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Simultaneous Quantitation of Eight Coumarins in *Radix angelicae dahuricae* by Micellar Electrokinetic Capillary Chromatography

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A new simple, rapid, sensitive and accurate quantitative detection method using micellar electrokinetic capillary chromatography for the measurement of eight coumarins levels in *Radix angelicae dahuricae* is described, namely scopoletin (1), xanthotoxol (2), xanthotoxin (3), bergapten (4), oxypeucedanin (5), imperatorin (6), cnidilin (7) and isoimperatorin (8). Analytes were separated on the buffer solution which contained 10 mM borax, 40 mM sodium dodecyl sulfate and 20 % (v/v) acetonitrile, 22 kV for the separation voltage at 20 ºC. 7 of the 8 coumarins (expect xanthotoxol) were simultaneously quantitated. The recovery of the 7 coumarins was in the range of 92.7-105.6 % with RSD values less than 3.6 %.

Key Words: *Radix angelicae dahuricae*, Coumarins, MECC, Separation, Quantitation, TCM.

INTRODUCTION

Radix angelicae dahuricae, dried radix of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. and *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. var. *formosana* (Boiss.) Shan et Yuan, belonging to Umbelliferae family, is a widely used traditional Chinese medicine (TCM). Its dry roots, Baizhi in Chinese have been frequently used as a common acesodyne, especially for headache and toothache[1]. In addition, it can be used for cough, asthma, coryza, hypertension, vitiligo, psoriasis, acne, herpes zoster, freckle, leucorrhea, rheumatism, *etc*. in Chinese clinics for over 2000 years. Coumarins are the main effective components responsible for their activities, such as antihistamine, photoallergy, promotion of lipometabolism, spasmolysis, antibacterial, inhibition of arachidonic acid-induced platelet aggregation, expansion of coronary vessels, excitation of motor and respiratory center, anticancer, *etc*.[2]. Coumarins are often used as reference standards in the quality control of *Radix angelicae dahuricae* and its products[3]. In the Chinese Pharmacopoeia, imperatorin has been used as the chemical marker for quality control of *Radix angelicae dahuricae*[1]. But quality control of traditional Chinese medicine must extensively represent its integral quality. So it is necessary to establish a method which can simultaneous quantitative analyze of active compositions.

As we know, capillary electrophoresis technique is a separation and determination method which is used capillary and high pressure electric field as separation channel and driving force, respectively. The electroosmotic flow is used as pumps in the high-performance liquid chromatography, so there is no need for complex mechanical pumps. But the flow profile is flat. Because of its advantageous high separation efficiency and fast analysis time, capillary electrophoresis (CE) has been proven to be a useful technique for the separation and determination. capillary electrophoresis is used widely for separation analysis, such as many inorganic or organic substance, amino acids, peptide, protein, nucleic acid, enantiomer, biological product and traditional Chinese medicine[4-12]. In term of quantitative analysis of *Radix angelicae dahuricae*, several analytical methods have been reported for the determinations of coumarins[13], including pressurized capillary electrochromatography[14], high-performance liquid chromatography coupled with mass spectrometry[15], cell membrane chromatography[16], high-speed counter-current chromatography[17,18], thin layer chromatography[19], column-switching high-performance liquid chromatography[20], gas chromatography coupled with mass spectrometry[21], capillary electrophoresis[9] and high-performance liquid chromatographic techniques[22-26]. However, all these methods, though attaining sometimes low detection limits or the requirement of tedious pretreatment, now rapid and low cost of analysis is increasingly being demanded in many areas especially on pharmaceutical analysis. We find an easy, rapid, sensitive and cheap method for the determination of eight coumarins levels in *Radix angelicae dahuricae*. Only a few

kinds of coumarins were reported to quantitate with this method before. The method of simultaneous quantitation several coumarins was even fewer and fewer[8-12]. In this paper, we have simultaneously separated 8 coumarins, scopoletin (1), xanthotoxol (2), xanthotoxin (3), bergapten (4), oxypeucedanin (5), imperatorin (6), cnidilin (7), isoimperatorin (8) and quantitated 7 of them (except xanthotoxol) in *Radix angelicae dahuricae* by micellar electrokinetic capillary chromatography (MECC) within 20 min. In this paper the theory of capillary electrophoresis and the influences of the buffer solution on the capillary electrophoresis separation was described. The structures of all the 8 compounds are shown in Fig. 1.

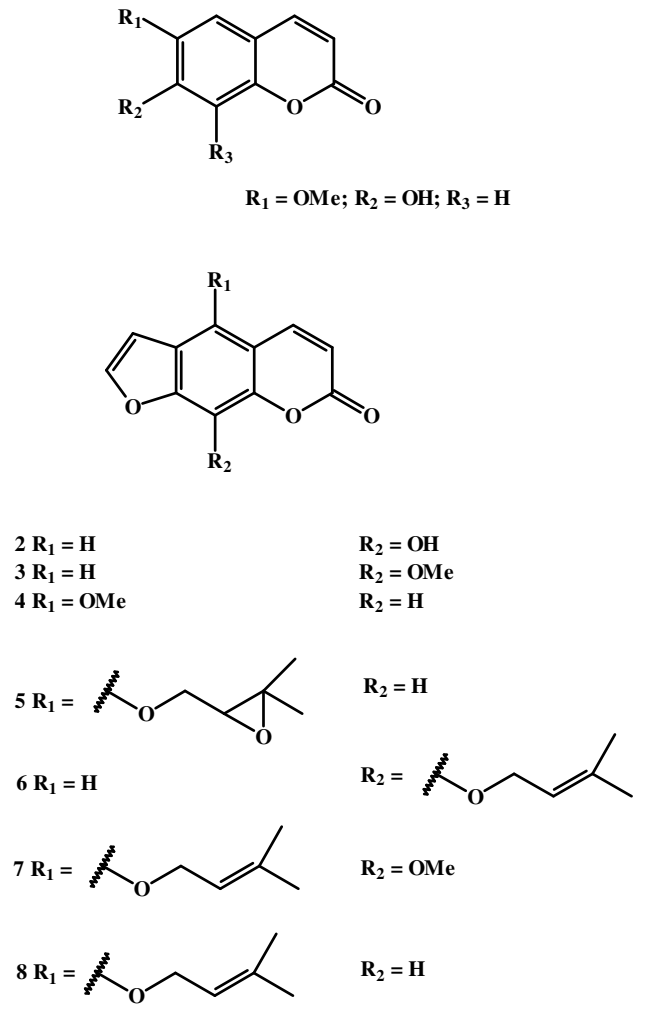

Fig. 1. Chemical structures of 8 compounds (1) scopoletin, (2) xanthotoxol, (3) xanthotoxin, (4) bergapten, (5) oxypeucedanin, (6) imperatorin, (7) cnidilin and (8) isoimperatorin

EXPERIMENTAL

Acetonitrile was HPLC grade and obtained from Tedia (Tedia, Fairfield, USA). Ultrapure water was produced by Heal-Force-PWVF Water System (Likang, Hongkong, China). Borax, sodium dodecyl sulfate and ethanol are all analytical reagent. Five batches of samples were collected from different fields of Hebei province in China. Scopoletin (1), oxypeucedanin (5), imperatorin (6), isoimperatorin (8) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Xanthotoxol (2), xanthotoxin (3) and bergapten (4) were bought from Shanghai Tauto Biotech (Shanghai, China). Cnidilin (7) was isolated by our laboratories from *Radix angelicae dahuricae* and its purity was no less than 98 % by HPLC analysis.

Electrophoretic separations were performed using a Beckman (Beckman Coulters, Fullerton, CA, USA) P/ACETM MDQ System equipped with an on-column UV detection system operating at 214 nm. The instrument control and data acquisition were carried out by a 32 Karat software package (Beckman Coulter). The separation voltage was 22 kV and the temperature was 20 ºC, controlled by a constant temperature control system. Separations were carried out using fused-silica capillary (Beckman Technologies, USA) of 60 cm total length (50 cm effective length) × 75 μm i.d. New capillaries were flushed with methanol for 5 min, ultrapure water for 5 min, 0.1 M HCl for 5 min, ultrapure water for 5 min, 0.1 M NaOH for 5 min and ultrapure water for 5 min in order to make them activation. Each day before the experiment, the capillary were flushed with ultrapure water, 0.1 M NaOH and ultrapure water for 3, 5 and 5 min, respectively. The capillary was washed with 0.1 M NaOH for 2 min, deionized water for 2 min and buffer for 2 min before each run. The injection mode was choosen pressure injection, (0.5 psi, 5 s). The electrolyte was buffer solution which contained 10 mM borax - 40 mM sodium dodecyl sulfate (SDS)-20 % acetonitrile. All buffer solutions were filtered through a 0.45 μm filter before use.

Standard solution preparation: After accurately weighed, each reference compound (scopoletin 2.01 mg/mL, xanthotoxol 2.41 mg/mL, xanthotoxin 3.09 mg/mL, bergapten 5.58 mg/mL, oxypeucedanin 4.80 mg/mL, imperatorin 3.60 mg/mL, cnidilin 4.84 mg/mL, isoimperatorin 2.26 mg/mL) was prepared in 10 mL methanol as stock solutions. The standard stock solution containing the 8 standards (scopoletin 40.08 μg/mL, xanthotoxol 57.06 μg/mL, xanthotoxin 14.02 μg/mL, bergapten 21.48 μg/mL, oxypeucedanin 243.72 μg/mL, imperatorin 126.16 μg/mL, cnidilin 92.42 μg/mL, isoimperatorin 96.70 μg/mL) was diluted with buffer solution to make 5 different concentrations to construct the calibration curves.

Sample preparation: 1.5 g dried powders of *Radix angelicae dahuricae* samples were extracted with 20 mL of 75 % ethanol in ultrasonic bath for 40 min and centrifuged at 1500 g for 5 min. Then the resultant solution was diluted with buffer solution to 1/5 of the original concentration. The solutions were filtered through 0.45 μm filters and the filtrates were injected directly into capillary electrophoresis. Peak identification was performed by standard addition method.

RESULTS AND DISCUSSION

Extraction conditions: We have studied the extraction method, solvent and time to optimize the extraction conditions. According to experimental result, the extraction efficiency of ultrasonic extraction was corresponded to refluxing and the former was more convenient than the latter. Under the same condition, there were more foreign material in the methanol

extractive than in the ethanol ones. Compared with different concentration of ethanol, 75 % ethanol, which was known as the omnipotent solvent was considered to be the best extraction solvent. To determine optimal extraction time, 1.5 g samples were extracted with 20 mL of 75 % ethanol by ultrasonic extraction for 10, 20, 30, 40, 50 and 60 min, respectively. It showed that the compounds were almost completely extracted within 40 min. Above all, extracting with 75 % ethanol in ultrasonic bath for 40 min was chosen to be the optimum extraction conditions. Fig. 2 showed the differences between the ultrasonic time and extraction efficiency.

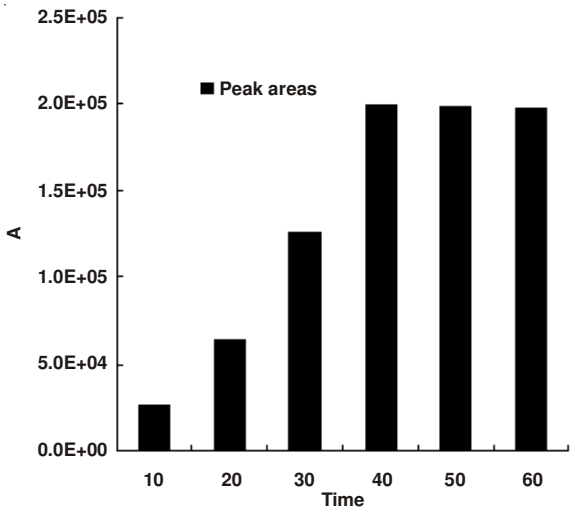

Fig. 2. Effect of time on extraction efficiency (displayed by the peak areas of oxypeucedanin)

Optimization of separation: In capillary electrophoresis, particle velocity depends on both electrophoresis flow and electroosmotic flow (EOF). Electrophoresis was the directional movement of charged particles in buffer solution under the influence of electric field. Electroosmosis is a phenomenon of relative movement between common buffer solution (pH > 2) and the charged capillary. The speed of electrophoresis (u_{ep}) and eletroosmosis (u_{os}) could be calculated by the following formula:

$$u_{ep} = \varepsilon\zeta_i E/4\pi\eta \qquad u_{os} = \varepsilon\zeta_{os} E/\eta$$

ε, η: dielectric constant and viscosity of mediator, E: electric field strength, ζ_i: electric potential of particles, ζ_{os}: electric potential of calillary. The apparent migration speed for the different iron was expressed as followed:

positive ion	$u_{ep} + u_{os}$
neutral molecule	u_{os}
negative ion	$u_{os} - u_{ep}$

In the structure of these 8 compounds, only scopoletin and xanthotoxol posses one hydroxyl group may be deprotonated generating a charged species. The charged property of the other compounds are similar, uncharged molecule. So the CZE separation mode was not suit to this study. And the experimental results was consistent with the theory. Some common buffer solution ($Na_2B_4O_7$, boric acid, NaH_2PO_4, Na_2HPO_4) were chosen as separation buffer, the migration speed of these compounds were the same, so we changed the separation mode.

In MECC, the micelle acted as the stationary phase in HPLC. There were affinities between the compound and micelle. The differences of relative affinities between the compound and micelle environment leaded to the different retention behaviour. The compound was distributed between water phase and micelle phase. The capacity factor $k_p = n_p/n_s$. n_p, n_s were the number of molecules distributed into the micelle phase and the water phase, respectively. In this study, SDS was added in the buffer solution and the centre of SDS is hydrophobe. So the lower polarity, the longer appearance time. While the SDS is a anionic surfactant and the apparent migration speed of the compound which can combine with the SDS is slower than positive ion and neutral molecule. In addition, SDS displays electronegativity in solution. In the structure of scopoletin and xanthotoxol, there is one hydroxyl group which can be deprotonated generating a charged species in alkalescent solution. The affinities of these two compounds and SDS is less than that of other 6 compounds and SDS and the appearance time of these 2 compounds are short. We thought this may be another reason about the appearance sequence. But it was not in the mainly position. In present experiment, after adding sodium dodecyl sulfate (SDS) in the borax, the separation efficiency was improved. The influence of the concentration of SDS on separation efficiency and the migration time was more powerful, which was shown in Fig. 3. The migration times of the compound became longer as the SDS concentration increased. The low concentration of SDS was not able to identificate the single peak. The peaks were wide and obtuse. But when the concentration of SDS was added to 40 mM, the 8 single peaks were clearly recognized. In order to obtain the optimum separation conditions, acetonitrile used as the organic modifier was added to buffer solutions to improve separation efficiency. Especially, the addition of acetonitrile could efficiently improve the shape of peaks, which was successful to separate the 8 peaks baseline. In this experiment the concentration of borax was studied, too. When the concentration of borax was 5mM in the buffer solution, the peak of 2, 3 seriously overlapped and the peak of 6, 7, 8 was not baseline separated. With the increasing of the concentration of borax, the separation efficiency was improved. But as we showed in Fig. 4, with the increasing of the concentration of borax, the influence to the migration time of the 8 compounds was not significant. But too much borax, SDS and acetonitrile were made the migration time too long and too much ion and organic solvent in buffer solution could produce more joule heat which produce air bubble to discontinue the electrophoresis.

The pH of buffer with 10 mM borax, 40 mM sodium dodecyl sulfate and 20 % acetonitrile was 9.80, which was just suit for the separation. Changing the pH of buffer (with NaOH or HCl) was not improved the separation efficiency, but increased the ionic strength of buffer even joule heat.

The temperature was investigated, too. With the increasing of temperature, the separation efficiency gradually improved, but the separation time and joule heat also increased. So based on the best separation efficiency, the lowest temperature, 20 ºC were chosen to be the optimum separation condition.

The separation voltage was investigated from 15 to 25 kV, the separation time became longer with the increasing of the voltage, while the separation efficiency was not improved

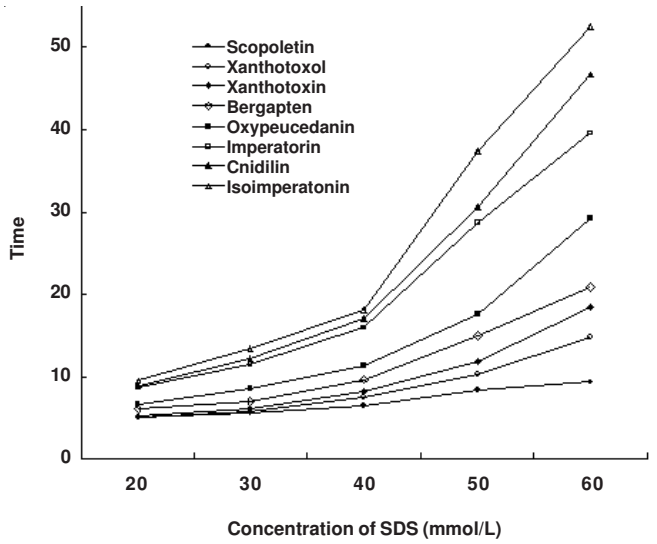

Fig. 3. Effect of concentration of sodium dodecyl sulfate (SDS) on the migration time

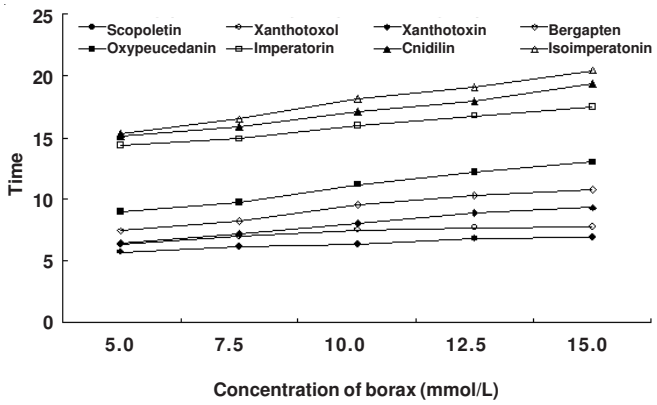

Fig. 4. Effect of concentration of borate on the migration time

significantly. 22 kV was chosen to be the optimum separation voltage.

Identification of 8 coumarins from *Radix angelicae dahuricae*: There are many coumarin in *Radix angelicae dahuricae*. Peak identification was performed by standard addition method, spiking every single standard analyte to the sample in order and then comparing the peak areas and migration time. Only those compounds which were exactly known about the peaks were quantitated. Eight coumarins were baseline separated and identificated, while the content of xanthotoxol was lower than the limits of quantification (LOQ), which was not quantitated in *Radix angelicae dahuricae*.

Calibration curves and limits of detection: Calibration curves were constructed by plotting the peak areas *versus* the concentration of standard solution. Five concentrations of the 8 analytes solutions were prepared as stated standard solution preparation. Limits of detection (LOD) was based on the signal to noise ratio of 3. All the analytes showed good linearity (r^2 > 0.994). The results are given in Table-1.

Precision, accuracy, repeatability and stability: The intra-day precision was obtained by tautologically injected concentrations at 1/8 of the standard stock solutions containing 8 coumarins for 6 times in a single day, while the inter-day variance was obtained by tautologically injected the same solution over 3 consecutive days. The RSD of migration times and peak areas were showed in Table-2.

TABLE-1
CALIBRATION CURVES OF THE 8 COUMARINS

Compounds	Calibration curves	r	Linear range (μg/mL)	LOD
Scopoletin	y = 2573x + 2853.4	0.9977	1.25-20.04	0.31
Xanthotoxol	y = 999.06x + 1034.1	0.9971	1.78-28.53	0.44
Xanthotoxin	y = 11842x + 1860.5	0.9981	0.44-7.01	0.30
Bergapten	y = 3206.5x + 15066	0.9996	0.67-10.74	0.28
Oxypeucedanin	y = 2575.7x + 28675	0.9992	7.62-121.86	0.23
Imperatorin	y = 4784.8x + 123123	0.9991	3.94-63.08	0.25
Cnidilin	y = 3617.7x + 19505	0.9989	2.89-46.21	0.36
Isoimperatorin	y = 3126.6x + 6479	0.9976	3.02-48.35	0.38

TABLE-2
INTRA-DAY AND INTER-DAY PRECISION OF THE 8 OUMARINS

Compounds	Migration times (n = 6) RSD (%)		Peak areas (n = 6) RSD (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Scopoletin	1.75	2.82	0.57	1.94
Xanthotoxol	2.68	3.01	0.79	2.39
Xanthotoxin	1.85	2.66	0.69	2.16
Bergapten	1.24	1.57	0.46	1.33
Oxypeucedanin	0.75	1.13	0.24	0.73
Imperatorin	0.82	1.03	0.19	0.68
Cnidilin	1.02	1.36	0.32	1.14
Isoimperatorin	1.33	2.58	0.47	0.93

About 50 % of the amounts of 7 coumarins (1, 3, 4, 5, 6, 7, 8) in the *Radix angelicae dahuricae* were added in known amounts of *Radix angelicae dahuricae* samples (0.3 g) and then the sample were extracted and analyzed with the established method. The recovery rates of these coumarins were in the range of 92.7-105.6 % with RSD values less than 3.6 %. The results were showed in Table-3.

TABLE-3
RECOVERIES OF THE 7 COUMARINS (n = 3)

Compounds	Initial amount (μg)	Added amount (μg)	Detected amount (μg)	Recovery (%)	RSD (%)
Scopoletin	117.72	58.29	183.57	105.6	3.6
Xanthotoxin	29.13	14.52	42.79	92.7	2.8
Bergapten	65.31	32.36	93.53	95.3	2.6
Oxypeucedanin	610.74	307.20	946.12	101.8	1.4
Imperatorin	404.04	201.60	595.38	98.6	2.5
Cnidilin	263.79	130.68	391.26	97.7	2.7
Isoimperatorin	264.75	133.34	418.64	103.5	3.1

Six samples of one batch were treated as stated in sample preparation. The RSD values of migration times of 7 compounds (1, 3, 4, 5, 6, 7, 8) were 3.8, 3.1, 2.7, 1.7, 2.4, 1.9 and 2.6 %, while the peak areas of them were 2.9, 2.5, 2.1, 0.6, 0.7, 1.6 and 1.3 %, which showed repeatability of this method.

The sample solution were prepared as stated in sample preparation and analyzed at 0, 2, 4, 6, 8, 10, 12 and 24 h, respectively. The RSD values of migration times of 7 compounds (1, 3, 4, 5, 6, 7, 8) were 3.5, 3.3, 2.5, 2.2, 1.9, 2.6 and 3.1 %, while the peak areas of them were 2.5, 2.8, 1.8, 0.4, 0.6, 1.2 and 1.5 %, which showed the stability of sample solution.

TABLE-4
CONTENTS OF THE 7 COUMARINS (n = 3)

Sample	Compounds (μg/g)						
	Scopoletin	Xanthotoxin	Bergapten	Oxypeucedanin	Imperatorin	Cnidilin	Isoimperatorin
1	369.7 ± 1.2	62.3 ± 1.5	177.5 ± 1.8	1869.9 ± 1.3	941.4 ± 1.2	682.4 ± 0.9	753.7 ± 1.8
2	209.8 ± 2.2	41.8 ± 1.8	158.7 ± 1.4	2069.1 ± 2.4	879.7 ± 1.4	719.8 ± 1.6	698.3 ± 2.2
3	408.9 ± 1.5	58.2 ± 1.0	166.9 ± 2.0	2092.5 ± 1.9	1018.6 ± 1.0	769.9 ± 1.8	813.6 ± 1.6
4	291.7 ± 1.1	32.6 ± 1.3	173.8 ± 1.6	2087.4 ± 2.1	1069.9 ± 1.1	799.5 ± 2.6	880.8 ± 1.7
5	392.4 ± 2.4	97.1 ± 2.6	217.7 ± 1.7	2035.8 ± 2.3	1346.8 ± 0.8	879.3 ± 1.4	882.5 ± 2.1

Sample analysis: The contents were calculated with external standard method. Oxypeucedanin was the highest component in all batches of sample, followed by imperatorin. The lowest component was xanthotoxin and the contents of xanthotoxol was lower than the limits of quantification (LOQ), which was not quantitated in *Radix angelicae dahuricae* in this experiment. The typical electropherograms of standard solution and sample were shown in Fig. 5. The contents of the 7 coumarins were summarized in Table-4.

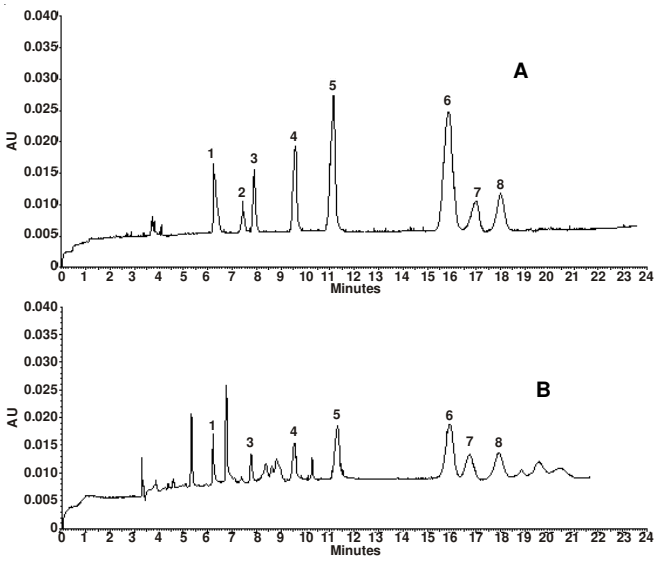

Fig. 5. Electropherograms of standard solution (A) and sample (B), (1) scopoletin, (2) xanthotoxol, (3) xanthotoxin, (4) bergapten, (5) oxypeucedanin, (6) imperatorin, (7) cnidilin and (8) isoimperatorin

Conclusion

The micellar electrokinetic capillary chromatography (MECC) method established has been applied successfully to simultaneous separation of eight coumarins and determination of seven of them in *Radix angelicae dahuricae*. This method was proven to be a powerful technique for the analysis of active components in Chinese traditional medicine. This was the first report on the simultaneous quantification of eight bioactive constituents in *Radix angelicae dahuricae* by MECC. This method can be used for extensively evaluation the integral quality of *Radix angelicae dahuricae*.

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