V t} \cdot \frac{t - t_0}{t_{mc} - t_0} \qquad (4)$$

With high reliability of qualitative analysis, μ_{eff} was employed to identify the peaks of the triterpenoids in TCMs.

Effect of SDS concentration on CE separation

MEKC, a newly fast-developing CE mode, makes the separation of neutral compounds possible for applications. SDS, a typical anionic surfactant, was taken as micella in MEKC in this study, which affected the separation of triterpenoids at several concentration points checked (Figure 2). It can be seen that the effective mobilities difference between BA and OA was large, and the effective mobilities difference between OA and UA was almost zero when the SDS concentration approached to 10 mmol L^{-1}. In addition, the effective electrophoretic mobilities and resolution of the triterpenoids increased as SDS concentration changed from 10 to 90 mmol L^{-1}. However, the trend reduced gradually. Take the migration time of triterpenoids into consideration, a buffer solution with 50 mmol L^{-1} SDS was selected.
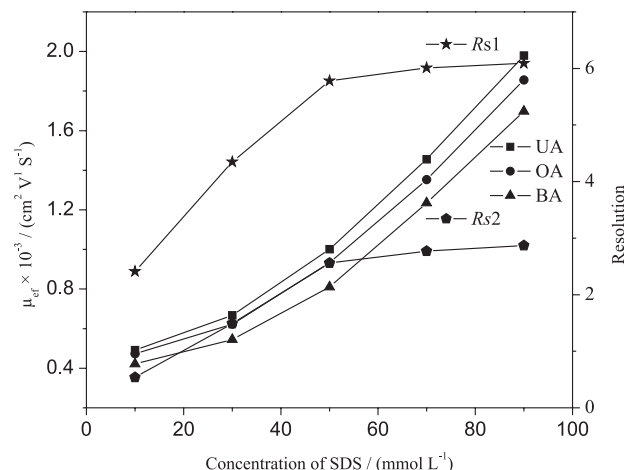

Figure 2. Effect of SDS concentration on CE separation. Conditions: 6% (v/v) methanol (pH 9.0) containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen phosphate and 15 mmol L^{-1} HP-β-CD; capillary, 58 cm (50 cm to detector) × 75 μm i.d.; applied voltage, 20 kV; detection, 210 nm. (Rs1, resolution of BA and OA; Rs2, resolution of OA and UA.).

Effect of borate-phosphate concentration on CE separation

The influence of borate-phosphate mixture on the separation of standard mixture of BA, OA and UA was investigated as listed in Table 1. When buffer concentration exceeded 20 mmol L^{-1}, the migration time of triterpenoids became longer obviously, because the value of zeta potential and EOF reduced as ionic strength increased. Meanwhile, the electric current and joule heat increased rapidly. Therefore, both sodium dihydrogen phosphate and disodium tetraborate concentrations were 10 mmol L^{-1} in the buffer.

Table 1. Effect of buffer composition on the separation of triterpenoids

No.	Buffer composition	Results
1	30 mmol L^{-1} A or B	Increasing migration time and poor resolution
2	20 mmol L^{-1} A or B	Shorter migration time, but poor peak shape
3	10 mmol L^{-1} A + 10 mmol L^{-1} B	Appropriate migration time and better peak shape

A stands for disodium tetraborate; B stands for sodium dihydrogen phosphate.

Effect of pH value on CE separation

The pH value of running buffer may be a key factor affecting the peak shape and efficiency in CE system as the triterpenoids are all weak acids (pKa of the acids were given in Figure 1). In this experiment, the effect of pH value on the separation of triterpenoids studied from 6.0 to 10.0 was shown in Figure 3. As pH increased, the effective electrophoretic mobility of triterpenoids decreased and resulted in increased migration time. However, the resolution of triterpenoids was slightly improved; pH 9.0 was finally selected.

Effect of organic modifiers on CE separation

Organic solvents can alter selectivity, reduce viscosity of background electrolyte, improve the resolution of the hydrophobic triterpenoids. Methanol, ethanol and acetonitrile at various concentrations were used in the running buffer for an attempt to improve the separation of the triterpenoids. However, as listed in Table 2, OA and UA can not be completely separated when ethanol or acetonitrile was used. Three sharp and symmetric peaks were achieved when a running buffer with 6% (v/v) methanol was used. When the concentration of methanol exceeded 6%, the resolution of the isomers changed very little.

Effect of HP-β-CD concentration on CE separation

The resolution of OA and UA by MEKC and the range of linearity were not satisfied.[15] We have known that the triterpene acids can form inclusion complexes with CDs. The stability of the complexes has obviously effects on the resolution and the elution order of triterpenoids.[23] Electrolyte systems containing HP-β-CD ranging from
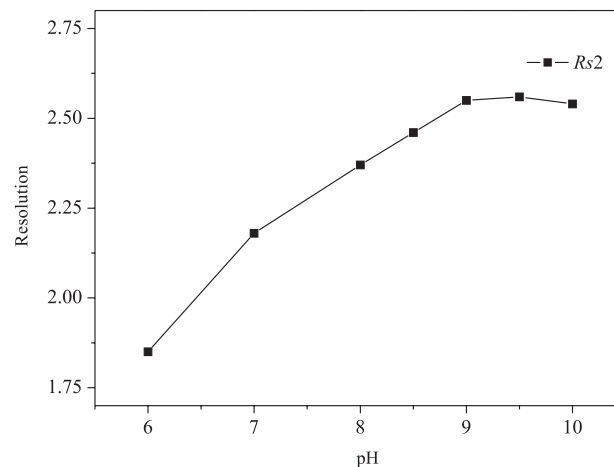

Figure 3. Effect of pH value on the resolution of the isomers. Conditions: 6% (v/v) methanol containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen phosphate, 50 mmol L^{-1} SDS and 15 mmol L^{-1} HP-β-CD; capillary, 58 cm (50 cm to detector) × 75 μm i.d.; applied voltage, 20 kV; detection, 210 nm. (Rs2, resolution of OA and UA.).

5 to 25 mmol L^{-1} were tested. The results obtained were given in Figure 4. It was apparent that the resolution of triterpene acids changed dramatically with increasing HP-β-CD concentration. The resolution of OA and UA was found to be 2.56 when 15 mmol L^{-1} HP-β-CD was used in the buffer.

Method validation

Linearity

Three triterpenoids were separated in the buffer (pH 9.0) of 6% (v/v) methanol containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen

Table 2. Effect of organic modifiers composition on the separation of triterpenoids

No.	organic modifiers composition	Results
1	5% (v/v) A	Unstable baseline, and the peaks of OA and UA overlapped.
2	5% (v/v) B	Relatively stable baseline, but two peaks still overlapped.
3	3% (v/v) C	More stable baseline, and two acids separated from each other.
4	6% (v/v) C	Relatively stable baseline and better resolution obtained.
5	9% (v/v) C	Changed little.

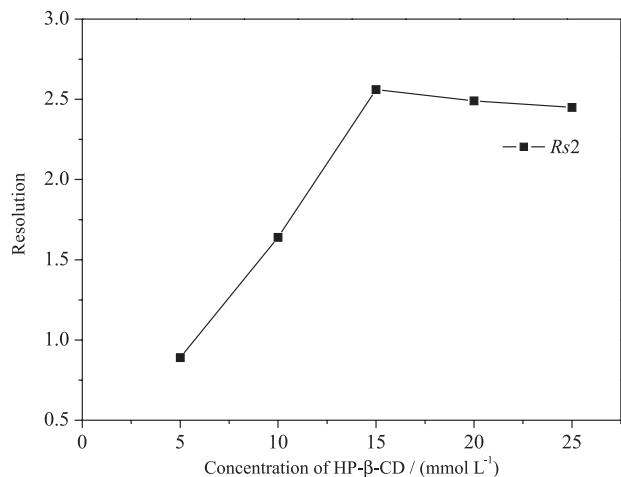A stands for acetonitrile; B stands for ethanol; C stands for methanol.

Figure 4. Effect of HP-β-CD concentration on the resolution of the isomers. Conditions: 6% (v/v) methanol containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen phosphate and 50 mmol L^{-1} SDS. Capillary, 58 cm (50 cm to detector) × 75 μm i.d.; applied voltage, 20 kV; detection, 210 nm. (Rs2, resolution of OA and UA.).

phosphate, 50 mmol L^{-1} SDS and 15 mmol L^{-1} HP-β-CD within 15 min. The electropherogram of standard mixture was shown in Figure 5a. The peak area (y / μV s) and the concentration (x / μg mL^{-1}) were fit to the linear functions: $y_{BA} = 2062 + 72.54x_{BA}$ (r = 0.9985); $y_{OA} = 1296 + 87.19x_{OA}$ (r = 0.9989); $y_{UA} = 2870 + 83.98x_{UA}$ (r = 0.9988). And the good linear relationships between the peak area and concentration were obtained in the ranges of 16.5-330 μg mL^{-1}, 18-360 μg mL^{-1} and 19.5-390 μg mL^{-1} for BA, OA and UA, respectively. The limits of detection (LOD) and limits of quantitation (LOQ) under the proposed CD-MEKC conditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The values of LOD and LOQ were 2.1 μg mL^{-1}, 8.2 μg mL^{-1} for BA, 2.2 μg mL^{-1}, 8.6 μg mL^{-1} for OA and 2.8 μg mL^{-1}, 10.3 μg mL^{-1} for UA, respectively.

Precision

The samples were prepared and determined by the method described above, the results of which were shown in Table 3, and the electropherogram of the sample B was shown in Figure 5b. The precision study was comprised of repeatability (intra-day precison) and reproducibility (inter-day precison) studies. The repeatability was established by analyzing each sample five times on the same day. The reproducibility was determined by analyzing each sample (one time/day) on a 5-day period. As listed in Table 3, we found that the relative standard deviation (R.S.D.) of intra-day precision was less than 2.32% for BA, 2.31% for OA and 2.48% for UA, while the R.S.D. of inter-day precision was less than 3.12% for BA, 2.81% for OA and 3.31% for UA, respectively.
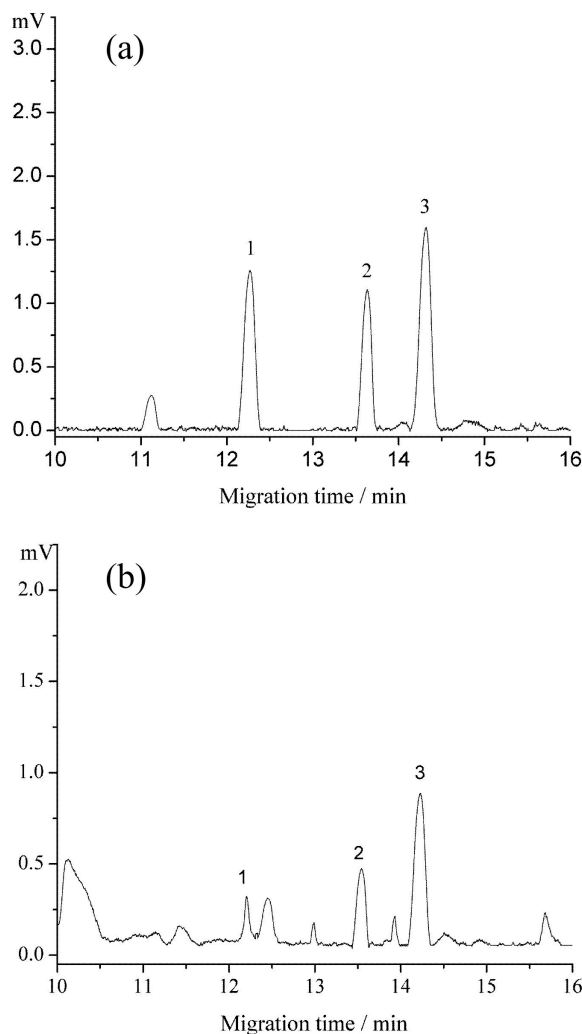

Figure 5. CD-MEKC electropherograms of standard mixure (a) and sample (B) (b) (1-BA; 2-OA; 3-UA). Buffer: 6% (v/v) methanol containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen phosphate, 50 mmol L^{-1} SDS and 15 mmol L^{-1} HP-β-CD. capillary, 58 cm (50 cm to detector) × 75 μm i.d.; applied voltage, 20 kV; detection, 210 nm.

Recovery and accuracy

The recoveries of the triterpene acids were determined by the method of standards addition. Suitable amounts of three acids were spiked into sample B, which were determined previously. The mixture was extracted and analyzed by using the proposed procedure. Table 4 showed the recoveries of BA, OA and UA applying a CD-MEKC method. As listed in Table 4, the recoveries were 93.3-96.0%, 95.2-102.9% and 93.8-103.1% for BA, OA and UA, respectively.

Comparison of MEKC with γ-CD CMPA-HPLC

OA and UA could not be well separated from each other by common HPLC on reversed column in my

Table 3. Statistical results of precision of the triterpene acids (n = 5)

		Intra-day Precision		Inter-day Precision	
		Content / (mg g⁻¹)	R.S.D / (%)	Content / (mg g⁻¹)	R.S.D / (%)
A	BA	#		#	
	OA	1.11	2.11	1.16	2.81
	UA	2.44	2.21	2.46	2.96
B	BA	0.29	2.32	0.31	3.12
	OA	0.48	2.19	0.48	2.77
	UA	1.58	2.25	1.61	3.15
C	BA	#		#	
	OA	1.23	2.31	1.25	2.72
	UA	4.22	2.48	4.25	3.31

not detected. A stands for *P. vulgaris*; B stands for leaves of *Diospyros kaki* L.f.; C stands for *C. pinnatifida*.

Table 4. Recoveries of BA, OA and UA in sample B applying a CD-MEKC method

Components	Cotent / (mg g⁻¹)	Added / (mg g⁻¹)	Found / (mg g⁻¹)	Recovery / (%)
BA	0.29	0.15, 0.21, 0.25	0.43, 0.49, 0.53	93.3, 95.2, 96.0
OA	0.48	0.21, 0.28, 0.35	0.68, 0.75, 0.84	95.2, 96.4, 102.9
UA	1.58	0.32, 0.52, 0.96	1.88, 2.09, 2.57	93.8, 98.1, 103.1

previous experiments. The resolution of the triterpenoids can be improved by CDs added to the HPLC mobile phase. Different concentrations of β-CD, HP-β-CD, DM-β-CD and γ-CD, used as the chiral additives, were added to the mobile phase to investigate the effect of CDs on the separation of the triterpene acids. However, OA and UA could still not be completely separated when β-CD, HP-β-CD and DM-β-CD were used. When 5.0 mmol L⁻¹ γ-CD was added to methanol/H₂O, pH 6.0, (75/25, v/v), the two acids were well resolved (Figure 6). γ-CD, with larger cavity and
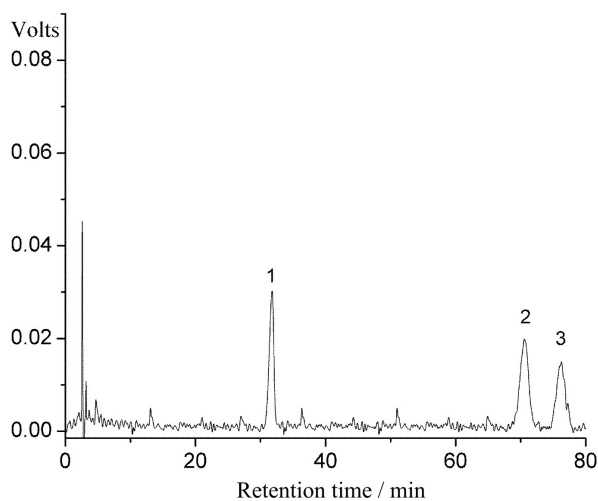
better water-solubility than other CDs, can form stable and hydrophilic inclusion complexes with the triterpenoids. Chromatographic properties of the triterpenoids, once included, may be dramatically affected. Furthermore, the elution order of OA and UA changed because it depends on the nature of the CDs.[23] As a result, the more stable γ-CD complexes are formed with UA compared to OA when 5.0 mmol L⁻¹ γ-CD was selected.

The CMPA-HPLC method appeared to cost time and suffer from complexity, whereas the CD-MEKC method described here was simple, robust and cost-effective while retaining sufficient analytical efficiency (Table 5). It took more than 75 min for a CMPA-HPLC analysis, but only 15 min for CD-MEKC analysis.

Figure 6. CMPA-HPLC chromatogram of standard mixure (1-BA; 2-UA; 3-OA). Column: Hedera ODS C₁₈; 250 mm × 4.6 mm i.d.(5 μm); flow-rate: 1 mL min⁻¹; injection volume: 10 μL; detection, 210 nm; mobile phase: methanol/H₂O, pH 6.0, (75/25, v/v) + 5 mmol L⁻¹ γ-CD.

Table 5. Number of theoretical plates (×10⁴) for triterpenoids analyzed with CMPA-HPLC and CD-MEKC

Method	BA	OA	UA
CMPA-HPLC	1.28	1.35	1.22
CD-MEKC	6.67	7.19	5.81

Conclusions

In this paper, a baseline separation of triterpenoids in TCMs was achieved by using MEKC with HP-β-CD as chiral selector. Three pentacyclic triterpene acids were baseline seperated in the buffer (pH 9.0) of 6% (v/v)

methanol containing 10 mmol L^{-1} disodium tetraborate, 10 mmol L^{-1} sodium dihydrogen phosphate, 50 mmol L^{-1} SDS, 15 mmol L^{-1} HP-β-CD within 15 min. A pathbreaking study was carried out comparatively. Compared with CMPA-HPLC, CD-MEKC has the advantages of rapidity, accuracy and high efficiency. It can be used for quantitative study and quality control of TCMs.

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