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Review

Separation methods for pharmacologically active xanthones

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Abstract

Xanthones, as a kind of polyphenolic natural products with many strong bioactivities, are attractive for separation scientists due to the similarity and diversity of their structures resulting in difficult separation by chromatographic methods. High performance liquid chromatography (HPLC) and thin layer chromatography (TLC) are traditional methods to separate xanthones. Recently, capillary electrophoresis (CE), as a micro-column technique driven by electroosmotic flow (EOF), with its high efficiency and high-speed separation, has been employed to separate xanthones and determine their physicochemical properties such as binding constants with cyclodextrin (CD) and ionization constants. Since xanthones have been used in clinic treatment, the development of chromatographic and CE methods for the separation and determination of xanthones plays an essential role in the quality control of some herbal medicines containing xanthones. This article reviewed the separation of xanthones by HPLC, TLC and CE, citing 72 literatures. This review focused on the CE separation for xanthones due to its unique advantages compared to chromatographic methods. The comparison of separation selectivity of different CE modes including capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic capillary chromatography (MEEKC) and capillary electrochromatography (CEC) was discussed. Compared with traditional chromatographic methods such as HPLC and TLC, CE has higher separation efficiency, faster separation, lower cost and more flexible modes. However, because of low sensitivity of UV detector and low contents of xanthones in herbal medicines, CE methods have seldom been applied to the analysis of real samples although CE showed great potential for xanthone separation. The determination of xanthones in herbal medicines has been often achieved by HPLC. Hence, how to enhance CE detection sensitivity for real sample analysis, e.g. by on-line preconcentration and CE-MS, would be a key to achieve the quantitation of xanthones.

Keywords: Review; Xanthones; Herbal medicines

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1. Introduction

Xanthones widely distributed in *Polygalaceae* family are a kind of natural products with polyphenolic structure, having many pharmacological effects such as MAO inhibition, antitumor activity, cytotoxicity, antibacterial activity, antifungal activity, anti-inflammatory properties, antioxidant activity and tuberculoatatic activity [1]. Due to their strong bioactivities, some herbal medicines in *Polygalaceae* family containing xanthones have been used as anti-inflammatory, anti-bacterial and anti-rheumatism agents in clinic [2]. Hence, to develop the separation and determination methods plays a very important role in the quality control of these herbal medicines. Existing methods for the analysis of xanthones involved high performance liquid chromatography (HPLC) [3,4] and thin layer chromatography (TLC) [5,6]. However, owing to the complicated components in Chinese herbal medicines, HPLC requires gradient elution that tends to be inconvenient, time consuming and often results in interference with other unknown constituents. TLC is not recommended for quantitative analysis due to its poor reproducibility and accurateness. The improvements in capillary electrophoresis (CE) are attractive for the studies of natural products, and illustrate good suitability for the analysis of Traditional Chinese Medicines because of high separation efficiency, short analysis time, less sample consumption, low cost, ease of mode change-over and column regeneration. There have been many publications in the literature [7–11] in this field. Recently, some new CE techniques have been developed, such as microemulsion electrokinetic capillary chromatography (MEEKC) and capillary electrochromatography (CEC) that can provide powerful tools for the separation of natural products [12,13]. Due to the unique characteristics of xanthone structures, it is also very interesting to use xanthones as model compounds to investigate the mechanisms of CE separation.

The separation of xanthones by CE has been investigated in our group in past 3 years, especially by the new techniques of CE. The separation for 10 xanthones (see Fig. 1 for the chemical structures of 10 xanthones) was mainly reviewed in this paper based on our work, combined with the studies of other researchers. The separations of xanthones by different CE modes were compared and the determinations of some physicochemical parameters of xanthones such as dissociation constants and binding constants with cyclodextrins were also introduced.

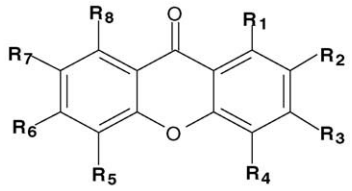
2. Chromatographic methods

2.1. High performance liquid chromatography (HPLC)

HPLC has been widely accepted as a routine chromatographic method for xanthone separation and many literatures have been involved in this field [3,4,14–22]. For most cases, the mixture of water and methanol was used as mobile phase, UV detector or MS, and C_8 or C_{18} column were adopted. These HPLC methods were applied to the analysis of xanthones in herbal medicines and drug preparations, and to monitor the process of extract, isolation and purification of xanthones.

2.2. Thin layer chromatography (TLC)

TLC is another important separation tool for xanthones with cheaper and simpler procedure than HPLC, although TLC has lower reproducibility and accurateness as compar-



No	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	*LogP
1	HO	MeO	MeO	H	H	H	HO	H	2.30
2	MeO	H	H	MeO	H	H	HO	H	2.69
3	MeO	HO	H	H	H	H	MeO	H	2.69
4	H	H	MeO	HO	H	H	MeO	H	2.69
5	MeO	HO	H	H	H	H	HO	H	2.17
6	HO	H	H	MeO	H	H	HO	H	2.17
7	HO	H	HO	MeO	H	H	HO	H	1.78
8	HO	MeO	HO	H	H	H	HO	H	1.78
9	HO	H	HO	H	H	H	HO	H	1.65
10	HO	MeO	MeO	H	H	HO	HO	H	1.91

Fig. 1. The chemical structure of 10 xanthones. log *P* denotes the logarithms of *n*-octane–water partition coefficient of xanthones.

ing to HPLC [5,23–28]. Cellulose, silicone, the moiety of monohydroxyphenyl and *o*-dihydroxyphenyl have been employed as separation mediums for xanthones in TLC [23–28].

2.3. Gas chromatography (GC)

As early as 1971s, Jefferson et al. [29] utilized gas chromatography (GC) to determine the derivatives of natural and synthetic xanthones on a packed column. Recently, Oba et al. [30] made a confirmation of the xanthone structures in thermally treated polycarbonates by reactive pyrolysis-gas chromatography–mass spectrometry (GC–MS). Nevertheless, due to tough pretreatment and analyte volatility limitation, GC has seldom been used for xanthone separation.

3. Electromigration methods

As a micro-column separation technique, CE has been just developed for xanthone separation recently. Yang et al. [31] determined the three xanthones in *Swertia przewalskii pissjauk* by CZE within 5 min with satisfactory recovery and linearity, using a running buffer of 25 mM disodium tetraborate at pH 9.0. In our laboratory, a series of CE methods have been established for the separate of 10 xanthones (Fig. 1) from *Securidaca inappendiculata Hassk* and the optimization of separation conditions has been systematically studied.

3.1. CZE separation of xanthones

Since its first introduction by Jorgenson and Lukacs [32], CZE becomes an important method for the separation of charged natural products, especially for the natural products with polyphenolic structures such as flavonoids, coumarin and organic acids, etc. [33–35]. A fast CZE method was reported in 2002 for the separation of 10 xanthones [36,37]. Based on the systematically optimization of such parameters as pH, concentration of running buffers, addition of sulfated β-CD, applied voltage and column temperature, the optimal separation was achieved for the 10 xanthones in less than 15 min with excellent separation efficiency, using a background electrolyte consisting of 200 mM borate (pH 9.5) and 10 mM sulfated β-CD under 30 kV applied voltage and 40 °C temperature, as shown in Fig. 2A. The study showed the addition of sulfated β-CD was essential to improve the overall resolution of 10 xanthones [37]. In addition, β-CD was also added to running buffer and showed totally different selectivity compared with sulfated β-CD (see Fig. 2B) [38].

In the last decade, nonaqueous CE (NACE) has been successfully employed to separate charged solutes with higher separation efficiency and better separation selectivity, especially for higher hydrophobic analytes [39–41]. However, our study demonstrated that the NACE provided with insufficient resolution for 10 xanthones, as shown in Fig. 2C [42]. It was speculated that the difference of dissociation constants of 10 xanthones in organic solvent tends to decrease, resulting in the poor resolution.

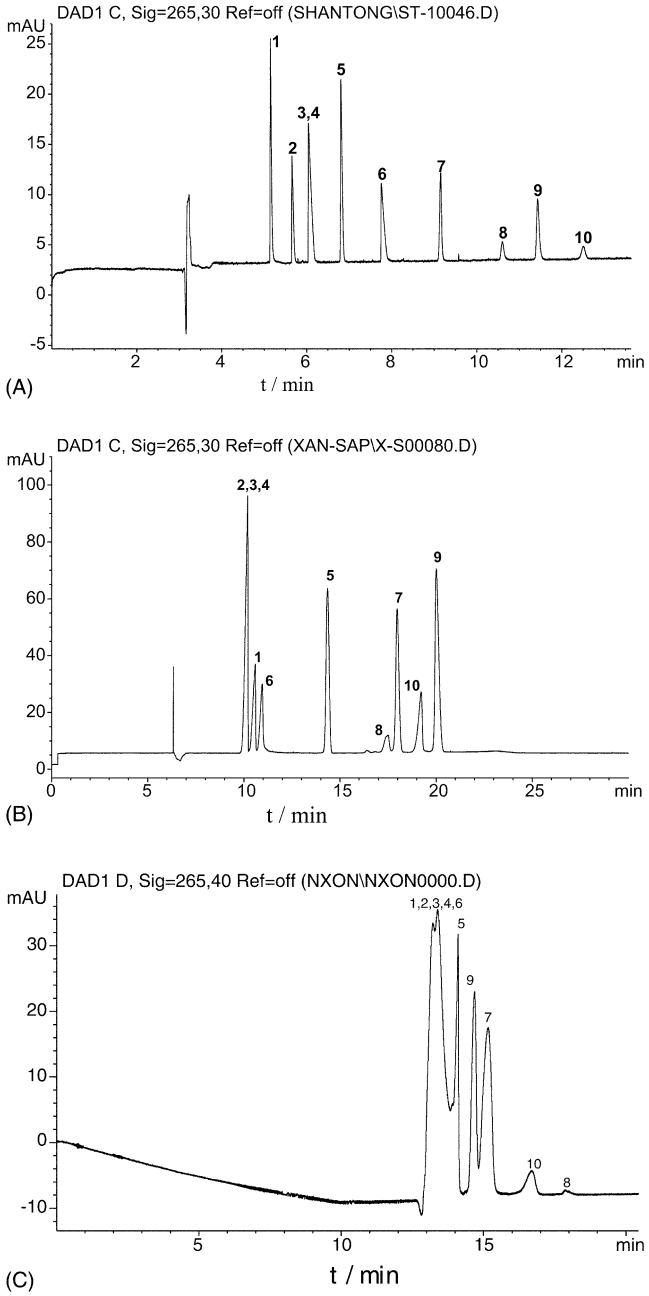


Fig. 2. The optimum electropherograms of 10 xanthones by CZE. (A) Sulfated β-CD as additive; (B) β-CD as additive; (C) NACE. Conditions: (A) buffer, 200 mM borate (pH 9.5) containing 10 mM sulfated β-CD; applied voltage 30 kV; temperature 40 °C; detection, UV at 265 nm. See Fig. 1 for peak identification. (B) Buffer, 30 mM borate (pH 10) containing 10 mM β-CD; applied voltage, 18 kV; temperature, 25 °C; detection, UV at 265 nm. See Fig. 1 for peak identification. (C) Methanol containing 12.5 mM acetate sodium and 1.5 mM acetic acid under applied voltage, 18 kV; temperature, 25 °C; detection, UV at 265 nm; 58.5 cm × 50 μm i.d. fused silica capillary for A and C; 58.5 cm × 100 μm i.d. fused silica capillary for B.

3.2. MEKC separation of xanthones

As a powerful analytical method, MEKC enables excellent separation of both charged and electrically neutral analytes [43,44]. The separation in MEKC is based on the difference

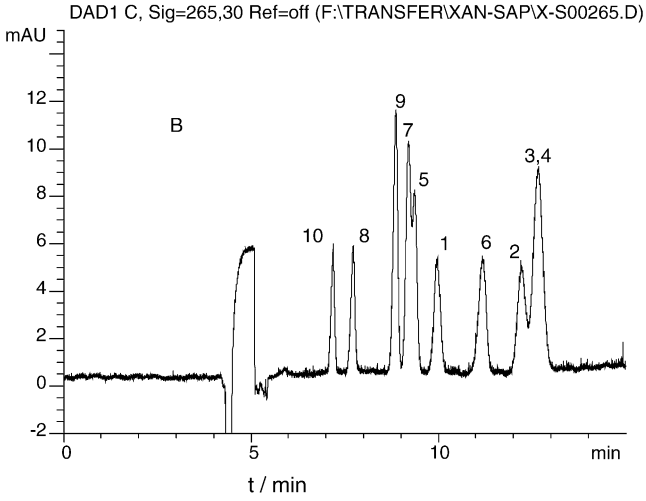


Fig. 3. MEKC separation for 10 xanthones with 5 mM β-CD as additive. Conditions: 58.5 cm × 50 μm i.d. fused silica capillary; 100 mM borate buffer (pH 10.5) containing 60 mM SDS; applied voltage, 20 kV; temperature, 35 °C; detection, UV at 265 nm. The peak numbers are correspondent to the xanthones numbered in Fig.1.

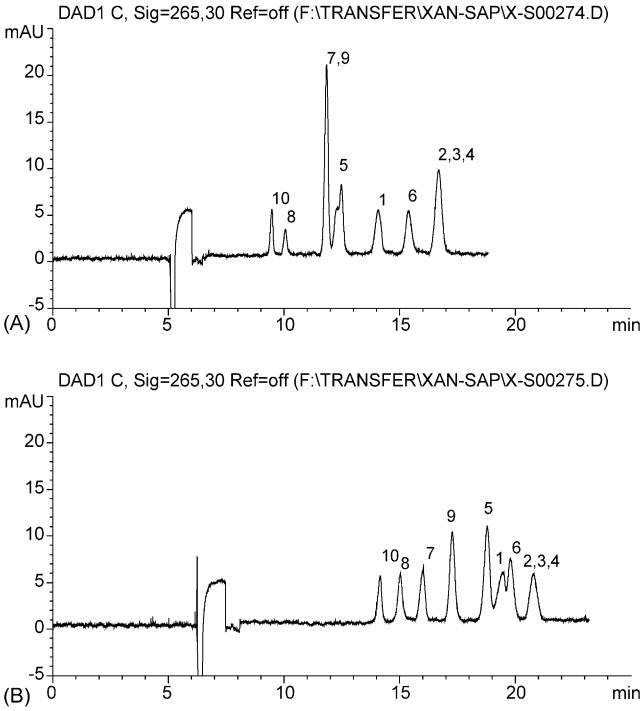


Fig. 4. Effect of the addition of methanol on the separation of xanthones with 5 mM β-CD as additive. (A) 5% (v/v) methanol; (B) 10% (v/v) methanol. Condition: 100 mM borate buffer (pH 10.5) containing 60 mM SDS and 5 mM β-CD; applied voltage, 20 kV; temperature, 35 °C; detection, UV at 265 nm. The peak numbers are correspondent to the xanthones numbered in Fig. 1.

in the partitioning of analytes between the micellar and aqueous phase. Thus, the separation selectivity can be manipulated by the modification of the micellar phase or aqueous phase [45,46]. To modify the aqueous phase, organic solvents and cyclodextrins are often added to the aqueous buffer system. On the other hand, the micellar phase can also be modified through the use of mixed micelles. In our laboratory, a MEKC method was also developed for the separation of xanthones [47]. The investigation indicated that 10 xanthones could be well separated within 15 min by using 100 mM borate buffer (pH 10.5) containing 60 mM SDS and 5 mM β-CD, although the overall resolution by MEKC was somewhat lower than that by CZE (see Fig. 3) [48]. It was observed that the additions of CDs and organic solvents played an important role in the separation of xanthones. Additions of 5 mM β-CD and 10% (v/v) methanol in the buffer system could achieve the baseline separation for four xanthones with lower hydrophobicity (see Fig. 4). The dependence of the migration order of those xanthones on their structures and solute–micelle-modifier interactions were also discussed in detail by using the logarithms of *n*-octane–water partition coefficient (log *P*) as a structural parameter [47]. The study showed that the hydrophobicity of 10 xanthones was closely associated with the MEKC separation, and the separation selectivity of these xanthones with lower hydrophobicity was easier to be affected by electrophoretic condition variations.

3.3. MEEKC separation of xanthones

Microemulsion electrokinetic capillary chromatography (MEEKC) is an electrodriven separation technique, which can generate highly efficient separations of both charged and neutral solutes [49–51]. This technique separates solutes based on their hydrophobicities and electrophoretic mobilities. The microemulsion buffers used in MEEKC are composed of minute water-immiscible oil droplets suspended in an aqueous buffer, and solutes partition between the oil droplet and the aqueous buffer phases. Water-insoluble compounds will favor inclusion into the oil droplet rather than into the buffer phase, making the partition of the solute possible in a chromatographic manner. Hydrophobic solutes will reside more frequently in the oil droplet than water-soluble solutes. The separation basis is similar to MEKC, but solutes are more easily able to penetrate the surface of the droplet than the surface of a micelle [52], allowing MEEKC to be applied to a wider range of solutes than MEKC.

The theory of MEEKC has been thoroughly reviewed by Altria [53]. Recently, a high-speed MEEKC separation has been reported to greatly reduce the analytical time [54]. The reported applications of MEEKC cover a wide range of analytes, such as derivatized sugars, polycyclic aromatic hydrocarbons, proteins, hop bitter acids, agrochemicals, vitamins, kentones, analgesics/cold medicine ingredients, steroids, basis and acidic drugs, pharmaceutical excipients, cardiac glycosides, natural products, chiral drugs, urine, dyes, and fatty acid esters [53,55,56]. It was also reported that some active components, e.g. saponins and isoquinoline alkaloids, in traditional Chinese medicines were successfully separated by MEEKC [57,58].

Ten xanthones were used in our laboratory to systematically study the influence of operation variables including the composition of emulsion, buffer pH, different modifiers, the capillary temperature and the applied voltage on the selectivity in MEEKC [59]. Some parameters including logarithm of *n*-octanol–water partition coefficient (log *P*), resolution (*R*), peak symmetry factor (*f*s) and theoretic plant number (*n*) were employed to evaluate separation selectivity under various operating conditions, and the separation mechanisms were elucidated and compared with MEKC [60]. Based on the optimization, the best separation for 10 xanthones was obtained by using 50 mM borate buffer (pH 9.5) containing 10% (v/v) *n*-butanol, 80 mM *n*-heptane, 5 mM sulfated β-CD and 120 mM SDS (see Fig. 5 C) [59]. The investigation showed 5 mM sulfated β-CD as an additive could greatly enhance the overall resolution of 10 xanthones with a moderate separation time and showed better separation than that with β-CD as modifier (see Fig. 5) [60]. What should be emphasized here is that operation variables could have important effects on the separation efficiency of xanthones [60]: (1) in all pH range studied from 8.0 to 10.5, the xanthone 7–10 with lower hydrophobicity kept good peak shapes and separation efficiency. However, at pH 8.0–9.0, the peak shapes of xanthone 1–6 with higher hydrophobicity were very poor, which were obviously improved with the increase of pH values. (2) For most xanthones, the addition of organic solvents such as methanol, acetonitrile, propan-2-ol and tetrahydrogenfuran could effectively increase the theoretical plate numbers of analytes but the resolution was not obviously improved. (3) 10 mM sodium cholate (SC), 10 mM cetyltrimethylammonium bromide (CTAB), 5% (v/v) Tween-80 and Triton X-100 were respectively added into the microemulsion system to form mixed surfactant for studying the changes of selectivity. Unfortunately, the addition of SC destroys the overall separation and the peak shape, resulting in very poor resolution of all xanthones. With the addition of CTAB, the migration times of xanthones decreased slightly because low concentration CTAB can reduce the charge density on the droplets. What is more, the addition of CTAB increased the theoretic plant number of all analytes to a great extent. The addition of Tween-80 obviously narrowed the detection window in MEEKC and decreased the migration times of xanthones 5–9. Tween-80 is a kind of neutral surfactant and can make these xanthones more soluble in the aqueous phase and reduce the affinity to the droplets, resulting in the decrease in migration times of xanthones 1–6. Adding Tween-80 greatly improved the theoretic plant numbers of the analytes with higher hydrophilicity such as xanthone 7–10. On the contrary, the addition of Tween-80 decreased the separation efficiency of xanthone 1–6 with lower hydrophilicity. Similarly, the addition of Triton X-100 deteriorated the separation of xanthone 1–6, but dramatically increased the theoretic plant number of xanthone 7–10. (4) Like in CZE, higher applied voltage represented higher efficiency for most analytes. However, the increase of capillary temperature reduced the separation efficiency for xanthones. (5) The study also showed that CDs as modifiers could affect the separation efficiency.

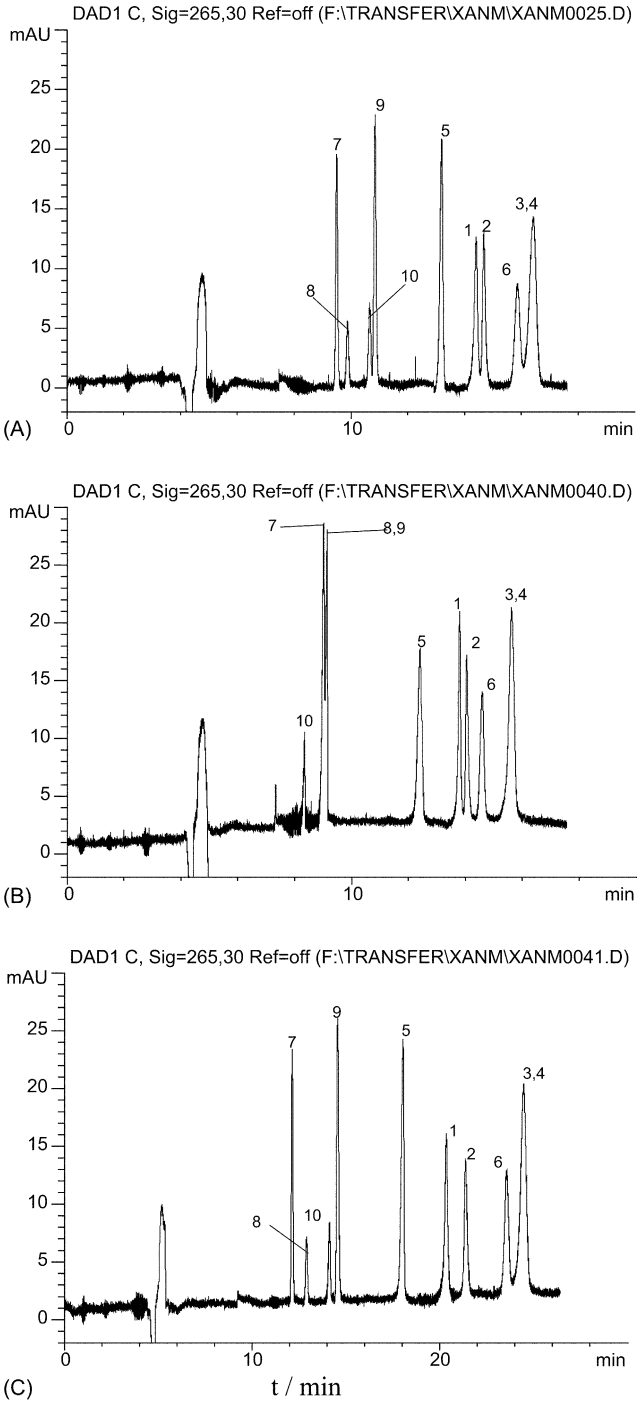


Fig. 5. MEEKC separation for 10 xanthones. (A) Without additive; (B) 5 mM β-CD as additive; (C) 5 mM sulfated β-CD as additive. Condition: 48.5 cm × 50 μm i.d. fused silica capillary; 50 mM borate buffer (pH 9.5) containing 10% (v/v) *n*-butanol, 80 mM *n*-heptane and 120 mM SDS under 25 °C temperature and 25 kV applied voltage. See Fig. 1 for peak identification.

In fact, the change of separation efficiency in MEEKC is closed related to the dynamic process. An equation used in

MEKC can be applied to the MEEKC:

$$H_{tot} = H_1 + H_m + H_{aq} + H_T + H_{ep}$$

where H_{tot} is overall plate height, and H_1, H_m, H_{aq}, H_T and H_{ep} are plate heights generated by longitudinal diffusion, sorption–desorption kinetics in micellar solubilization, inter-micelle mass transfer in the aqueous phase, radial temperature gradient effect on the electrophoretic velocity of the micelles and dispersion of electrophoretic mobilities of the micelles, respectively. Among these five factors, H_1, H_m and H_{ep} were found to contribute significantly to band broadening [61]. The longitudinal diffusion decreases with an increase in the EOF, whereas H_m and H_{ep} increase linearly with the increase in the EOF [61]. The same discussion is also applicable to MEEKC. What is dominant would determine the change of separation efficiency when operation conditions were changed.

3.4. CEC separation of xanthones

Capillary electrochromatography (CEC) is a powerful separation technique combining, in principle, advantages of HPLC and capillary electrophoresis [62–65]. CEC uses electroosmotic flow to drive the mobile phase that transports the solutes through the chromatographic column. Separation can be achieved by differential partition between two phases, differential electromigration or a combination of these two. However, much work will still be necessary if CEC can be accepted as a routine analytical technique and a viable alternative to CE and HPLC [66,67]. We explored the possibility of separating 10 xanthones by CEC on a C18 column [48,68]. The best separation was achieved by using 25 mM acetic acid buffer solution (pH 4) with 50% acetonitrile. In addition, the study revealed that percentage of acetonitrile in mobile phase was very important for the separation. Unfortunately, compared with CZE, MEKC and MEEKC, CEC did not provide acceptable separation for 10 xanthones.

3.5. Comparison of different CE modes for xanthone separation

3.5.1. Separation selectivity

According to the descriptions above, there exist great differences in separation selectivity and analysis time for xanthones when adopting different CE modes. MEEKC showed the longest analysis time and the largest detection window (analysis time scale from first emerging peak to last one) compared with others. The baseline separation of nine xanthones can be successfully completed by CZE and MEEKC. Especially for CZE, the fastest analysis was achieved within 12.5 min. As for MEKC and CEC, however, the baseline separation cannot be achieved. It was also demonstrated that the peak order was greatly varied when using different CE modes due to different separation mechanisms. It should be noted that MEEKC showed stronger separation ability for xanthones than MEKC.

It can be seen that peak order is fluctuated between different separation modes. The reason is the separation mechanisms are more or less different from each other. In CZE separation, the difference in xanthone mobilities resulted from the dissociation constants discrepancy of phenolic hydroxyl groups and the difference in binding constants between xanthones and sulfate β-CD. Therefore, xanthone 7–10 with three hydroxyl groups were more negatively charged than xanthone 1–6 in the running buffer, thus were eluted at last. Whereas, in MEKC and MEEKC, hydrophobicity, which can be represented by log P, plays essential role in the separation, in addition to the ionization of analytes. The xanthones can be divided into two groups according to their log P. The xanthones 1–6 with higher log P values had slower mobility due to their stronger affinity to the oil droplet or micelle. Whereas, the xanthones 7–10 with lower log P values eluted faster because of less reaction with the oil droplet or micelle. MEEKC showed better separation ability for xanthones than MEKC, especially for those with similar hydrophobicity. It can be well explained that the surface of the droplet was much more easily penetrated by solutes than the surface of a micelle, since the latter is more rigid than the former, allowing MEEKC to be applied to a wider range of solutes than MEKC. CEC is a recently developed technique, but it could not show better separation power for xanthones.

3.5.2. Separation efficiency

Theoretic plate number and symmetry factor by using different CE modes for xanthone separation were compared and the data are listed in Table 1. The data indicated that CZE showed the highest theoretic plate numbers for xanthones but obtained the lowest symmetry factor, compared with other CE modes. MEKC demonstrated the lowest separation efficiency with theoretic plate number below 100,000/m. MEEKC showed higher theoretic plate numbers similar to CZE, and better symmetry factors. However, as a combination of CE and HPLC, CEC has not show any advantage in the respect of separation efficiency.

Table 1
The theoretic plant number (m^{-1}) and symmetry factor of nine xanthones by using different CE methods under optimal conditions

Xan	CZE	MEKC	MEEKC	CEC
1	300700 (0.29)[a]	29014 (1.16)	210658 (1.30)	–
2	208392 (0.22)	25698 (0.68)	223113 (0.72)	26578 (0.92)
3, 4	73676 (0.12)	21274 (1.12)	81820 (1.31)	–
5	307802 (0.29)	54134 (0.71)	187565 (0.90)	140088 (0.94)
6	132989 (0.14)	26904 (0.94)	125463 (0.89)	–
7	397038 (0.97)	39095 (1.12)	252080 (0.99)	22588 (2.03)
8	231604 (0.76)	50749 (1.25)	193655 (0.97)	31785 (2.14)
9	325794 (0.82)	49642 (1.45)	228473 (0.79)	152898 (1.26)
10	195496 (1.01)	59222 (1.34)	192090 (1.47)	146633 (0.89)

[a] The data in the parentheses denotes symmetry factor. See Fig. 1 for xanthone number. See Section 3 for the optimal conditions of different CE modes.

3.5.3. Repeatability of migration or retention time

The repeatability of migration or retention times in CZE separation was tested by using a pair of BEG vials for consecutive analysis. The investigation demonstrated that the relative standard deviation (R.S.D.) for migration times of xanthones was below 1%. MEKC, MEEKC and CEC also demonstrated satisfactory R.S.D. (<7%).

3.5.4. Comparison between CE and HPLC

A HPLC method for the separation of 10 xanthones was developed for comparison with CE methods [48,69]. As shown in Fig. 6, the baseline separation of xanthones was achieved using linear gradient elution within 40 min, but the analytical time is much longer than CE methods mentioned above, demonstrating that CE is a more suitable tool than HPLC for the separation of xanthones. However, the detection limit of CE is not as high as that of HPLC because the on-line detection used in CE has narrow capillary diameter. Therefore, it will be interesting to investigate on-line preconcentration techniques for CE to improve its detection sensitivity. On the other hand, Yang [70] used TLC with silica as stationary phase and a mixture of methanol and chloroform as elution solvent for the separation for xanthones, showing an elution sequence of 10 xanthones as follows: 3, 4, 1, 6, 2, 10, 7, 8, 5 and 6 [70]. It was very interesting that xanthone 3 and 4, which could not be separated by any mode of CE and reversed-phase HPLC, could be separated by TLC. Consequently, two-dimensional chromatography or on-line coupling of HPLC and CE would be studied to completely separate these xanthones.

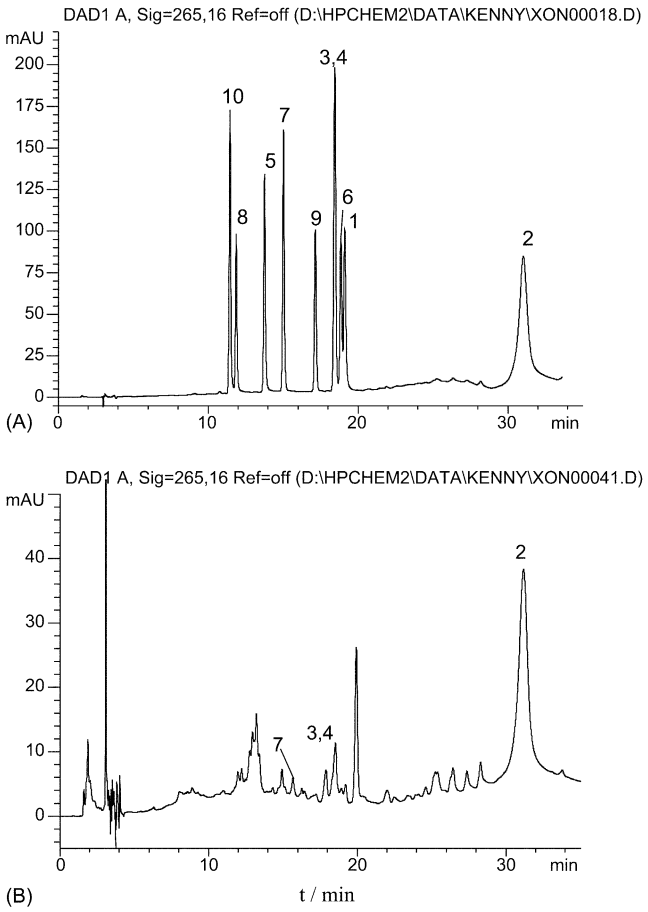


Fig. 6. HPLC separation for xanthones. (A) Xanthone standards solution; (B) real sample solution. Condition: A ZORBAX RX-C_8 (4.6 mm × 25 cm, 5 μm) (Agilent Technologies, CA, USA) was used; UV detection was set at 265 nm; the mobile phase was composed of two solvents, water and acetonitrile, and a linear gradient program was developed; the volume percentage of acetonitrile was linearly changed from 5 to 95% within 30 min, and then 95% acetonitrile was maintained for 5 min; the flow-rate and the column temperature were set at 1.0 ml/min and 25 °C, respectively. See Fig. 1 for peak identification.

3.6. Mechanism elucidation of xanthone separation in CE

As described above, the addition of sulfate β-CD or β-CD in running buffer can obviously enhance the separation selectivity for 10 xanthones in CZE, MEKC and MEEKC. Hence, to investigate the separation mechanism in presence of sulfate β-CD or β-CD would be significantly important. Bo et al. [71] studied the quantitative structure-electromigration relationships (QSER) to elucidate the separation mechanism in CZE with sulfate β-CD or β-CD as additives. The binding constants (K) of CDs and xanthones are varied with different structures of CDs and xanthones, as shown in Tables 2 and 3 [37,38]. In addition, the structural descriptors of xanthone molecules in term of quantum chemical indices and calculation chemistry were calculated in our work, listed in Table 4 [71]. Computer was used to simulate the interaction process between sulfated β-CD or β-CD and xanthones on the basis of molecular mechanics and molecular dynamics, from which the interaction energy between analyte-selector was obtained. Among the descriptors above, interaction energy (INE), the logarithms of octanol–water partition coefficient (log P) and total energy (TE) of xanthone molecular were selected to describe molecular recognition process between

Table 2
Apparent binding constants between sulfated β-CD and studied xanthones

Xanthone	Binding constant (K, L mol^{-1})	Linear regression equation	Correlation coefficient
1	72.29	$Y = 143.90 + 10.69X$	0.9895
2	87.47	$Y = 65.85 + 5.76X$	0.9844
3	73.03	$Y = 74.70 + 5.46X$	0.9684
4	73.03	$Y = 74.70 + 5.46X$	0.9684
5	125.3	$Y = 111.28 + 13.94X$	0.9115
6	342.2	$Y = 19.59 + 6.70X$	0.9966
7	116.4	$Y = 71.55 + 8.32X$	0.9970
8	276.9	$Y = 66.55 + 18.42X$	0.9702
9	364.1	$Y = 54.73 + 19.92X$	0.9786
10	–	–	–

X stands for $[L]$ (mmol L^{-1}), Y for $[L]/(\mu f–\mu i)$ (mmol s kV L^{-1} cm^{-2} × 10^{-3}). Conditions: buffer, 200 mM borate, pH 9.5; applied voltage, 18 kV; temperature, 25 °C; detection, UV at 265 nm; capillary inner diameter, 50 μm.

Table 3
Apparent binding constants between β-CD and studied xanthones

Xanthone	Binding constant (K, L mol^{-1})	Linear regression equation	Correlation coefficient
1	16.19	$Y = 5.960 + 0.096X$	0.9002
2	246.0	$Y = 1.256 + 0.309X$	0.9900
3	447.4	$Y = 0.742 + 0.332X$	0.9822
4	447.4	$Y = 0.742 + 0.332X$	0.9822
5	177.7	$Y = 1.049 + 0.186X$	0.9115
6	659.6	$Y = 0.382 + 0.252X$	0.9921
7	−7.45	$Y = 4.804 - 0.036X$	0.9970
8	375.4	$Y = 0.371 + 0.139X$	0.9740
9	386.0	$Y = 0.310 + 0.119X$	0.9844
10	934.2	$Y = 0.170 + 0.159X$	0.9912

X represents $[L]$ (mmol L^{-1}), Y represents $[L]/(\mu f–\mu i)$ (mmol s kV L^{-1} cm^{-2} × 10^{-3}). Conditions: buffer, 30 mM borate, pH 10; applied voltage, 20 kV; temperature, 25 °C; detection, UV at 265 nm; capillary inner diameter, 100 μm.

xanthone and CDs in CZE. The multiple regressions of log K with INE, log P and TE showed different separation mechanisms when sulfated β-CD or β-CD was added to the running buffer. For sulfated β-CD, it can be reached that the electric and hydrogen bond effects between sulfated β-CD and xanthones are the most important factors for the molecular recognition. The electric and hydrogen bond effect is benefit to the interaction and decides the stability of inclusive-complex between sulfated β-CD and xanthones. On the contrary, the van der Waals force effect between sulfated β-CD and xanthones, and the stronger hydrophobicity of analytes are not benefit to the inclusive-complexes formation. It can be well explained that the sulfonic acid group of sulfated β-CD has extremely electric effect (including hydrogen bond effect) on the xanthones, so the electric and hydrogen bond effects are dominant in the molecular recognition. As for β-CD, it can be concluded that the van der Waals forces effect between β-CD and xanthones is the most essential factor for the molecular recognition. Stronger the van der Waals forces are, the more stable the inclusive-complex becomes. Unlike sulfated β-CD, β-CD is electrically neutral and the resulted hydrogen bond with xanthones is much weaker than that of sulfated β-CD, so under this circumstance the van der Waals forces becomes dominant, and the phenomenon observed above with sulfated β-CD as a additive cannot be happened. What is more interesting is that log P exerts negative contribution to the interactions between CDs and xanthones, which is opposite to the traditional theory about CDs. It was showed that calculated log K from multiple regression equation and observed log K from experiment can be compared, so the CZE behavior of xanthones could be predicted by this QSEQ study.

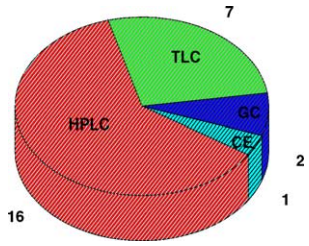


Fig. 7. The number of publications about xanthone determination in real samples by means of different chromatographic methods based on SCI.

Besides binding constant, dissociation constant is an important parameter that can determine the CZE behaviors and the interactions between solutes and micellar or droplet phase in MEKC or MEEKC. Furthermore, dissociation constant can have an essential impact on the transportation of solutes across protein or liposome membrane. CZE can serves as an effective tool to determine dissociation constant. Yang et al. [31] determined the dissociation constants of three xanthones *Swertia przewalskii pissjauk*. In our study, the dissociation constants of 10 xanthones shown in Fig. 1 were determined by CZE and provided important data for studying CE separation mechanism of xanthones [72].

4. Quantitation and validation

Although xanthones are attractive natural products with strong activities, the quantitation of xanthones in real samples has been still limited due to their extremely low contents. For most cases, chromatographic methods such as HPLC, TLC, and CE have been just used for the

Table 4
The descriptors of analytes for QSRR

Xan	log P	TE	INE 1	INE 2	μ	HOMO	LUMO
1	2.30	−1.024	−0.308	−0.271	5.662	−0.2849	0.0678
2	2.69	−0.9493	−0.114	−0.203	4.726	−0.2829	0.0811
3	2.69	−0.9493	−0.155	−0.090	5.105	−0.2875	0.0704
4	2.69	−0.9493	−0.104	−0.193	3.027	−0.3011	0.0738
5	2.17	−0.9103	−0.259	−0.268	5.425	−0.2901	0.0690
6	2.17	−0.9103	−0.337	−0.108	5.317	−0.2838	0.0642
7	1.78	−0.9852	−0.209	−0.136	3.778	−0.2967	0.0672
8	1.78	−0.9852	−0.234	−0.300	6.936	−0.2852	0.0670
9	1.65	−0.8713	−0.142	−0.193	6.515	−0.3046	0.0675
10	1.91	−1.099	−0.101	−0.219	5.770	−0.2908	0.0760

log P denotes the logarithms of octanol–water partition coefficient. TE denotes total energy (unit: 10^3 hartree). INE 1 denotes interaction energy (unit: 100 kcal/mol) of β-CD. INE 2 denotes interaction energy (unit: 100 kcal/mol) of sulfated β-CD. μ denotes dipole moment (unit: Debye) of xanthone. HOMO denotes the energy of the highest occupied molecular orbital (unit: hartree). LUMO denotes the energy of the lowest unoccupied molecular orbital (unit: hartree).

separation, purification of xanthones from complicated matrices, and the physicochemical study on xanthones. As for the determination of xanthones in real samples, HPLC is a main chromatographic method used at present based on science cited index (SCI) shown in Fig. 7. The contents of xanthones in real sample such as nanocapsules [14], herbal medicine [69], human body fluidity [73] were successfully determined by HPLC-UV with good recovery, wide linear range and higher detection sensitivity [69]. Recently in order to increase detection sensitivity and identify the xanthones online, HPLC-MS has been applied to xanthone analysis in real samples. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the positive mode are mainly used method [4,74–76]. As for TLC, it is not recommended for quantitative analysis because of the poor reproducibility and accurateness [5,6]. Our work introduced above showed an attractive potential for separating xanthones by CE, but unfortunately, CE has not widely applied to determine xanthones in herbal medicines due to the low sensitivity of UV detector. Just one paper [31] has been involved in this field with satisfactory recovery and linearity. Three xanthones in *Swertia przewalskii pissjauk* (1,7-*O*-beta-D-glucopyranosyl-8-hydroxy-3,7-dimethoxyxanthone, 1,8-dihydroxy-3,7-dimethoxy-xanthone, 1,7-dihydroxy-3,8-dimethoxyxanthone) have been determined by CZE and their dissociation constants have been calculated with the values of 9.04, 8.94, and 8.59, respectively.

5. Prospect for future study

At present, the CE method for the separation of xanthones faces a problem of the lower detection sensitivity of UV detector when it is applied to real samples. UV detection in CE seems to be enough for the determination of xanthones in some drug preparations and extract with higher contents. However, for herbal medicines, in which the content of xanthones is usually very low, this problem becomes more prominent. Hence, the research in this field should focus on how to enhance detection sensitivity by using CE-MS and preconcentration online or offline such as stacking technique in future. Although CEC and NACE showed worse separation than CZE, MEKC and MEEKC, these two modes are suitable for being coupled to MS since volatilized salts are often used in CEC and NACE. What is more, ESI–MS–MS has a stronger ability to distinguish impurity peaks, compensating the disadvantage of poor resolution.

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References

[1] Jiangsu Institute of Botany, Chinese Academy of Medical Sciences and Kunming Institute of Botany, Xinhua bencao gangyao, vol. 1, Shanghai Science and Technology Press, 1988, p. 292.
[2] A. Marston, M. Hamburger, I. Sordat-Diserens, et al., Phytochemistry 33 (4) (1993) 809.
[3] D.A.G. Cortez, A. Morston, K. Hostettmann, Chromatographia 50 (1/2) (1999) 7.
[4] A. Schieber, N. Berardini, R. Carle, J. Agric. Food Chem. 51 (17) (2003) 5006.
[5] J.B. Harborne, Phytochemical Methods, third ed., Chapman & Hall, 1998.
[6] C. Leuckert, V. Ahmadjian, C.F. Culberson, A. Johnson, Mycologia 82 (3) (1990) 370.
[7] X.D. Su, T. Bo, R.K. Li, K.A. Li, H.W. Liu, Chromatographia 55 (1/2) (2002) 63.
[8] T. Bo, K.A. Li, H.W. Liu, J. Pharm. Biomed. Anal. 31 (5) (2003) 885.
[9] S.X. Gong, X.D. Su, T. Bo, X. Zhang, H.W. Liu, K.A. Li, J. Sep. Sci. 26 (6/7) (2003) 549.
[10] H.T. Feng, S.F.Y. Li, J. Chromatogr. A 973 (1/2) (2002) 243.
[11] X.H. Sun, C.L. Gao, W.D. Cao, X.R. Yang, E.K. Wang, J. Chromatogr. A 962 (1/2) (2002) 117.
[12] R. Pomponio, R. Gotti, B. Luppi, V. Cavrini, Electrophoresis 24 (10) (2003) 1658.
[13] Q.L. Tang, M.L. Lee, TrAC Trends Anal. Chem. 19 (2000) 648.
[14] M. Teixeira, C.M.M. Afonso, M.M.M. Pinto-Madalena, C.M. Barbosa, J. Chromatogr. Sci. 41 (7) (2003) 371.
[15] L. Li, D.N. Zhu, Y.Q. Yan, Zhongguo Yaoke Daxue Xuebao 33 (1) (2002) 42.
[16] Z.X. Shi, F.Z. Hu, M. Liu, Fenxi Huaxue 30 (12) (2002) 1532.
[17] M.A. Lacaille-Dubois, K. Galle, H. Wagner, Planta Med. 62 (4) (1996) 365.
[18] P.A. Mourier, C.R. Vitry, S. Rhone-Poulenc, Vitry-sur-Seine, Analusis 17 (1/2) (1989) 67.
[19] H. Kanamori, I. Sakamoto, Hiroshima-ken Eisei Kenkyusho Kenkyu Hokoku 34 (1987) 1.
[20] I.M. Jalal, S.S. Sa'sa, A.W. Rjoob, H.S. Khalil, J. Liq. Chromatogr. 10 (11) (1987) 2525.
[21] M.J. Pettei, K. Hostettmann, J. Chromatogr. 154 (1) (1978) 106.
[22] M. Teixeira, M.M. Afonso-Carlos, M.M.M. Pinto-Madalena, C.M. Barbosa, J. Chromatogr. Sci. 41 (7) (2003) 371.
[23] G. Kitanov, Acta Pharm. 50 (1) (2000) 69.
[24] P. Nedialkov, G. Kitanov, J. Tencheva, Acta Pharm. 48 (3) (1998) 211.
[25] X.H. Xu, J.T. Stewart, J. Planar Chromatogr. Mod. TLC 11 (3) (1998) 222.
[26] G. Bottura, M.A. Pavesi, Microchem. J. 35 (1) (1987) 112.
[27] H. Kanamori, I. Sakamoto, M. Mizuta, O. Tanaka, Chem. Pharm. Bull. 32 (12) (1984) 4942.
[28] K. Hostettmann, M. Hostettmann-Kaldas, O. Sticher, J. Chromatogr. 202 (1) (1980) 154.
[29] A. Jefferson, C.I. Stacey, F. Scheinmann, J. Chromatogr. 57 (2) (1971) 247.
[30] K. Oba, H. Ohtani, S. Tsuge, Polym. Degrad. Stab. 74 (1) (2001) 171.
[31] Y.F. Yang, Z.X. Liao, L. Guo, Y. Chen, J. Liq. Chromatogr. Related Technol. 26 (8) (2003) 1219.
[32] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[33] T. Bo, K.A. Li, H.W. Liu, Anal. Chim. Acta 458 (2) (2002) 345.
[34] T. Bo, H.W. Liu, K.A. Li, Chromatographia 55 (9/10) (2002) 621.
[35] T. Bo, K.A. Li, H.W. Liu, J. Liq. Chromatogr. Related Technol. 25 (17) (2002) 2601.
[36] T. Bo, X.D. Yang, F. Gao, H.W. Liu, K.A. Li, L.Z. Xu, Chin. Chem. Lett. 13 (3) (2002) 269.

[37] T. Bo, X.D. Yang, F. Gao, H.W. Liu, K.A. Li, L.Z. Xu, Chromatographia 55 (3/4) (2002) 217.
[38] T. Bo, Y.F. Huang, X.D. Yang, K.A. Li, H.W. Liu, L.Z. Xu, J. Chromatogr. Sci. 41 (4) (2003) 182.
[39] S.P. Porras, M.L. Riekkola, E. Kenndler, J. Chromatogr. A 905 (2001) 259.
[40] S.P. Porras, P. Jyske, M.L. Riekkola, E. Kenndler, J. Microcol. Sep. 13 (2001) 149.
[41] S.P. Porras, M.L. Riekkola, E. Kenndler, Chromatographia 53 (2001) 290.
[42] T. Bo, Applications of capillary electrophoretic technique in the analysis of active compounds in Chinese traditional medicines, Ph.D. thesis, Peking University, 2003.
[43] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[44] B. Maichel, E. Kenndler, Electrophoresis 21 (2000) 3160.
[45] S. Katsuta, K. Saitoh, J. Chromatogr. A 780 (1997) 165.
[46] T. De Boer, R.A. De Zeeuw, G.J. De Jong, K. Ensing, Electrophoresis 20 (1999) 2989.
[47] T. Bo, X.D. Yang, F. Liu, K.A. Li, L.Z. Xiu, H.W. Liu, Anal. Chim. Acta 474 (1/2) (2002) 37.
[48] T. Bo, F. Liu, K.A. Li, H.W. Liu, J. Liq. Chromatogr. Related Technol. 26 (6) (2003) 993.
[49] K.D. Altria, B.J. Clark, P.E. Mahuzier, Chromatographia 52 (11/12) (2000) 758.
[50] C.W. Klampfl, Electrophoresis 24 (10) (2003) 1537.
[51] H. Siren, A. Karttunen, J. Chromatogr. B 783 (1) (2003) 113.
[52] S. Terabe, N. Matsubara, Y. Ishihama, Y. Okada, J. Chromatogr. 608 (1992) 23.
[53] K.D. Altria, J. Chromatogr. A 892 (1/2) (2000) 171.
[54] P.E. Mahuzier, J.B. Clark, S.M. Bryant, K.D. Altria, Electrophoresis 22 (17) (2001) 3819.
[55] K.D. Altria, J. Chromatogr. A 844 (1999) 371.
[56] K.D. Altria, P.E. Mahuzier, B.J. Clark, Electrophoresis 24 (3) (2003) 315.
[57] Y.Q. Luo, T. Bo, M. Li, S.X. Gong, K.A. Li, H.W. Liu, J. Liq. Chromatogr. Related Technol. 26 (11) (2003) 1719.
[58] T. Bo, L. Zhong, M. Li, Y.Q. Luo, K.A. Li, H.W. Liu, D.A. Guo, Chromatographia 56 (11/12) (2002) 709.
[59] T. Bo, X.D. Yang, F. Liu, K.A. Li, L.Z. Xu, H.W. Liu, J. Sep. Sci. 26 (1/2) (2003) 133.
[60] T. Bo, S.X. Gong, X.D. Yang, W. Li, K.A. Li, H.W. Liu, Chin. J. Chromatogr. 21 (2003) 439.
[61] S. Terabe, K. Otsuka, T. Ando, Anal. Chem. 61 (1989) 251.
[62] B. Chankvetadze, G. Blaschke, J. Chromatogr. A 906 (1/2) (2001) 309.
[63] U. Pyell, J. Chromatogr. A 892 (1/2) (2000) 257.
[64] J.I. Ding, P. Vouros, J. Chromatogr. A 887 (1/2) (2000) 103.
[65] I.S. Krull, A. Sebag, R. Stevenson, J. Chromatogr. A 887 (1/2) (2000) 137.
[66] K.D. Altria, J. Chromatogr. A 856 (1/2) (1999) 443.
[67] T. Helboe, S.H. Hansen, J. Chromatogr. A 834 (1999) 315.
[68] T. Bo, S.X, Gong, X.D. Yang, K.A. Li, H.W. Liu, Chin. J. Chromatogr., in press.
[69] T. Bo, X.D. Yang, K.A. Li, H.W. Liu, L.Z. Xu, B.Q. Che, J. Liq. Chromatogr. Related Technol., in press.
[70] X.D. Yang, Study of constitutes and bioactivities of Securidaca inappendiculata Hassk, Ph.D. thesis, Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences, 2002.
[71] T. Bo, J. Ren, X.D. Yang, S.X. Gong, K.A. Li, H.W. Liu, Chin. J. Chromatogr. 21 (2003) 535.
[72] X.M. Wu, S.X. Gong, T. Bo, K.A. Li, H.W. Liu, J. Chromatogr. B, in preparation.
[73] J.X. Kelly, R.W. Winter, A. Cornea, D.H. Peyton, D.J. Hinrichs, M. Riscoe, Mol. Biochem. Parasitol. 123 (1) (2002) 47.
[74] C. Terreaux, M. Maillard, M.P. Gupta, K. Hostettmann, Phytochemistry 40 (6) (1995) 1791.
[75] T. Rezanka, V.M. Dembitsky, J. Chromatogr. A 995 (1/2) (2003) 109.
[76] C.T. da Costa, J.J. Dalluge, M.J. Welch, B. Coxon, S.A. Margolis, D. Horton, J. Mass Spectrom. 35 (4) (2000) 540.