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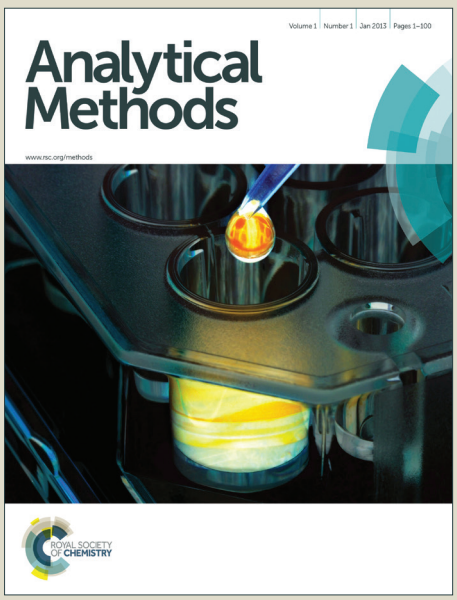

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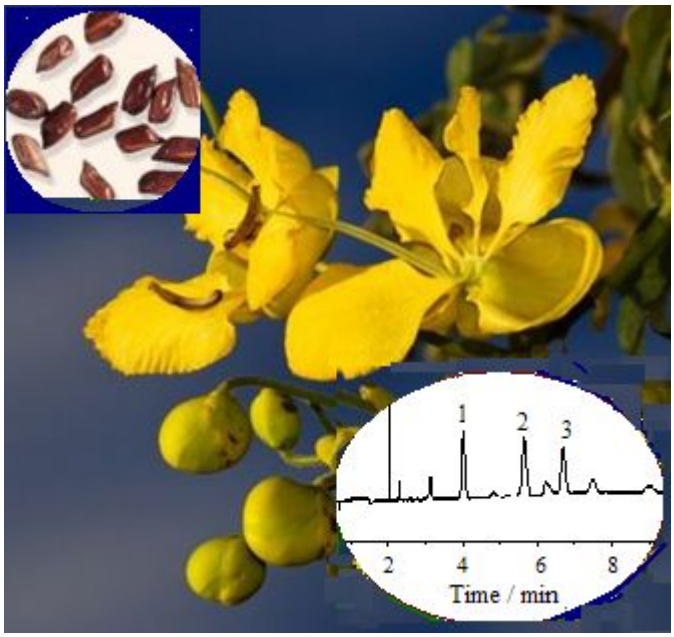

A novel method using nonaqueous capillary electrophoresis-UV detection was used for the effective separation and rapid simultaneous determination of anthraquinones aurantio-obtusin, emodin and rhein in semen cassiae and cassia seed tea

A novel nonaqueous capillary electrophoresis method for effective separation and simultaneous determination of aurantio-obtusin, emodin and rhein in semen cassiae and cassia seed tea

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Anthraquinones (aurantio-obtusin, emodin and rhein) could not be determined simultaneously by capillary zone electrophoresis because of aurantio-obtusin with low solubility in water. A novel nonaqueous capillary electrophoresis (NACE) method with diode-array detection (DAD) for effective separation and simultaneous determination of the three anthraquinones is developed. A 40 mM NaAc–40 mM NH_4Ac–40 mM NaOH in methanol was used as a background electrolyte solution for the separation of the three anthraquinones. The effects of ultrasonic extraction conditions and NACE conditions on separation were investigated in detail. Under the optimized conditions, the analytes can be effectively separated in 7 min. The standard curves of three anthraquinones showed good linearity

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with the correlation coefficients r＞0.999. The detection limit (S/N=3) of aurantio-obtusin, emodin and rhein were 0.25, 0.12 and 0.19 μg mL^{-1}, respectively. Their average intra- and inter-day analytical precisions (relative standard deviation, RSD) were less than 2.8% and 4.0%, respectively. The recoveries at the three spiked levels of aurantio-obtusin, emodin and rhein from semen cassiae and cassia seed tea samples were in the range of 86.6–106%. The proposed method provides the speediness, selectivity, sensitivity, linearity and accuracy as well as low reagents consumption necessary for simultaneous analysis of the test anthraquinones. The ultrasonic extraction–NACE method was used for the analysis of aurantio-obtusin, emodin and rhein in semen cassiae and cassia seed tea with satisfactory results, and could be applied for quality control of semen cassiae and cassia seed tea.

Keywords: Nonaqueous capillary electrophoresis; Aurantio-obtusin; Emodin; Rhein; Semen cassiae;Cassia seed tea

Introduction

Semen cassiae is the seed of the plant Cassia tora L. (Leguminosae). It contains a variety of bioactive anthraquinones substances. Modern pharmacological research has revealed that it has multiple functions, such as liver protection, bacteriostasis, catharsis and immune adjustment, eyesight improvement, diuresis, antitumor, and antioxidation [1]. The commercial products of semen cassiae include both unroasted and roasted samples. The

water extract of unroasted semen cassiae might have a potential health activity on the cancer chemoprevention. The bioactive constituents of semen cassiae are anthraquinones, including aurantio-obtusin, chryso-obtusin, obtusin, etc [2]. They do benefit our body but can also cause health ailments like obesity, irritable bowel syndrome and psoriasis. Thus, it is necessary to select anthraquinones as the index components for quality control. Chrysophanol and aurantio-obtusin were selected as markers for semen cassiae in the Chinese Pharmacopoeia (2010 Edition)[3]. However, chrysophanol can be found in many other herbs, it is not a specific effective component of semen cassiae. Therefore, the determination of the active components including the markers in semen cassiae is required for the evaluation of its quality.

Among the analytical methods of anthraquinones for traditional Chinese medicines, high performance liquid chromatography (HPLC) is still most popular[4-9]. However, the retention time of som anthraquinones was too long, for example, from about 54 min for rhein to about 97 min for emodin in rhubarbs [4] and from 500 to 1520 min for five anthraquinones in Rhizoma Rhei [5]. HPLC method with fluorescence detection is more suitable for the analysis of the anthraquinones than HPLC UV methods [6]. Some HPLC methods suffer from low resolution or large consumption of organic solvent. There were few reports for determination of aurantio-obtusin in herbal medicine [8,9].

Capillary electrophoresis (CE) has become a powerful tool in natural product analysis, due to its high resolution, short analysis time, and low solvent and sample consumption. Capillary electrochromatography (CEC) mode of CE has been used for analysis of some anthraquinones in herbs [10-13]. Capillary zone electrophoresis (CZE) mode has been proposed for the analysis of some anthraquinones in herbal medicines [14-18]. In all of above works, determination of aurantio-obtusin was not achieved in CE, it is possible due to its low solubility in water. Nonaqueous capillary electrophoresis (NACE) is a process similar to CZE which is useful in the separation of compounds that are insoluble in water as it relies mainly on the use of organic solvents[19]. It can be used for the effective separation and fast determination of both uncharged and charged analytes. A fast method to examine the content of aurantio-obtusin and other anthraquinones in semen cassiae would be desirable.

The main purpose of of the present study is to develop a rapid, sensitive and accurate NACE analytical method for effective separation and simultaneous determination of aurantio-obtusin, rhein and emodin in semen cassiae. Figure 1 shows the structures of these anthraquinones. Validation parameters of the NACE method, such as sensitivity, linear range, precision and accuracy, have been determined. Finally, the newly developed method was successfully applied to quantify three anthraquinones in semen cassiae and cassia seed tea.

Figure 1

Experimental

Instrumentation

All experiments were performed with an Agilent HP^{3D} CE system with air-cooling and a diode-array detector (Agilent, Waldbronn, Germany). Data were collected with the Agilent Chemstation version A10.02 chromatographic data system. An ultrasonic cleaner (Ultrasonic Instrument Co., Kunshan, China) working at 40 MHz with an output power of 40 W was employed as ultrasonic source. A TGL-16M centrifuge (Xiangyi centrifuge Co., Hunan, China), RE-2000A rotary evaporator (Shanghai Yarong Biochemistry Instrument Co.), and PHS-3C pH meter (Shanghai Precision & Scientific Instrument Co., Shanghai, China) were used in sample treatment.

Chemicals and solutions

Emodin, Rhein and autrantio-obtusin(purity: >99% for each) were purchased from National Institutes for Food and Drug Control (Beijing, China). Ammonium acetate (NH_4Ac), sodium acetate (NaAc) and sodium hydroxide (NaOH) were of analytical greade. Methanol, acetonitrile, chloroform and ethanol were HPLC grade, and obtained from Beijing Chemical Factory (Beijing, China).

The background electrolyte solution (NaAc-NH_4Ac-NaOH of 40 mM for each in methanol) was prepared by dissolving 1.64 g NaAc, 1.54g NH_4Ac and 0.80 g NaOH with methanol. Single stock standards of emodin, rhein and autrantio-obtusin were prepared by dissolving the compound with methanol, their concentrations were 204, 180 and 224 μg mL^{-1}, respectively. The stock solutions stored at 4 ˚C were stable for at least six months. Mixed standard working solutions were prepared by diluting the standard stock solution with methanol just before use.

Sample preparation

Semen cassiae and cassia seed tea were obtained from Baoding medicinal herbs store**.** 0.2 g powders of the sample and 30 mL chloroform were added into the extraction vessel. A PVC film was floated on the extraction solvent in the extraction vessel to restrain the formation of the ultrasonic fountain. Ultrasonic extraction was carried out at 25˚C for 20 min.

After being extracted once, the residue was extracted again under the same conditions. The combined extract was filtrated and evaporated at 40 ˚C until it was dried, and the remain was redissolved by methanol, the extract was transferred into a 10 mL flask, and diluted with methanol to the mark. The solution was vortexed, and filtered through a 0.45μm cellulose acetate membrane filter before NACE analysis.

Electrophoresis conditions

A 35 cm (26.5cm to the detector) 75 μm i.d. and 365 μm o.d. uncoated fused silica capillary (Yongnian Optical Fabric Factory, Handan, China) was utilized. Prior to its use every day, the capillary was consecutively flushed with 0.1 M NaOH for 10 min, distilled water for 10 min and the background electrolyte solution for 10 min. After each analysis run the capillary was flushed for 1 min with the the background electrolyte solution to maintain the reproducibility of the analysis. Sample introduction was made at the anodic capillary side using 50 mbar pressures for 5 s. The high-voltage power supply was set at 18 kV. The 40 mM ammonium acetate–40 mM sodium acetate–40 mM sodium hydroxide–methanol was used as a background electrolyte solution. Capillary temperature was kept at 20˚C. DAD was employed at a wavelength of 254 nm.

Results and discussion

Optimization of NACE conditions

Choice of background electrolyte solution. The organic solvents applied in nonaqueous capillary electrophoretic separations must meet certain requirements. They should dissolve the analytes and the electrolytes used in the separation. Low dynamic viscosity is preferred in CE because it allows high mobilities of the solvated analyte ions and hence separations in a reasonable time-frame. Relative permittivity (dielectric constant) is an important parameter because it describes the strength of the interactions between ions in the solvent[20]. In this work, some organic solvents

including ethanol, acetonitrile, acetone and methanol were used as nonaqueous media, respectively, and the separation effect of three anthraquinones was compared. The result showed that three analytes could not be baseline separated using ethanol or acetonitrile, while no rhein was observed using acetone. And using methanol three analytes could be baseline separated, with shortest analytical time, it is possible due to that lower viscosity (η= 0.55 mPa·S) and higher dielectric constants (ε=33.64) of methanol improved both sample ion mobility and the level of electro osmotic flow[20].

The selection of suitable electrolytes is very important for CE. Addition of electrolyte in organic solvent make having certain electric conductivity. Electrolyte cation also has a clear effect on the capillary electrophoretic selectivity in nonaqueous media. It is an important condition for achieving nonaqueous capillary electrophoresis separation, and correct selection of electrolyte could improve peak shape and separation effect. The initial experiments were conducted using borate, tris(hydroxymethyl)metyl aminomethane**,** ammonium acetate and sodium acetate as electrolyte, respectively. Test results indicated that emodin, rhein and autrantio-obtusin could not be separated well in each of these electrolyte solutions. However, when a mixture of ammonium acetate and sodium acetate were employed, the separation of the three analytes can be achieved, and stable baseline and excellent peak shape were obtained. The effects of concentration ranged

from 30 to 50 mM for ammonium acetate and sodium acetate on the CE separation were investigated.

Figure 2

Figure 2 shows that the migration time of the three drugs decreased with the increasing of the electrolyte concentration from 30 to 40 mM, and increased with the increasing of the electrolyte concentration from 40 to 50 mM. It is because that the thickness of electric double layer between the capillary walls and the buffer solution decreased with the increase of electrolyte concentration. Otherwise, high electrolyte concentration could increase the coverage of sites on the silica surface, resulting in the decrease of electroosmotic flow (EOF) because of the decrease of the silicon hydroxyl [21]. Finally, 40 mM ammonium acetate–40 mM sodium acetate was used as electrolyte for the separation of three analytes.

With the aim of getting higher accuracy, better resolution and reproducibility in the separation, different concentrations of NaOH were tested.

Figure 3

Figure 3 shows the DAD electropherograms obtained in each case. The resolution between peaks improves when the NaOH concentration decreased, but it is also noticed that migration time increased, probably due to the lower ionization of the compound and, as a consequence, its slower migration velocity to the anode. Therefore, finally, 18 mM NaOH

concentration was selected as the optimum. To sum up, 40 mM ammonium acetate, 40 mM sodium acetate and 40 mM NaOH in methanol medium were selected as optimum BGE.

Separation voltage. With the increase of separation voltage the EOF increased and the analytical time decreased. However, the high voltage led to Joule heating and affected the separation of the analytes. The effect of voltages on the separation of the three drugs was investigated. By increasing separation voltage from 13 to 21 kV, the migration times of the three drugs decreased due to the increase of both EOF and mobility of the compounds. However, the higher voltage caused the decrease in peak areas of the analytes, and would cause higher current and lead to more Joule heating, which produces radial temperature gradient and decreases separation efficiency. When voltage at 18 kV the peaks of the three drugs could be baseline separated within 7 min. When voltage at 21 kV rhein and aurantio-obtusin coulnot be separated. Therefore 18 kV voltage was selected in this work with higher sensitivity and shorter analytical time.

Effect of separation temperature. The effect of the capillary temperature on separation was investigated at 15, 20, 25, and 30 ˚C. The result showed that migration time decreased by 2.4 min with the increasing capillary temperature from 15 to 30˚C, while resolution decreased slightly. When capillary temperature was set at 20˚C, shorter separation time was

observed, along with high sensitivity and good peak shape. Thereby capillary temperature of 20˚C was selected in this work.

Optimization of extraction conditions

Effect of extraction solvent and its volume. The experiments for selection of extraction solvent were conducted at the power of 35 W for 30 min with 30 mL of different solvents, such as methanol, ethanol, acetonitrile and chloroform. The results are shown in Fig. 4.

Figure 4

It can be seen that the methanol offered a interference of impurity to aurantio-obtusin peak, while ethanol and acetonitrile could not extract rhein. Use of chloroform can extract simultanously emodin, rhein and aurantio-obtusin with better extraction effect. The effects of solvent volume were tested. A large solvent volume can dissolve the constituents more selectively, and can lead to an enhancement of the extraction yield. 5–35 mL chloroform were tested. The result showed that the maximal signal was observed till up to 30 mL, but the signal decreases for too large volume. The too much extraction solvent can restrain the cavitation effect. So 30 mL of chloroform was selected in the work.

Effects of extraction time on extraction yields. Effects of extraction time on extraction yields of the three anthraquinones were studied from 2.5 min to 30 min when other conditions were constant.

Figure 5

The results shown in Fig. 5 indicate that the extraction yields of aurantio-obtusin increase with the increase of the extraction time from 2.5 to 10 min, and slightly increases from 10 to 30 min. extraction yields of emodin increase with the increase of the extraction time from 2.5 to 10 min, and then present a platform for longer extraction time. The extraction yield of rhein increased with the increase of extraction time from 2.5 to 20 min, and then present a platform for longer extraction time. The results may be related to the decomposition of analyte caused by overlong extraction time. To obtain the highest total extraction yield for the three analytes, 20 min was selected in the next steps.

Effects of extraction temperature on extraction yields. Effects of extraction temperature on extraction yields of the three anthraquinones were studied from 5 to 40 ˚C when other conditions were constant.

Figure 6

The results shown in Fig. 6 indicate that the extraction yields of aurantio-obtusin and rhein increase with the increase of the extraction temperature from 5 to 25˚C, and for aurantio-obtusin slightly decreases and for rhein slightly increases from 10 to 30 ˚C. Extraction yields of emodin increase lightly with the increase of the extraction temperature from 5 to 25˚C, and then present a platform for longer extraction time. The results may be related to the decomposition of analyte caused by overlong

extraction temperature. To obtain the highest total extraction yield, 25˚C was selected in the next steps.

Performance of the method

Selectivity. Under optimized conditions, selectivity was determined for each analyte in the assay. The electropherograms of real semen cassiae and cassia seed tea samples and standards were obtained, as shown in Fig. 7.

Figure 7

The migration time of 4.1, 5.6 and 6.6 min for emodin, rhein and aurantio-obtusin, respectively, was obtained for standards, and same migration time was observed for semen cassiae and cassia seed tea samples. Otherwise, it was shown that the three drugs could be baseline separated. Some unknown peaks appearing in Fig. 7 do not interfere with the separation of the three analytes. There was no interference peak in real samples. The results show that good consistency of migration time and effective baseline separation were achieved.

Linearity and detection limit. Linearity of the NACE method was evaluated by determining the analytes in the 6 concentration points, that is 0.40, 2.00, 8.00, 20.00, 40.00, and 80.00 μg mL^{-1} for emodin, 0.63, 3.15, 6.30, 12.60, 31.50, and 63.33 μg mL^{-1} for rhein, 0.83, 1.66, 4.15, 8.30, 16.60, and 41.67μg mL^{-1} for aurantio-obtusin. The mitrix-calibration curves of the peak area toward analyte concentration were constructed by least-squares linear regression. The equations of calibration curves obtained based on

three parallel measurements are listed in Table 1. It can be seen that the linearity is very satisfactory.

The limit of detection (LOD) was considered the minimum analyte concentration yielding an S/N = 3, and the limit of quantification (LOQ) was considered the minimum analyte concentration yielding an S/N=10. The LOD and LOQ values for the two analytes were determined, which are listed in Table 1. The LOD value of emodin and rhein for this method is lower than that of CZE-diode-array detection[15].

Table 1

Precision. The precision of the method was investigated at room temperature by analyzing emodin, rhein and aurantio-obtusin, concentration of which in sample was 629, 582 and 468 μg g^{-1}, respectively. The average intra-day variability (RSD) of peak area obtained for 5 determinations was 1.6%, 2.8% and 0.5% for emodin, rhein and aurantio-obtusin, respectively. The test solution stored in a refrigerator at 4˚C was determined by 5 times per a day within 3 days. The inter-day RSD of peak area was 4.0% for emodin, 3.4% for rhein and 1.2% for aurantio-obtusin.

Above results indicate that the proposed method provides the selectivity, sensitivity, linearity and precision necessary for selective and simultaneous analysis of the test anthraquinones.

Application

The optimized conditions were applied to the separation and determination of the three analytes. The samples were treated according to the procedure described above. The results are listed in Table **2**. The content of emodin, rhein and aurantio-obtus in semen cassiae samples was obtained to be 629, 582 and 468 μg g^{-1}, with the RSD (n=3) of 0.84, 1.91 and 2.99%, respectively. For cassia seed tea sample, content of aurantio-obtusin is about twice as high as its level of the other anthraquinone.

To examine the method accuracy, recovery test of the assays for the three analytes was determined by adding known amounts of these anthraquinones to the samples. The data in Table 2 showed that the average recovery of emodin, rhein and aurantio-obtusin for semen cassiae sample was 90.5, 86.6 and 101%. A high recovery for both semen cassiae and cassia seed tea shows that the method can be used for the accurate determination of marker aurantio-obtusin, emodin and rhein in semen cassiae and cassia seed tea.

Table 2

Conclusions

A new method for effective separation and simultaneous determination of three anthraquinones by NACE is developed. The present method offers advantages of being speediness, simplicity, sensitivity and accuracy as well as low consumption of reagents. The NACE method can be used for effective separation and simultaneous determination of aurantio-obtusin,

rhein and emodin and for quality control of semen cassiae and cassia seed tea.

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List of Figures

Table 1 Analytical performance of the method

Analyte	Linear range ($\mu g\ g^{-1}$)	Linear equation	Correlation coefficient (r)	LOD ($\mu g\ g^{-1}$)	LOQ ($\mu g\ g^{-1}$)
Emodin	0.40-80.0	Y=0.0678+14.6804X	0.99987	0.12	0.40
Rhein	0.63-63.3	Y=-0.1205+18.7917X	0.99935	0.19	0.63
Aurantio-obtusin	0.83-41.7	Y=5.5674+16.6981X	0.99959	0.25	0.83

Table 2 Determination of three anthraquinones in semen cassiae and cassia seed tea

Sample	Analytes	Content (RSD)* ($\mu g\ g^{-1}$)	Added ($\mu g\ g^{-1}$)	Found ($\mu g\ g^{-1}$)	Recovery/ %	Average recovery （%）
Semen cassiae	Emodin	629 (0.84)	304.5	910	92.4	90.5
			587.3	1142	87.3	
			900.9	1456	91.8	
	Rhein	582(1.91)	291.0	834	86.7	86.6
			561.4	1059	85.0	
			861.1	1341	88.1	
	Aurantio-obtusin	468(2.99)	222.9	701	105	101
			429.9	877	95.2	
			659.5	1144	103	
Cassia seed tea 1#	Emodin	604 (1.88)	295.1	864	87.9	90.8
			602.4	1151	90.8	
			886.9	1434	93.6	
	Rhein	602(3.47)	282.1	852	88.7	88.3
			575.8	1095	85.2	
			847.8	1369	90.5	
	Aurantio-obtusin	1224 (0.91)	594.0	1853	106	106
			1212	2481	104	
			1786	3162	109	
Cassia seed tea 2#	Emodin	562 (2.69)	300.6	819	85.4	89.0
			606.8	1124	92.6	
			894.7	1358	89.0	
	Rhein	621(2.79)	287.3	878.8	89.7	88.3
			580.1	1136	88.8	
			855.3	1360	86.4	
	Aurantio-obtusin	1363 (1.03)	660.1	2005	97.3	103
			1332	2768	105	
			1965	3469	107	

* The data in the brackets refer to the RSD (%) for three samples

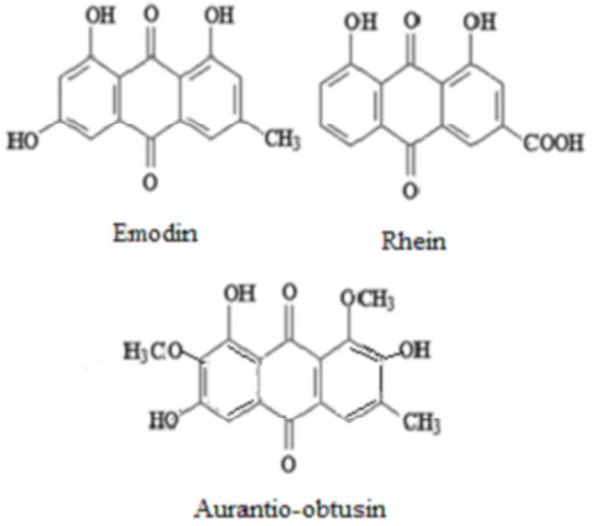

Fig.1
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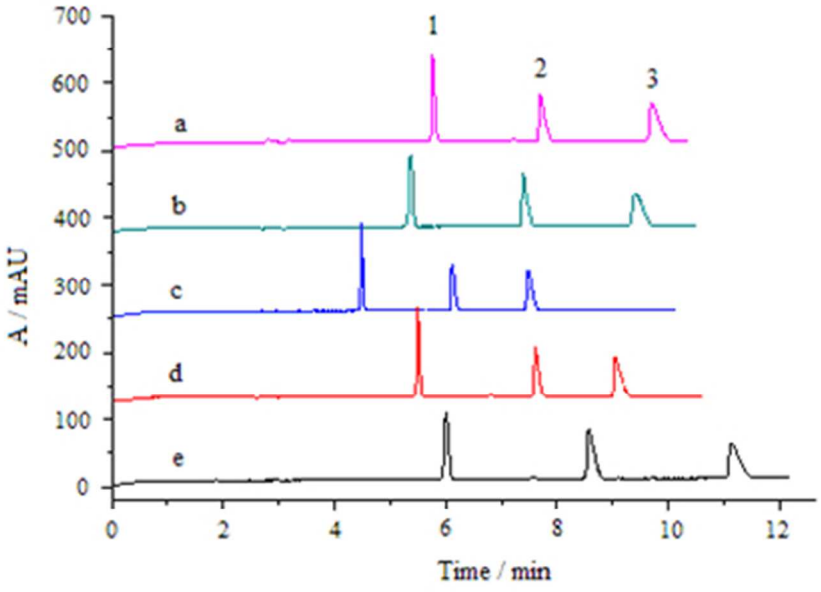

Fig. 2
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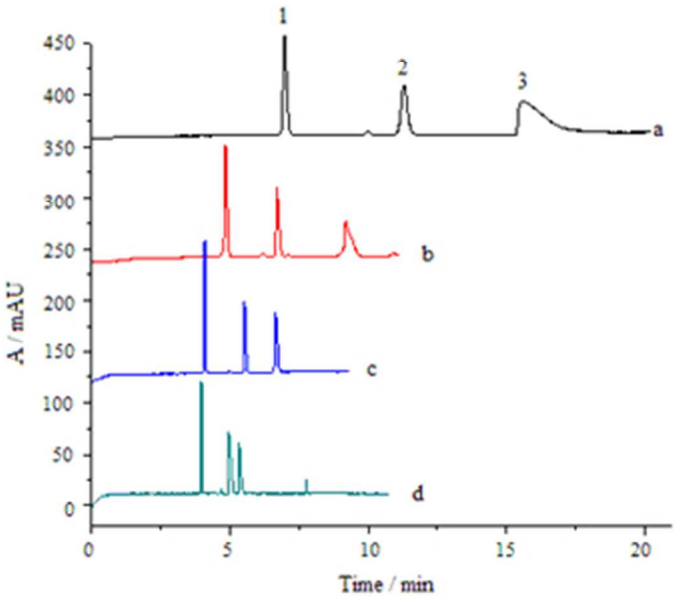

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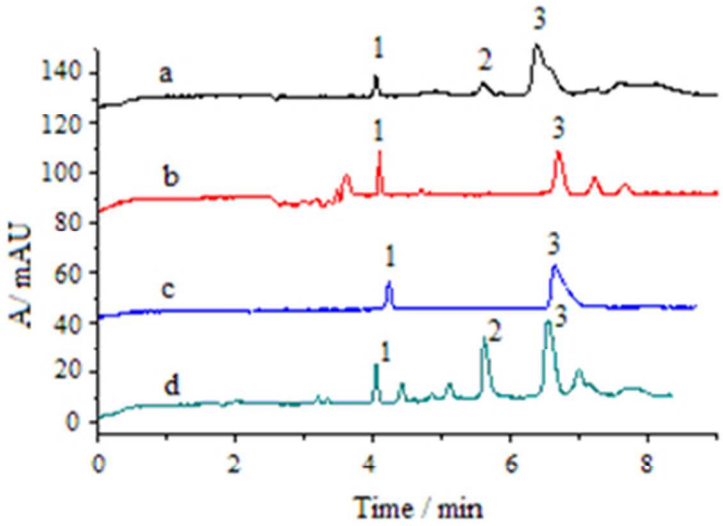

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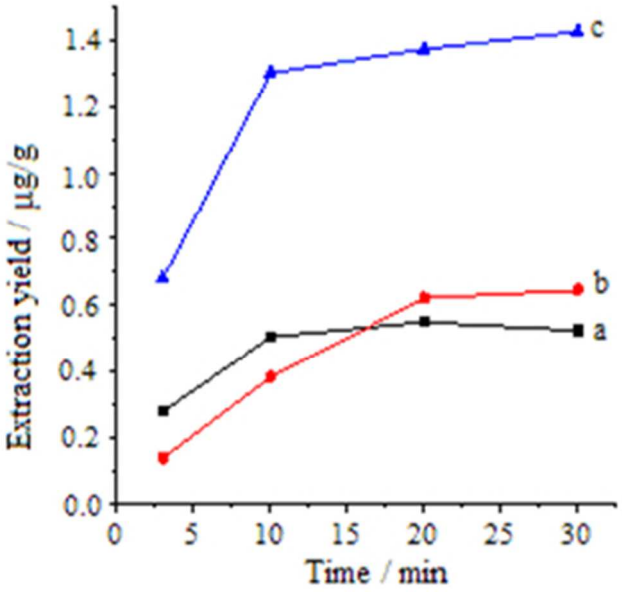

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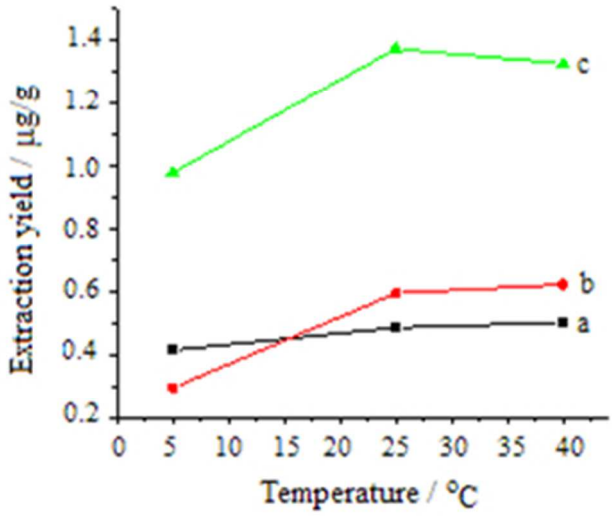

Fig.6
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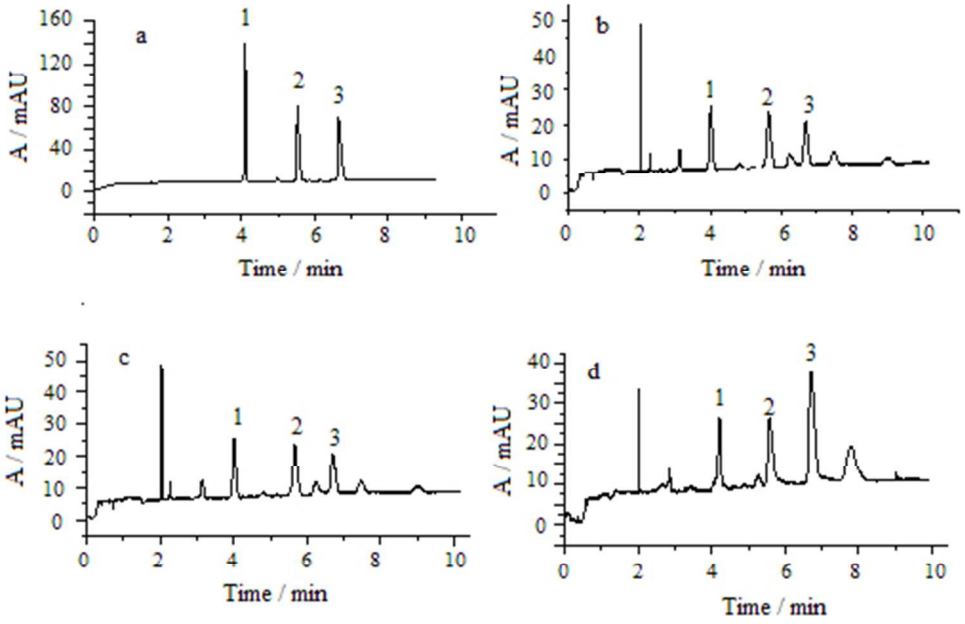

Fig.7
139x94mm (96 x 96 DPI)