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Simultaneous Separation and Analysis of Two Bioactive Xanthones in the Tibetan Medicinal Plant *Gentianopsis paludosa* (Hook. f.) Ma by Micellar Electrokinetic Capillary Chromatography

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Summary. 1,7-Dihydroxy-3,8-dimethoxyxanthone (X1) and 1,8-dihydroxy-3,7-dimethoxyxanthone (X2) are two important xanthones of the Tibetan medicinal plant *Gentianopsis paludosa* (Hook. f.) Ma. They are very similar in structure, the only difference being exchange of OH and OCH_3 at the 7 and 8 positions. By calculations based on the geometry of the molecules using the MM+ force field, the different distances between the hydroxyl groups of the two xanthones were obtained (4.64774 Å for X2 and 7.19412 Å for X1), therefore, the two hydroxyl groups of X1 should freely interact with more water molecules than those of X2 in aqueous solution. In other words, X2 is more hydrophobic than X1. Micellar electrokinetic capillary chromatography (MEKC) was therefore chosen for separation of the compounds. The optimum separation conditions were: 20 mM borate + 20 mM SDS (pH 9.8) as running buffer, 17.5 kV applied potential, and detection wavelength 260 nm. The two xanthones were well separated in 9.0 min, with Gaussian peak shapes. The repeatability of the MEKC method (expressed as RSD) for X1 and X2 was 0.9 and 1.1%, respectively, for migration time, and 3.1 and 1.4% for peak area. The limits of detection ($S/N = 3$) were 0.41 μg mL^{-1} for X1 and 0.82 μg mL^{-1} for X2. The recovery of the MEKC method for the two xanthones was also satisfactory.

Key Words: xanthone, *Gentianopsis paludosa,* micellar electrokinetic capillary chromatography, MM+ force field

Introduction

Gentianopsis paludosa (Hook. f.) Ma (Chinese name Shisheng Bianlei), belonging to the family of Gentianaceae, is an annual herb which grows at an altitude of 1100–4900 m in China, India, and Nepal, among others [1]. It is also called 'ji he dou' by local people in the northwest region of China. The whole herb is used for treatment of conjunctivitis, hypertension, hemorrhoids, hepatitis, nephritis, gastroenteritis, dyspepsia, fever, influenza, and

diarrhea in indigenous medicine [2]. Phytochemical studies show that *Gentianopsis paludosa* contains xanthones, terpenoids, and flavonoids [3–6]. Flavonoids may be the active constituents responsible for the anti-diarrheal activity of *Gentianopsis paludosa* [7]. Xanthones are natural products with a polyphenolic structure which have many pharmacological effects, for example antitumor activity, cytotoxicity, antibacterial activity, antifungal activity, anti-inflammatory properties, antioxidant activity, and tuberculostatic activity [8]. Among these xanthones, 1,7-dihydroxy-3,8-dimethoxyxanthone has apparent inhibitory effects on *Mucoraceae*, *Phytophthora capsici*, and *Fusarium axysporum* [9]. It is, therefore, very important to develop methods for separation and analysis of xanthones for quality control of these herbal medicines and exploitation of new medicine resources.

1,7-Dihydroxy-3,8-dimethoxyxanthone (X1) and 1,8-dihydroxy-3,7-dimethoxyxanthone (X2) are two important xanthones of *Gentianopsis paludosa*, which are very similar in structure, the only difference being exchange of OH and OCH_3 at positions 7 and 8. Thin-layer chromatography (TLC) [10] has been used to determine the X1 content of Bian Lei Chong Ji made from *Gentianopsis paludosa*. High-performance liquid chromatography (HPLC) [11, 12] has also been used for simultaneous determination of X1 and luteolin in *Gentianopsis paludosa*. None of these methods has been used to separate X1 and X2, however.

In the last 10 years capillary electrophoresis (CE) has been introduced for separation of xanthones [13–16], owing to the advantages of high separation efficiency, short analysis time, less sample consumption, low cost, ease of mode change-over, and column regeneration. Bo et al. developed different CE methods for separations of ten xanthones, including capillary zone electrophoresis (CZE) [13, 14], micellar electrokinetic capillary chromatography (MEKC), and microemulsion electrokinetic capillary chromatography (MEEKC) [15, 16]. X1 and X2, however, were not among the ten xanthones from *Securidaca inappendiculata* used as model analytes.

Yang et al. used CZE for analysis of X1, X2, and 1,7-O-β-D-glucopyranosyl-8-hydroxy-3,7-dimethoxyxanthone in *Swertia przewalskii pissjauk*; separation was achieved in 5 min by use of 25 mM borate (pH 9.0) as running buffer [17]. The sensitivity achieved for X1 and X2 was quite low, however, especially for X1. In the work reported here a sensitive and accurate MEKC method was established for analysis of X1 and X2 and applied to analysis of these compounds in the Tibetan medicinal plant *Gentianopsis paludosa*.

Experimental

Reagents and Chemicals

Standards of 1,7-dihydroxy-3,8-dimethoxyxanthone (X1) and 1,8-dihydroxy-3,7-dimethoxyxanthone (X2) *(Fig. 1)* were gifts to the authors; the compounds were characterized spectroscopically (NMR, IR, MS) by the State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical and Physics, Chinese Academy of Sciences, Lanzhou, China. The electropherograms obtained from standard solutions of each analyte contained one peak only.

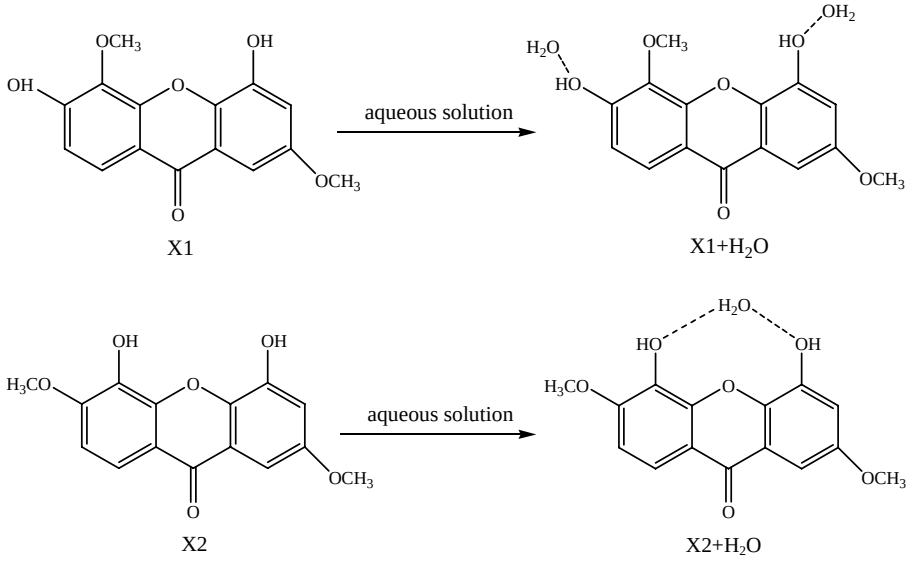

Fig. 1. The molecular structures of the two xanthones and depiction of the interactions between their hydroxyl groups and water molecules in aqueous solution.
X1, 1,7-dihydroxy-3,8-dimethoxyxanthone; X2, 1,8-dihydroxy-3,7-dimethoxyxanthone

Other chemicals were of analytical-reagent grade and used as received. *Gentianopsis paludosa* (Hook. f.) Ma was purchased from the local Tibetan hospital in Huangzhong County, Qinhai Province, China, and identified by Associate Professor Shijian Xu, State Key Laboratory of Arid Agroecology, School of Life Science, Lanzhou University.

Stock standard solutions (1000 μg mL^{-1}) of X1 and X2 were prepared in methanol and stored under refrigeration (4°C) when not in use. Standard solutions at different concentrations were prepared by appropriate dilution of the stock solutions with methanol.

Sample Preparation

Air-dried flowers (or stems) of *Gentianopsis paludosa* (12.5 g) were separately crushed into powder and immersed in 62.5 mL ethanol overnight. The powder was then extracted three times with ethanol at 70°C for 2 h each time. The extracts were combined and concentrated to dryness under vacuum. Distilled water (30 mL) was added to the residue, the mixture was extracted with chloroform (3 × 30 mL), and the extracts were combined and concentrated to dryness under vacuum. The residue was then extracted with 25 mL methanol for 10 min in an ultrasonic bath. The sample stock solutions of both flowers and stems obtained in this way were stored under refrigeration (4°C) when not in use. Sample working solutions were obtained by diluting the stock solutions 20-fold with methanol, then passed through a 0.45-μm filter, and finally injected directly into the CE system.

Electrophoresis

MEKC was performed with a high-performance capillary electrophoresis (CE) apparatus (CL1030; Beijing Cailu Instrumental, Beijing, China) equipped with a power supply (up to a constant potential of 30 kV), an HW-2000 chromatography workstation, and a UV–visible detector (double light beams, 190–720 nm, set at 238 or 260 nm).

The running buffer used for MEKC was prepared daily from 100 mM borate and 100 mM SDS, and then adjusted to the desired pH by addition of either 0.2 M NaOH or 0.2 M HCl. A PHS-3B meter (Shanghai Precision and Scientific Instrument, Shanghai, China) was used for pH measurement. All solutions for CE were filtered through a 0.45-μm syringe filter (Shanghai Xinya Purification Apparatus Factory, Shanghai, China) before use.

Compounds were separated in fused silica capillary tubing of 75 μm i.d., 375 μm o.d. and 50.0 cm long (41.5 cm to the detector) (Yongnian Photoconductive Fiber Factory, Hebei Province, China). Before the first use the capillary was rinsed with 0.1 M NaOH for 5 min, distilled water for 5 min, and running buffer for 5 min. Between electrophoretic runs, the capillary was rinsed successively with distilled water (2 min), 0.1 M NaOH (2 min), distilled water (2 min), and then running buffer (2 min). The buffer was renewed after every three runs for good repeatability.

Samples were introduced from the anodic end of the capillary by hydrodynamic injection in which the sample vial was raised by 15.5 cm for 5 s. All procedures were conducted at room temperature.

Validation of the Method

Calibration plots were obtained by fourfold analysis of each of seven calibration solutions. The limit of detection (LOD) was calculated as the analyte concentration for which the signal (peak area)-to-noise ratio was 3. To assess the repeatability of migration times and peak areas of the method under the optimum conditions, precision (reported as RSD, %) was tested by analysis (n = 5) of a standard mixture containing 100 μg mL^{-1} X1 and X2. Recovery was determined by the method of standard additions. X1 and X2 were added to extracts of *Gentianopsis paludosa* of known concentration and analysis of the extracts was repeated.

Results and Discussion

Development of the Analytical Method

The dissociation constants (pK_a) of X1 and X2 were determined as 8.59 and 8.94, respectively [17], so CZE should be a very appropriate method for separation. Yang et al. had determined X1 and X2 in *Swertia przewalskii pissjauk* by using 25 mM borate (pH 9.0) as running buffer [17], but in our preliminary experiment separation of X1 and X2 could not be achieved by CZE. The electropherogram obtained from a standard mixture of X1 and X2 by using 20 mM borate (pH 9.8) as running buffer is shown in *Fig. 2*. The peak

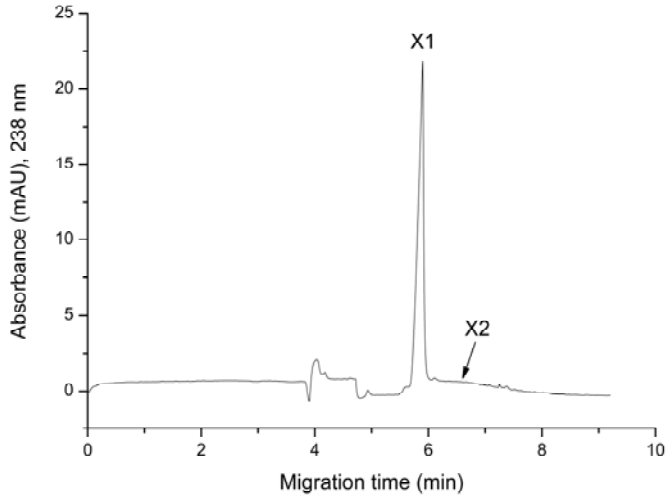

Fig. 2. Electropherogram obtained from a standard mixture of 200 μg mL^{-1} X1 and X2 in methanol by CZE. The electrophoretic buffer was 20 mM borate, pH 9.8, and the applied potential was 15 kV

obtained for X1 was obviously Gaussian in shape, and therefore satisfactory, but that obtained for X2 was very wide and only slightly higher than the baseline. Adjustment of the pH and concentrations of the borate solution did not improve the peak shape of X2. It was therefore concluded that CZE was unsuitable for separation of these two xanthones.

Nonaqueous capillary electrophoresis (NACE) was also attempted as separation method. With 10 mM ammonium acetate and 0.5% acetic acid in methanol as running buffer, Gaussian peaks were obtained for both X1 and X2, but their migration times were identical and their poor resolution could not be improved by changing the concentrations of ammonium acetate and acetic acid.

To develop an appropriate method for separation of X1 and X2, calculations were performed to find the difference between the two xanthones. From the geometries of the two molecules optimized by molecular mechanics using the MM+ force field we found the distance between 1-OH and 8-OH of X2 was 4.64774 Å, but that between 1-OH and 7-OH of X1 was 7.19412 Å. These different distances between the hydroxyl groups of the two xanthones should lead to different interactions with water molecules in aqueous solution *(Fig. 1)*; the two hydroxyl groups of X1 can freely interact with more water molecules than those of X2. This explain why X2 is more hydrophobic than X1. The different peak shapes in *Fig. 2* confirm this deduction. MEKC should, therefore, be a more suitable method for separation of the two xanthones with different hydrophobicity in aqueous solution, and MEKC resulted in clear improvement of the peak shape of X2, as a result of adding SDS to the running buffer electrolyte.

Effect of SDS Concentration

The effect of SDS concentration in the range 5 to 40 mM on the migration behavior of the two xanthones was investigated by use of 20 mM borate (pH 9.8) as the buffer electrolyte. The effect of SDS concentration on the peak heights of X1 and X2 is illustrated in *Fig. 3*, from which it is clearly apparent that increasing the concentration of SDS resulted in improvement of the peak shape of X2, with gradually increasing peak height, whereas that of X1 became broader and deteriorated seriously. The migration time of X2 was greater than that of X1. These experimental results indicated there was greater affinity between X2 and the SDS micelles, which was consistent with the above deduction based on the geometry of the two molecules.

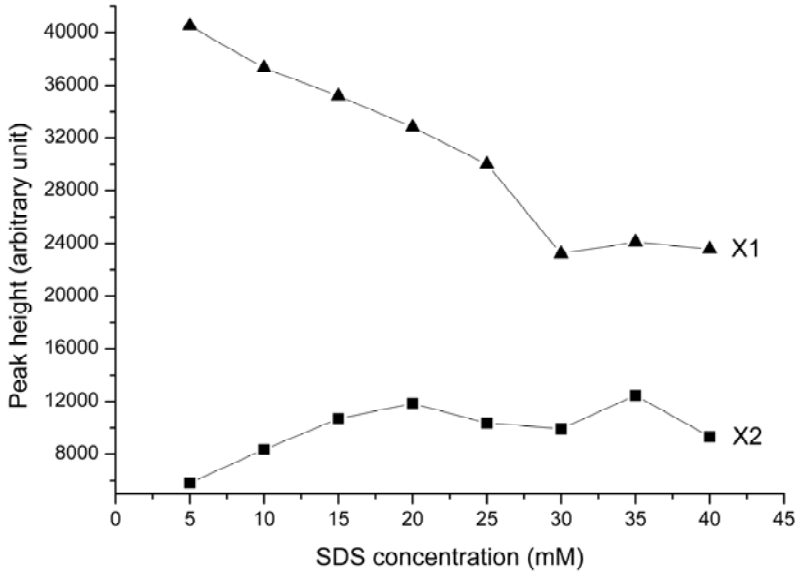

Fig. 3. Effects of SDS concentration on the peak heights of X1 and X2. Electrophoretic buffer, 20 mM borate, pH 9.8; capillary, 75 μm i.d. × 375 μm o.d. × 50 cm length (41.5 cm to detector); applied potential, 15 kV; detection wavelength, 238 nm; hydrodynamic injection, 5 s (capillary is raised to 15.5 cm); room temperature

The migration times of the xanthones increased rapidly with increase in SDS concentration from 5 to 25 mM, then decreased slightly when the SDS concentration was greater than 25 mM. As a compromise between peak height and migration time, 20 mM SDS was chosen for further study.

Effect of Running Buffer pH

The pH of the running buffer had an important effect on the migration behavior of the ionic components. The effect of buffer pH in the range 8.3–10.3 on the efficiency of separation of the two xanthones is illustrated in *Fig. 4*. At pH < 9.3 the migration times of X1 and X2 increased slowly; at pH > 9.3, however, they increased dramatically, possibly because increasing ionization of X1 and X2 with increasing pH resulted in an increase in the absolute mobility to the anode, thereby increasing the migration time to the cathode. Buffer pH also had an obvious effect on the sensitivity (expressed as peak heights) of X1 and X2. At pH < 9.8 peak areas and peak heights increased with increasing buffer pH, whereas when the pH was >9.8, sensitivity dete-

riorated seriously. Resolution of X1 and X2 was clearly improved by increasing buffer pH. To obtain short migration time, and high sensitivity and resolution, 9.8 was chosen as the optimum pH.

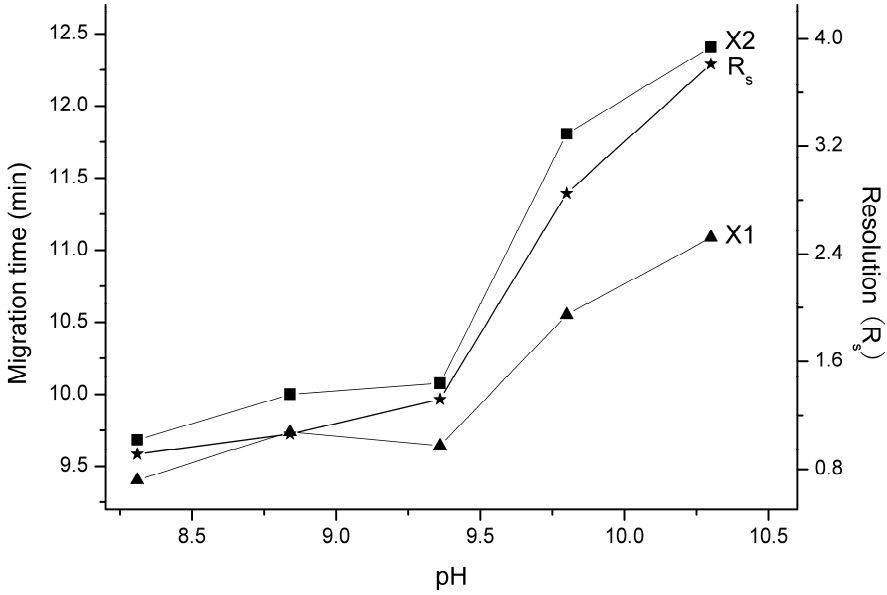

Fig. 4. Effect of pH on migration times and resolution (R_S) of X1 and X2. The electrophoretic buffer was 20 mM borate + 20 mM SDS; other conditions are as for *Fig. 3*

Effect of Borate Concentration

The concentration of the buffer solution had a substantial effect on the separation owing to its effect on viscosity and electroosmotic flow. The effect of borate concentration in the range 10 to 35 mM on the migration behavior of X1 and X2 is illustrated in *Fig. 5*. It is clearly apparent that the migration times of the two xanthones increased with increasing borate concentration. Resolution also increased with increasing borate concentration, but sensitivity decreased gradually, owing to high Joule heating. Finally, 20 mM borate was selected for subsequent study.

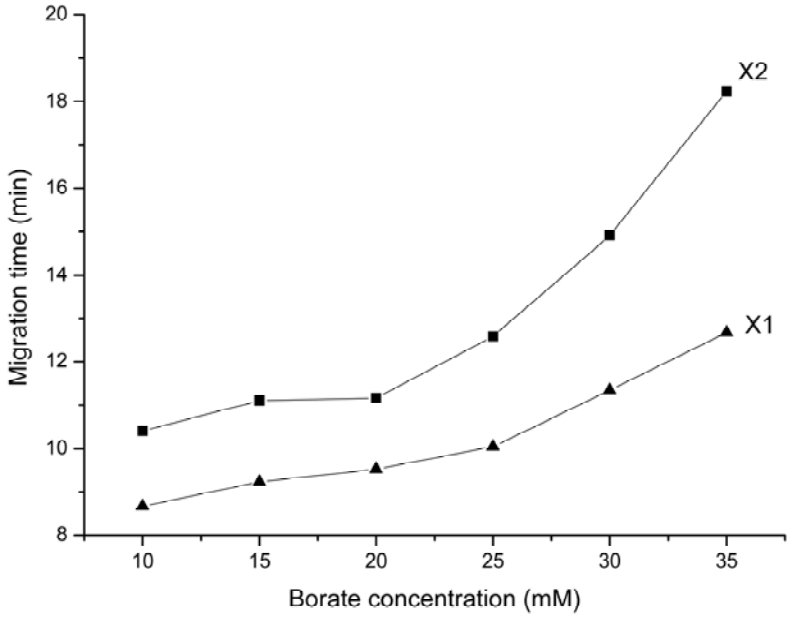

Fig. 5. Effect of borate concentration on the migration times of X1 and X2. The buffer was 20 mM SDS, pH 9.8; other conditions are as for *Fig. 3*

Effect of Applied Potential

The effect of the applied potential on the migration times and resolution of X1 and X2 was also studied. With increasing potential, migration times decreased, but sensitivity and resolution also decreased. Finally, 17.5 kV was selected as the appropriate separation potential.

Effect of UV Detection Wavelength

Maximum UV absorbance of both X1 and X2 is at 238 and 260 nm [4]. In a preliminary study, 238 nm was selected as the detection wavelength, but when this wavelength was used for analysis of real samples of *Gentianopsis paludosa,* too many unknown interfering peaks were observed, so 260 nm was eventually adopted as the detection wavelength for analysis of real samples.

On the basis of the experiments reported above, the optimum separation conditions for X1 and X2 were: 20 mM borate + 20 mM SDS (pH 9.8) as running buffer, applied potential 17.5 kV, and detection wavelength

260 nm. A typical electropherogram obtained from a standard mixture containing 100 μg mL^{-1} X1 and X2 under the optimum conditions is illustrated in *Fig. 6*A. The two xanthones were well separated within 9.0 min, and their peak shapes were quite satisfactory.

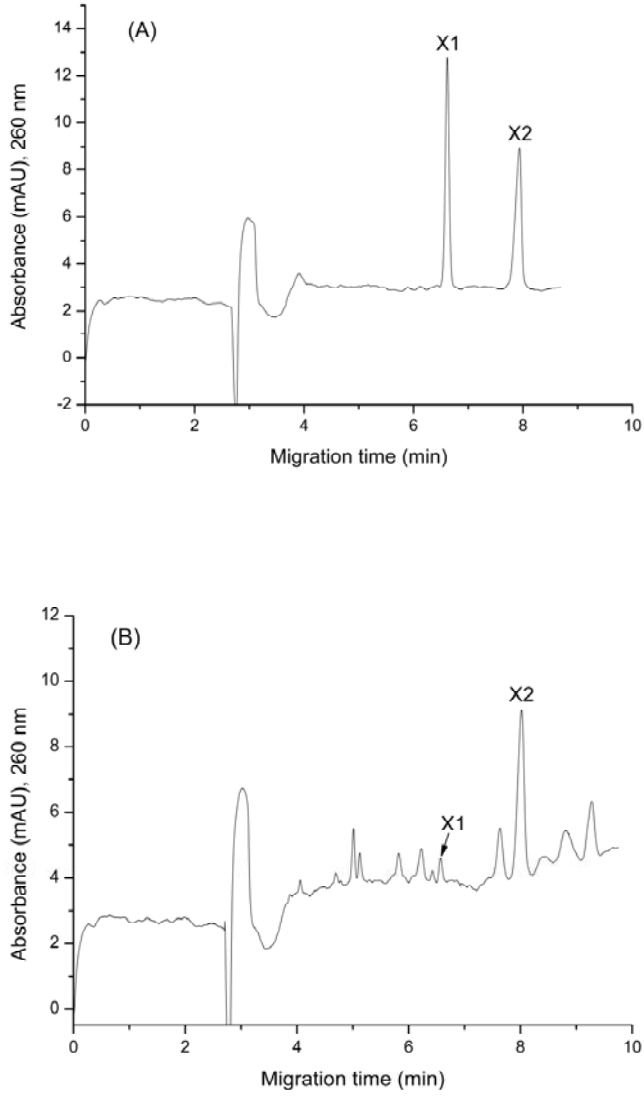

Fig. 6A, B

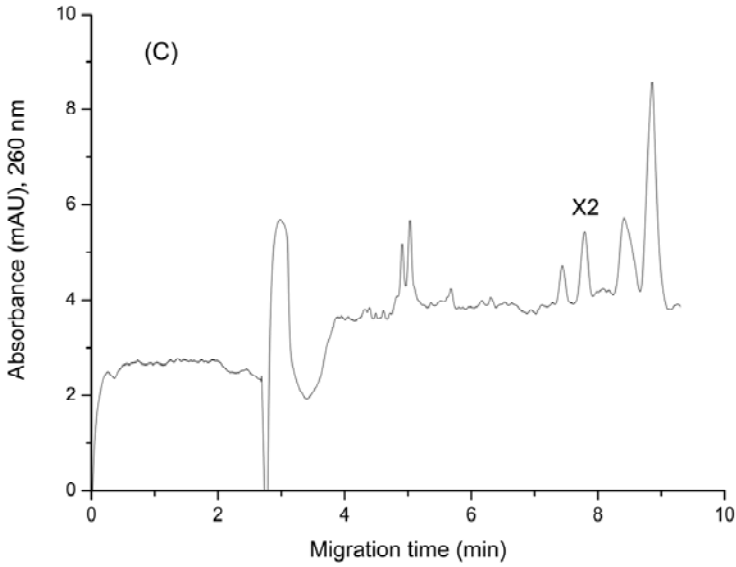

Fig. 6. Electropherograms obtained from: (A) mixed standard (100 μg mL^{-1}) of X1 and X2; (B) extract of flowers of *Gentianopsis paludosa* diluted 20-fold with methanol; (C) extract of stems of *Gentianopsis paludosa* diluted 20-fold with methanol. Electrophoretic buffer, 20 mM borate + 20 mM SDS, pH 9.8; applied potential, 17.5 kV; detection wavelength, 260 nm; other conditions are as for *Fig. 3*

Validation of the Method

The regression equations obtained from calibration, other regression data, and other results from method validation are summarized in *Table I*. The calibration results are indicative of an excellent linear relationship ($r > 0.9996$) between peak area and concentration of the standard. In this study, the linear ranges for X1 and X2 were 1.56–200.00 and 0.78–200.00 μg mL^{-1}, respectively; in the reported CZE method [17], the linear ranges were 50–372 and 5-500 μg mL^{-1}, respectively. This MEKC method clearly enables more sensitive analysis of both X1 and X2. The LOD were 0.41 and 0.82 μg mL^{-1} for X1 and X2, respectively.

Results from determination of recovery are listed in *Table II*. These data confirm the suitability of this MEKC method for application to real samples.

Table I. Performance characteristics of the MEKC method for analysis of X1 and X2 in standard solutions

	X1	X2
Calibration range[a] (μg mL^{-1})	1.56–200.00	0.78–200.00
Limit of detection (μg mL^{-1}) (S/N = 3)	0.41	0.82
Regression equation[b]		
Intercept (a)	−1821	−1724
Slope (b)	1034	1171
Correlation coefficient (r)	0.9996	0.9998
Repeatability (expressed as RSD)[c]		
Migration time	0.9%	1.1%
Peak area	3.1%	1.4%

[a]Seven data points, four replicates at each concentration

[b]$y = a + bx$, where y and x denote peak area and the concentration (μg mL^{-1}) of the two xanthones, respectively

[c]Five replicates

Table II. Results from determination of the recovery of X1 and X2 from *Gentianopsis paludosa* (n = 4)

Extract		Content (mg g^{-1})	Amount added (μg mL^{-1})	Recovery (%)
Flower	X1	0.033	7.00	98.9
			3.00	98.7
			1.00	101.0
	X2	1.43	80.00	103.5
			40.00	108.2
			20.00	91.9
Stem	X2	0.29	12.00	101.2
			25.00	97.4
			50.00	102.7

Application of the Method

This MEKC method was used for analysis of X1 and X2 in extracts of the flowers and stems of the Tibetan medicinal plant *Gentianopsis paludosa*. The peaks were identified by spiking the extracts with small amounts of standard solutions of each xanthone, and by comparing their migration times

with those of the standards. Typical electropherograms obtained from the flowers and stems of *Gentianopsis paludosa* are shown in *Figs 6B* and *6C*, respectively. The amounts of X1 and X2 measured, with RSD (n = 5), are listed in *Table III*. Obviously, amounts of X1 and X2 in the flowers were greater than those in the stems. The flowers of *Gentianopsis paludosa* are, therefore, a better source of X1 and X2.

Table III. X1 and X2 content of *Gentianopsis paludosa*, with RSD (n = 5)

Extract	X1		X2	
	Content (mg g^{-1})	RSD (%)	Content (mg g^{-1})	RSD (%)
Flower	0.033	3.0	1.43	2.1
Stem	–[a]	–[b]	0.29	2.3

[a]Not found
[b]Not calibrated

Conclusions

For the first time, a sensitive, accurate, and rapid MEKC method has been developed for simultaneous separation and analysis of the xanthones 1,7-dihydroxy-3,8-dimethoxyxanthone (X1) and 1,8-dihydroxy-3,7-dimethoxyxanthone (X2) in the Tibetan medicinal plant *Gentianopsis paludosa* (Hook. f.) Ma. On the basis of the different distances between the hydroxyl groups of the two xanthones, calculated by use of the MM+ force field, the two hydroxyl groups of X1 can freely interact with more water molecules in aqueous solution than those of X2, so X2 is more hydrophobic than X1. As a result, MEKC was an appropriate separation method, unlike CZE and NACE. The sensitivity of the method was higher than that of another reported method [17]. Assessment of precision and accuracy revealed the method was highly suitable for analysis of X1 and X2 in *Gentianopsis paludosa*. In addition, the results showed that the flowers of *Gentianopsis paludosa* were better sources of the two xanthones than the stems.

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