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Novel and simple nonaqueous capillary electrophoresis separation and determination bioactive triterpenes in Chinese herbs

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Abstract

Three bioactive triterpenes ursolic acid, oleanolic acid and 2α,3β,24-trihydroxy-urs-12-en-28-oic acid were simultaneously separated by nonaqueous capillary electrophoresis (NACE) with methanol:acetonitrile (65:35 v/v) mixture containing 90 mm trishydroxymethylaminomethane (Tris) at an applied voltage of +25 kV and a hydrodynamic injection of 5 s. The effect of solvent composition, electrolyte nature and concentration on the electrophoretic behavior of the analytes were systematically studied. Separations were carried out in a fused-silica capillary tube with UV detection at 214 nm. Good separation and correlation coefficients were obtained. Meanwhile, the method was applied to separation and determination the three components in six Chinese herbs extraction. It is concluded that this method could be used for speedy and accurate qualitative and quantitative analysis of bioactive triterpenes in Chinese herbs.

Keywords: Nonaqueous capillary electrophoresis; Ursolic acid; Oleanolic acid; 2α,3β,24-Trihydroxy-urs-12-en-28-oic acid; Chinese herbs

1. Introduction

Ursolic acid (UA) and oleanolic acid (OA) were reported to have the effects of anti-inflammatory [1,2], protecting liver, relieving pain and anti-tumor action through enhancing the production of both nitric oxide and tumor necrosis factor-α [3]. More pharmacology effect of UA is being found gradually such as restraining ulcer, controlling blood-fat and treating diabetes, etc. [4] and they are non-toxic [5]. UA and OA are widely coexisted in many Chinese herbs. *Rabdosia japonica* (Burm.f.) Hara var. Glaucocalyx (Maxin.) Hara (Chinese name: xiang-cha-cai) is a commonly used folk herb drug in china for many years. It possesses anti-enteritis, analgesic, anti-mastitis and anti-inflammatory effects and is also used as a kind of tumor inhibitory and honey resource plant [6]. UA and OA are its effective components, besides the two bioactive compounds, 2α,3β,24-trihydroxy-urs-12-en-28-oic acid (TA) was an important component in this herb. Up to know there has no analysis methods for the determination of the three components in this herb.

The qualitative and quantitative analysis methods of OA and UA include thin layer chromatography (TLC) [7,8], gas chromatography (GC) [9], high-performance liquid chromatography (HPLC) [10–12]. Van der Doelen et al. analyzed fresh triterpenoids resins and aged triterpenoids varnishes used high-performance liquid chromatography-mass spectrometry (HPLC–MS) [13]. Although TLC and HPLC methods can be employed to analyze herbs and medicines, these traditional methods need a large amount of samples. Analysis of terpenoids by capillary electrophoresis (CE) has been recognized only a few years ago. The micellar electrokinetic capillary chromatography (MEKC) was in the most common use [14]. And in Oleszek's review [15], separation

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of the triperpenoid in *Silene otites* was also done by MEKC. And for UA and OA, because their structures are very similar, it was not obtained very big resolution even the MEKC was used [14].

In recent years, nonaqueous capillary electrophoresis (NACE), which is based on the use of electrolyte solutions prepared from pure organic solvents, has become an active area of study. NACE offers a number of attractive features such as alteration of selectivity, reduced electrophoretic currents, and improved mass spectrum compatibility, solubility and stability of hydrophobic compounds. One of the most attractive features of organic solvents is that they greatly differ in physical and chemical properties (viscosity, dielectric constant, polarity, auto-protolysis constant, electric constant conductivity, etc.) between themselves and water, so changing the organic solvents or varying the proportions of two solvents allows a simple selective manipulation in NACE [16–18]. Accordingly, NACE was successfully applied to analyze a large number of pharmaceuticals, including acidic and basic drugs, chiral compounds, peptides, ions and preservatives [19–22].

Up to now, there have been no NACE methods were used to analysis these triterpenes. Because their solubility is poor in aqueous while better in nonaqueous solvent, the NACE would be more suitable for analysis them. To evaluate the quality of the three triterpenes in the different Chinese herbs, a simple, rapid and accurate NACE method was developed in this paper.

2. Experimental

2.1. Apparatus

The experiment was performed on a Waters Quanta 4000 Capillary system (Millipore, Waters Chromatography Division of Milford, MA, USA) with a built in 0–30 kV high voltage power supply, a fixed wavelength UV detector near the cathodic end and a forced-air cooling system. Capillary electrophoresis was performed using a 50.0 cm (42.4 cm to the detector) × 75 μm i.d. uncoated fused silica capillary (Yong Nian Optical Fibre Factory, Hebei Province, China). The data acquisition was carried out with a Maxima 820 Chromatography Workstation. All experiments were performed in cationic mode (anode at the inlet and cathode at the outlet). Samples were introduced from the end of the capillary by hydrodynamic injection where the sample vial was raised by 10.0 cm for 5 s. Direct UV detection was employed at a wavelength of 214 nm. The capillary was preconditioned prior to its first use by consecutively flushing with 0.5 M NaOH for 10 min, deionized water for 10 min and the running electrolytes for 10 min. After each run the capillary was rinsed with 0.5 M NaOH for 2 min, distilled water for 2 min, and then running electrolytes for 2 min. The buffer was renewed after every three runs for the good repeatability. To avoid buffer and sample evaporation, the buffer and sample reservoir must have lids. All operations were at 20.0 ± 0.5 °C.

2.2. Materials and reagents

The standard components of ursolic acid, oleanolic acid were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and 2α,3β,24-trihydroxy-urs-12-en-28-oic acid (their chemical structures were shown in Fig. 1) was gifts to the authors and its characterization by NMR, IR, MS were obtained from the College of Life Sciences, Northwest Normal University (Lanzhou, China). The material of *R. japonica* (Burm.f.) Hara var. glaucocalyx (Maxin) Hara was collected from Tianshui, Gansu province, China. And it was identified by Dr. K. Sun with voucher specimens deposited in College of Life Sciences, Northwest Normal University, Lanzhou, China. And the other Chinese herbs were purchased from Lanzhou Fuxinghou pharmaceutical company (Lanzhou, China). They were also identified by Dr. K. Sun with voucher specimens deposited in the same place. All chemicals used in this study were analytical reagents. Tris was purchased from Shanghai Shanpu Chemical Corporation (Shanghai, China). Acetonitrile and methanol were purchased from Tianjin Secondary Chemical Factory (Tianjin, China).

Tris was directly dissolved in methanol with acetic acid added to prepare stock solutions of 0.2 M (1.5% acetic acid). Working solutions were obtained by diluting the stock solutions with methanol just prior to use.

2.3. Sample preparation

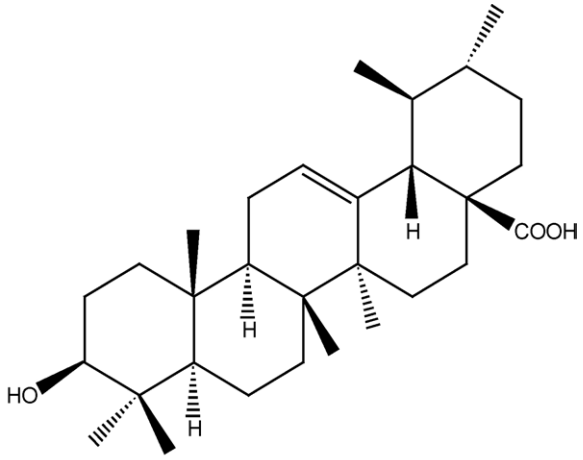
The samples powder (1 g) was weighed exactly into a 25 ml sample vial, respectively. Then 20 ml ethanol was added into it and the sample vial was put in an ultrasonic bath and extracted for 1 h. Extraction was repeated three times. Then the ethanol was evaporated. The residue was dissolved with methanol and diluted to 10 ml. All the solutions were passed through a 0.45 μm filter (Shanghai Xinya Purification Apparatus Factory, Shanghai, China) before being injected into the capillary electrophoresis system.

2.4. Data treatment

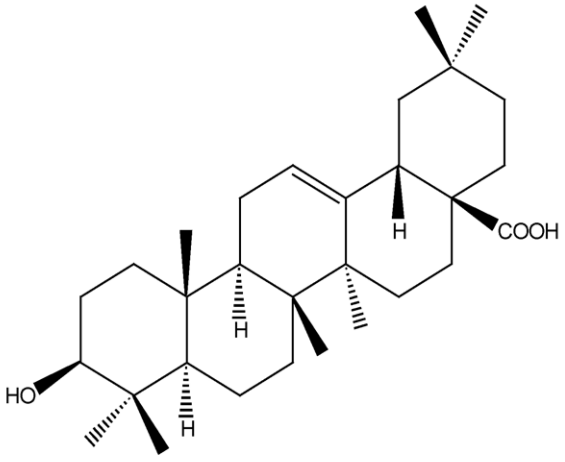
The electroosmotic mobility was determined by the migration time of acetone. In the chose electrolyte system we believe that acetone remains neutral and can be used as an EOF marker. From the experimental data, electrophoretic mobilities corrected for electroosmotic flow variations were calculated according to the following equation:

$$\mu_{ep} = \frac{L_t L_d}{V}\left(\frac{1}{t_r} - \frac{1}{t_{eo}}\right) \quad (1)$$

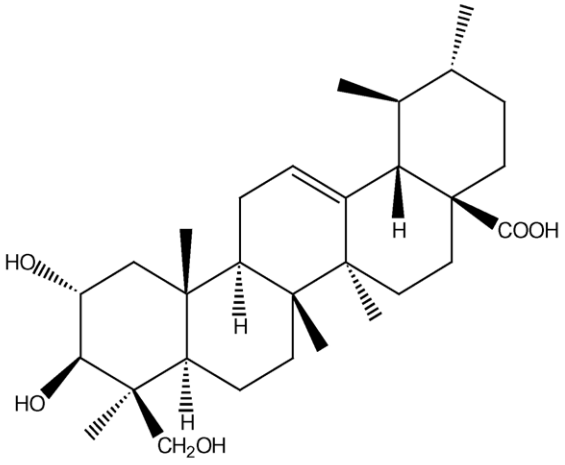
where L_t is the total length of the capillary, L_d the effective length (i.e., distance between points of injection and



1 ursolic acid



2 oleanolic acid



3 2α,3β,24-trihydroxy-urs-12-en-28-oic acid

Fig. 1. Structures of the analytes, with their identification numbers.

detection) of the capillary, t_r represents the migration time of the analyte, t_{eo} the migration time due solely to electroosmotic flow, and V is the applied voltage across the capillary.

3. Results and discussion

3.1. Method development and effect of acetonitrile concentration

The most polar organic solvents like methanol, acetonitrile, formamide, *N*-methylformamide, *N*,*N*-dimethylformamide, dimethylsulfoxide and a mixture of methanol and acetonitrile possess of a good dissolving power towards the electrolytes that were commonly used in NACE. Among the different requirements for the successful use of NACE, the organic solvents should present a low UV absorbance at the wavelength of interest. Publications reported that methanol and acetonitrile are the best solvents when UV detection is selected [23–25]. Using neat methanol will result in longer migration times, since the dielectric constant to viscosity ratio is much lower in methanol (60.6) than in water (89.9) or acetonitrile (110.3). In addition, electrophoretic medium containing a mixture of solvents was found particularly advantageous to achieve high selectivity. Therefore, the investigations were carried out using a mixture of methanol and acetonitrile. And to evaluate the role of the electrolytes on the electrophoretic separation, different electrolytes, namely ammonium acetate, sodium cholate and Tris were used at first. It was found the analytes were not separated in ammonium acetate and sodium cholate. When the Tris was used the separation was obtained. So it was used for the further investigation.

In the publication reported on the application of NACE to the analysis of pharmaceutical drugs the organic solvent composition has a critical effect on resolution, efficiency and migration time [24,25]. In this study there was no peak separation when the concentration of acetonitrile in methanol was lower than 20% (v/v). Thus, concentration of acetonitrile in methanol ranging from 25 to 60% (v/v) was tested. It was found that no separation was obtained when concentration of acetonitrile exceeded 45% (v/v). And the migration time and resolution decreased slightly with increasing acetonitrile concentration. As shown in Fig. 2, the electrophoretic mobilities of the three investigated compounds were considerably increased with increasing of the acetonitrile percentage in methanol. This behavior is mainly due to the modification of the dielectric constant to viscosity ratio. Since the electrophoretic mobility is directly proportional to the ε/η ratio, methanol–acetonitrile mixtures with lower ε/η values exhibit lower electrophoretic mobilities. In addition, the higher acetonitrile percentage volatilization may even cause the formation of bubbles, which can result in the current breakdown during the experiment work. Considering the total analysis time and resolution 35% (v/v) acetonitrile was chosen as the optimum.

3.2. Effect of concentration of Tris

In the capillary electrophoresis separation buffer concentration plays a significant role. Tris buffer may minimize

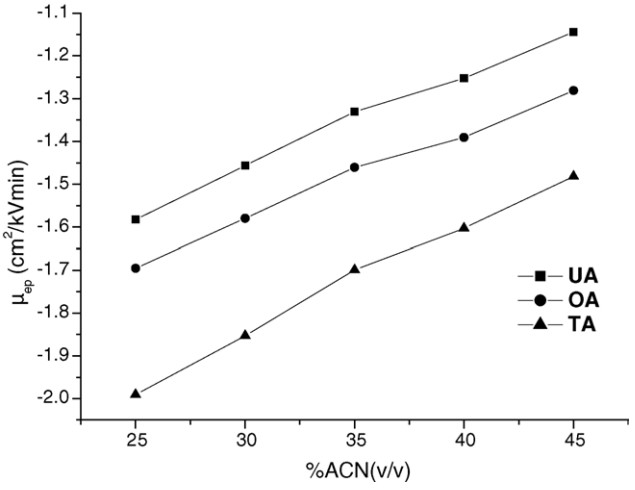


Fig. 2. Effect of acetonitrile concentration on the electrophoretic mobility of the analytes. Running electrolytes: 25–45% (v/v) acetonitrile in methanol, 90 mM Tris. Capillary: 50.0 cm (42.4 cm to detector) × 75 μm i.d. Applied voltage, 25 kV. Cartridge temperature: 20.0 ± 0.5 °C. Detection: 214 nm.

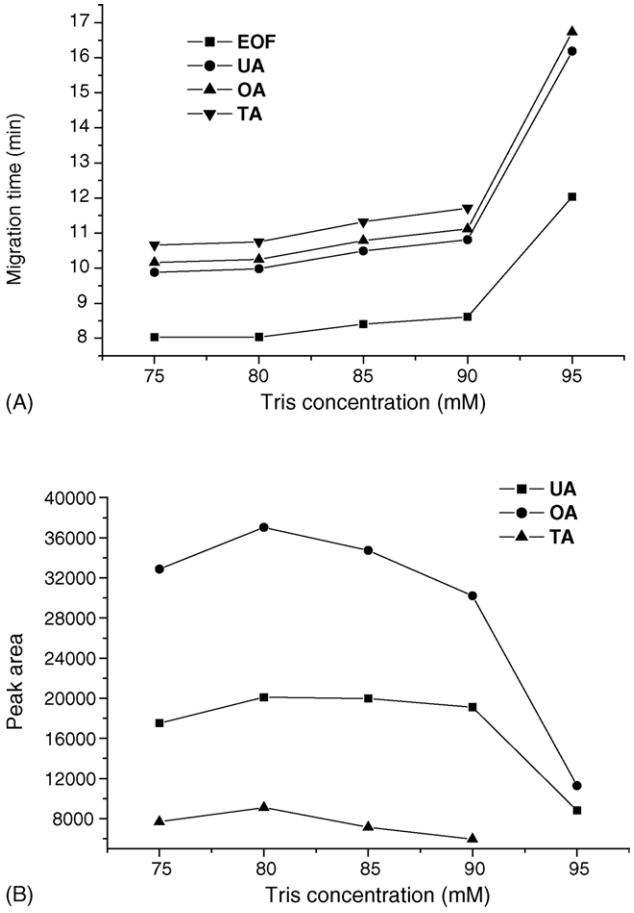


Fig. 3. Effect of Tris concentration on the migration time (A) and peak area (B) of the analytes. Running electrolytes: 75–95 mM Tris, 35% acetonitrile. The experiment conditions are same as in Fig. 2.

solute–wall interaction by shielding the capillary surface charge [26]. The higher the Tris concentration the more effective charge shielding was obtained. For this reason, the separation was performed with higher Tris concentration. To verify the effect of Tris concentration (60–95 mM) on migration behavior of the analytes, experiments were performed with 35% (v/v) acetonitrile in electrophoretic medium (Fig. 3). As shown in Fig. 3, the separation of UA and OA is less affected by the change of Tris in the studied concentration range. The resolutions of the analytes changed slightly with the increasing Tris concentration. UA and OA were not separated when the Tris concentration was lower than 75 mM, and the resolution was good at the higher Tris concentration (>80 mM). Meanwhile, the migration time and the peak width were increased also. And at higher Tris concentrations peak width became larger, presumably as a result of increased current generation and Joule heating. What's more, the peak height and area of analytes that refer to the detection sensitivity was weaker at the higher Tris concentration (>90 mM), especially for the analyte 3 (its peak was not observable at 95 mM). At 95 mM the migration time of the analytes was abruptly become longer. And in the range of Tris concentrations (75–90 mM) for the separation of UA and OA in the real samples, 90 mM was best. Considering the total analysis time, resolution, the detection sensitivity and the separation of real samples 90 mM was chosen as the optimum. So running electrolyte containing 90 mM Tris and a moderate acetonitrile concentration was selected in this system as electrolyte for further investigation.

3.3. Effects of pH^* and applied voltage

It is well known the pH of buffer is an important factor to the separation of the analytes. Although Tris was directly dissolved in methanol with 1.5% acetic acid added, the stock solution was 0.2 M. In the range of investigation (60–95 mM) the change of acetic acid was slight. What is important that in NACE the pH value is different from that in aqueous capillary electrophoresis. And the apparent pH (pH^*) was used to indicate the buffer pH of NACE. In the experiment, the pH^* was adjusted by added acetic acid or triethylamine, a series of buffers consist of 0.2–0.8 vol.% acetic acid or triethylamine were investigated under the buffer condition of 60 mM Tris and ACN:methanol (50:50 v/v). It was found that the pH^* was changed the separation of UA and OA was not improved greatly, changes of pH^* did not improve separation of them due to similarities of their structures; OA possesses additional CH_3 group as compared to UA, so the pH^* was not investigated further in this study.

Attempt was made to optimize the separation conditions by using different applied voltages ranging from 17.5 to 30 kV. The separation voltage determines the migration time directly and influences the resolution. The migration time decrease with increasing applied voltage and the resolution was decreased. And the higher applied voltage may even cause the formation of bubbles, which can result in the cur-

rent breakdown during the experiment work. Based on the migration time, resolution and the experiments work, 25 kV was selected as the applied voltage to accomplish a good compromise.

According to the factors mentioned above, the best resolution was obtained with an electrolyte containing 90 mM Tris, 35% acetonitrile in methanol medium and 25 kV applied voltage. The typical electropherogram for UA, OA and TA mixture under the optimum condition was shown in Fig. 4A.

3.4. Linearity, repeatability and detection limits

The linear relationship between the peak area and concentration of analytes was obtained by using the corresponding peak area (y) of the three standards (peak area provided by the chromatography workstation) versus their concentration (c). The linear regression equations, linear range, correlation coefficient, and detection limits were shown in Table 1. The results indicated that high linearity between y and c was attainable over the concentration range studied. Repeatability tests based on five injections of the three standard analytes were performed. The R.S.D. ($n = 5$) of the migration time and the peak area were 0.54 and 1.7% for UA, 0.45 and 2.24% for OA, 0.84 and 3.54% for TA, respectively. The limit of detection (LOD) was obtained as the analyte concentration that caused a peak with a height three times the baseline noise level and the limit of quantification (LOQ) was calculated as 10 times the baseline noise level. Thus, the LOQs were 12.3 μg/ml for UA, 4.6 μg/ml for OA and 13.3 μg/ml for TA. The LODs for the components quantified were in the range of 1.4–4.1 μg/ml (based on a 5 s injection), which was lower than the amounts found in the herb extraction.

3.5. Application

The method was used to the analysis of UA, OA and TA in *R. japonica* (Burm.f.) Hara var. glaucocalyx (Maxin.) Hara extraction. The typical electropherogram was illustrated in Fig. 4B. The peaks were identified by comparison the migration times and by spiking the standards to the sample solution. The main components in the extraction were quantified in duplicate by the present method. The result contents of the three compounds in this sample were given in Table 2. The recoveries of the method were determined with the standard addition method for the three analytes in the sample solution, respectively. The results and the R.S.D.% were also listed in Table 2. And UA and OA are widely coexisted in the following Chinese herbs: *Ligustrum lucidum* Ait. (Chinese name: nü-zhen-zi) is an important crude herb used in Chinese medicine. *Verbena officinalis* L. (Chinese name: mabian-cao), a popular herb in folk medicine for the treatment of rheumatism and bronchitis and for use as a diuretic, is widely grown in all temperate regions of the earth. *Piper kadsura* (Choisy) Ohwi (Chinese name: shi-nan-ye) are useful in cases of rheumatic arthritis and rheumatoid arthritis with joint pain, it has also been used for the relief of muscular contraction and ankylosis. *Lycopus lucidus* Turcz. var. hirtus Regel. (Chinese name: ze-lan) and *Prunella vulgaris* L. (Chinese name: xia-ku-cao) are also widely used to relieving liver pain and controlling high blood-fat. In order to validate the application of the present method, the method was used to

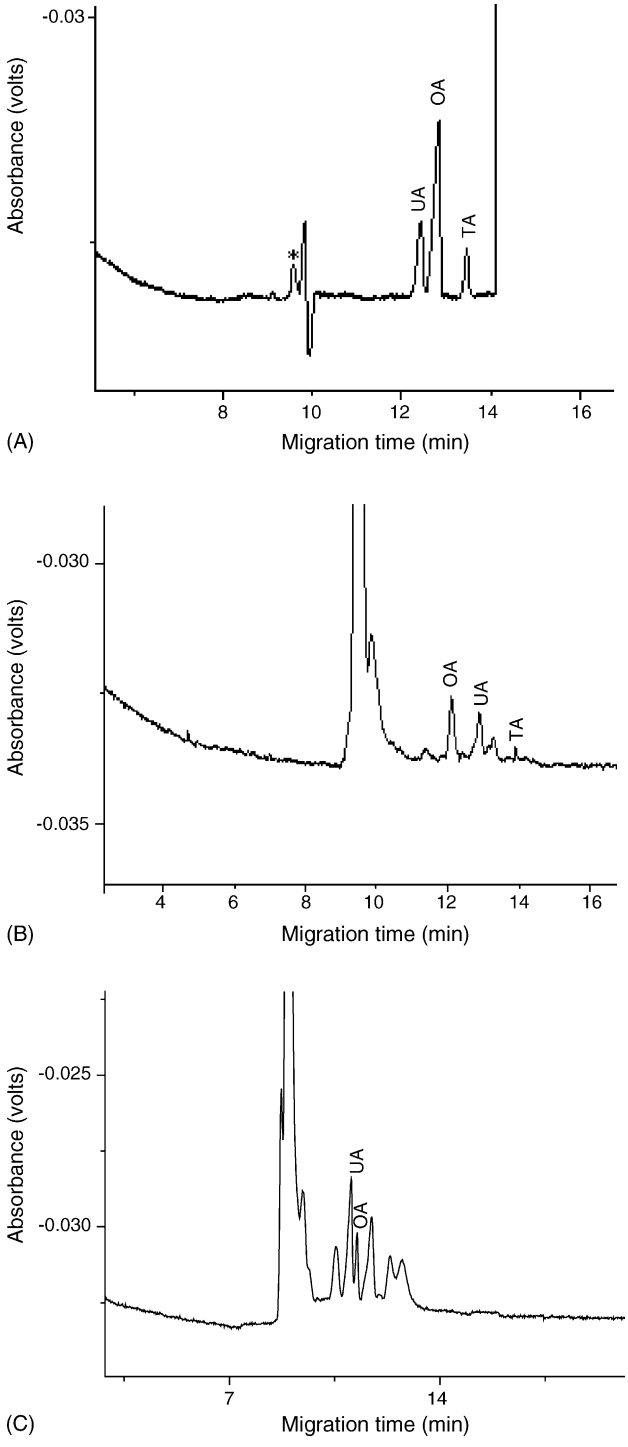


Fig. 4. The electropherogram of the standards analytes mixture (A) and real samples: (B) *Rabdosia japonica* (Burm.f.) Hara var. glaucocalyx (Maxin.) Hara, (C) *Piper kadsura* (Choisy) Ohwi (Chinese name: shi-nan-ye). *Acetone, running electrolytes: 90 mM Tris, 35% (v/v) acetonitrile in methanol. Other conditions are same as in Fig. 2.

Table 1
The results of regression analysis on calibration curves and the detection limits

Analyte	Regression equation, $y = a + bc$[a]	Correlation coefficient	Linear range (μg/ml)	Detection limit (μg/ml)[b]
UA	$y = -58.92 + 11.08x$	0.9987	12.5–660	3.8
OA	$y = -263.9 + 30.38x$	0.9999	12.5–400	1.4
TA	$y = 32.5 + 71.56x$	0.9996	12.5–400	4.1

[a] y denotes peak area of the analyte; c denotes the concentration (μg/ml) of the analyte.
[b] The detection limit was defined as the concentration where the signal-to-noise ratio is 3.

Table 2
Contents of the three analytes in (B) *R. japonica* (Burm.f.) Hara var. glaucocalyx (Maxin.) Hara extraction

Sample	UA	R.S.D. (%)	OA	R.S.D. (%)	TA	R.S.D. (%)
Total grass (mg/g)	2.09	1.4	0.755	2.9	0.523	3.7
Recovery (%)	96.5	1.9	95.1	3.8	94.0	4.3

Table 3
Contents of the ursolic acid and oleanolic acid in real samples and comparison of the contents to others

Samples	Ursolic acid			Oleanolic acid		
	Content (mg/g)	Recovery (%)	Content of Ref.	Content (mg/g)	Recovery (%)	Content of Ref.
C	13.4	93.6	15.05 [29][a] 79.8 [30][b]	3.54	91.5	6.351 [29] 27.9 [30]
D	5.42	91.3	1.31–1.98 [28][a]	1.04	90.4	1.0–1.08 [28]
E	6.74	89.9	3.1 [27][a]	1.00	88.7	1.2 [27]
F	4.71	95.8	4.7 [27][a] 20.7 [14][b]	24.05	90.3	9.1 [27] 10.83 [31][b] 78.3 [14]
G	3.33	90.0	3.5 [27][a]	0.495	91.1	1.5 [27]

C, *Piper kadsura* (Choisy) Ohwi (Chinese name: shi-nan-ye); D, *Lycopus lucidus* Turcz. var. hirtus Regel. (Chinese name: ze-lan); E, *Prunella vulgaris* L. (Chinese name: xia-ku-cao); F, *Ligustrum lucidum* Ait. (Chinese name: nü-zhen-zi); G, *Verbena officinalis* L. (Chinese name: ma-bian-cao).
[a] The contents of the analytes were determined by HPLC.
[b] The contents of the analytes were determined by CE.

analyze UA and OA in these Chinese herbs, the related results were listed in Table 3. And the determined results were compared to others (Table 3). From it we can find the results were satisfied. And most of them are consistent with the literatures except *Piper kadsura* (Choisy) Ohwi and *Ligustrum lucidum* Ait. This maybe the sample was collected from the different places or in different time, so the contents of the UA and OA was accordingly different.

4. Conclusion

This paper developed a novel, simple and applicable nonaqueous capillary electrophoresis method using Tris as running electrolyte with methanol:acetonitrile (65:35 v/v) mixture for the analysis of bioactive triterpenes in Chinese herbs extraction. In conclusion, the newly established NACE method was fit for the analysis of the main bioactive triterpenes and the quality control of the Chinese herbs. This is a promising feature in terms of the identification and analysis of bioactive triterpenes in Chinese herbs, especially ursolic acid and oleanolic acid. In addition, the NACE system will be useful for separation of many other bioactive substances. Related study is in progress.

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