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Separation and determination of the anthraquinones in *Xanthophytum attopvensis pierre* by nonaqueous capillary electrophoresis

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Abstract

A nonaqueous capillary electrophoresis (NACE) method with direct on-column UV detection has been developed for the separation of the pharmaceutically important anthraquinones from the total grass of *Xanthophytum attopvensis pierre* extract. The separation of three main anthraquinones (1-hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D-glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**)) was optimized with respect to concentration of sodium cholate (SC) and acetic acid, addition of acetonitrile (ACN), and applied voltage. Baseline separation was obtained for the three analytes within 5 min using a running buffer containing 50 mM sodium cholate (SC), 1.0% acetic acid and 40% ACN in methanol. The method of NACE for the separation and determination of bioactive ingredient in traditional Chinese medicines was discussed.

Keywords: *Xanthophytum attopvensis pierre*; Anthraquinones; Nonaqueous capillary electrophoresis

1. Introduction

Capillary electrophoresis (CE), because of its high resolution, minimal sample volume, **short** analysis time and high separation efficiency [1], has been an effective tool for drug quality control [2–10]. But, the most bioactive ingredients of drugs are insolvable in water, so it is necessary to introduce organic solvents in CE [11–13]. In fact, organic solvents are often employed to improve selectivity and resolution, increase solubility of hydrophobic compounds, change micelle properties and modulate the separation window. However, at high organic solvent level so contradictory results concerning erratic migration times and electric breakdown can occur. Therefore, the content of organic modifier was often lowed below 40% [13,14].

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 spectrometric 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.) with water, allowing a simple selective manipulation in NACE by changing the organic solvents or varying the proportions of two solvents [15,16]. Accordingly, NACE was successfully applied to analyze a large number of pharmaceuticals, including acidic and basic drugs, chiral compounds, peptides, ions and preservatives [17–20].

Xanthophytum attopvensis pierre belongs to the family of Rubiaceae plants. Rubiaceae plants have been shown to cool the blood, promote blood circulation, stop bleeding, remove blood stasis, and relieve pain activities due to the presence of many bioactive ingredient, especially anthraquinones [21]. Anthraquinones are best known for their antioxidant activity [22], anti-tumor promoters, Epstein–Barr virus activation [23], anti-human cytomegalovirus activity [24]. In addition, their extracts have been used for antitussive and expectotant, treating leukopenia caused by radiation, chemicals and unknown causes, and bacteriostatic in vitro [21]. So it has been used as folk medicine in China for the treatment

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of icterus hepatitis [25]. To better exploit these plants, we investigated the chemical constituents of *X. attopvensis pierre*, and found that it contains anthraquinones such as 1-hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D-glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**), among other compounds. *X. attopvensis pierre* may be a resource for extracting these active compounds. Anthraquinones have been analyzed by many techniques, including spectrophotometry [26], high-performance liquid chromatography (HPLC) [27], thin-layer chromatography (TLC) [28], micellar electrokinetic capillary chromatography (MEKC) [7,28–33], zone capillary electrophoresis (CZE) [34,35], capillary electrochromatography (CEC) [36] and nonaqueous capillary electrophoresis [37]. However, the method of TLC cannot be applied to simultaneously determine several components in single crude herb or in a medicinal preparation; HPLC suffers from limitation such as consumption of materials and time, and the number of prior steps are often required to obtain the species of interest from the sample matrix. Those investigations dealing with the qualitative analysis of anthraquinones by CZE and MEKC are time-consuming; analysis time of CEC is short, it is needed a packed column, however. So NACE is an optimum method for separation anthraquione compounds because it is rapid, easy and useful. Up to now, there is no report on the simultaneous determination of these three main anthraquione compounds in *X. attopvensis pierre*. Thus it is necessary to develop a simple, economical and efficient method for the simultaneous determination of these compounds in the Rubiaceae plants.

In this paper, a highly selective NACE system was developed for the separation and determination of the three active compounds in *X. attopvensis pierre*. The effects of other organic solvent composition, the concentrations of electrolyte, acetic acid and applied voltage were investigated.

2. Experiments

2.1. Instruments

A waters quanta 4000 capillary electrophoresis system (Milford, MA, USA) controlled by a personal computer was used. Capillary electrophoresis was performed using a 40.0 cm (32.5 cm to the detector) × 75 μm i.d. fused silica capillary (Yongnian Photoconductive Fibre Factory, Hebei Province, China). Samples were introduced from the anodic end of the capillary by hydrodynamic injection by rasing the sample vial 10.0 cm for 5 s. Direct UV detection was employed at a wavelength of 254 nm. Data acquisition was carried out with a maxima 820 chromatography workstation. The capillary was conditioned prior to use with 0.1 M NaOH for 10 min and distilled water for 5 min, followed by the electrophoresis buffer for 5 min. After each run the capillary was rinsed with 0.1 M NaOH for 2 min, distilled water for 2 min and then the running buffer for 2 min. All operations were performed at 21.0 ± 0.5 °C. To avoid buffer and sample evaporation, the buffer and sample reservoirs were capped.

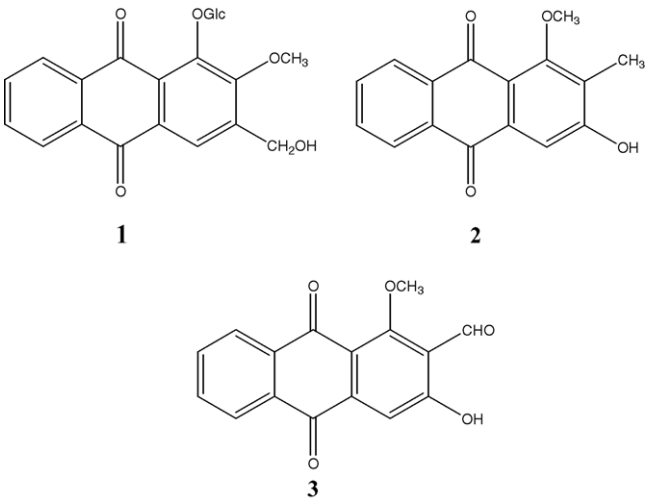

Fig. 1. The structures of the analytes.

2.2. Reagents

The three anthraquinones used as standards were gifts to the authors and their characterization by NMR, IR, MS were obtained from the state key laboratory of OSSO, Lanzhou Institute of Chemical Physics. They were first isolated from the whole plants of *X. attopvensis pierre* (see Fig. 1 for the structures). SC was purchased from Serva Feinbiochemica (Heidelberg, New York). Acetic acid was purchased from Tianjin First Chemical Factory. ACN was purchased from Tianjin Secondary Chemical Factory. Methanol was purchased from Shanghai Zhenxing First Chemical Factory. All of the chemicals used were of analytical grade. *X. attopvensis pierre* was collected from Hekou city, Yunnan province, China in July 2002 and identified by Prof. Wang Wenjiu, Southwest Forest College, Kunming, China.

2.3. Sample perpration

Stock solutions of **1** (250 mg L^{-1}), **2** (250 mg L^{-1}), and **3** (250 mg L^{-1}) were prepared in the running buffer. Solutions of lower concentration were prepared by dilution of the stock solutions in the appropriate running buffer. The running buffer solutions were prepared by adding 25 ml of 200 mM SC (methanol medium), 1.0 ml acetic acid and 40 ml ACN in a 100 ml flask and diluting to 100 ml with methanol. All solutions for CE were filtered through a 0.45 μm filter.

The powdered total grass (1.0 g) of *X. attopvensis pierre* was extracted with 7.0 ml methanol for one hour in an ultrasonic bath. Extraction was repeated three times. The extracts were combined and evaporated to near dryness, and then the residue dissolved using 25 ml of running buffer.

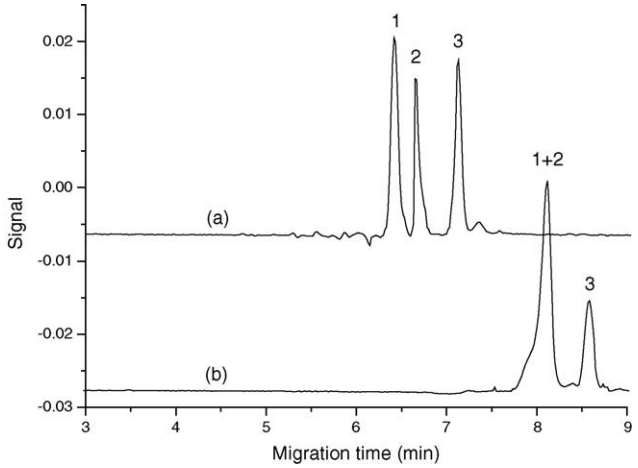

Fig. 2. Effect of different electrolyte on the migration behaviors of **1**, **2** and **3**. Buffer: 20% ACN, 1% acetic acid and (a) 50 mM SC, (b) 50 mM ammonium acetate. Capillary: 40.0 cm (32.4 cm to detector) × 75 μm i.d. Applied voltage: 25 kV. Cartridge temperature: 21.0 ± 0.5 °C. Detection: 254 nm. 1-Hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D-glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**).

The solution was passed through a 0.45 μm filter, and was injected directly into the capillary electrophoresis system.

3. Results and discussion

3.1. Choice of organic solvent and electrolyt

The most polar solvents, like methanol, ACN, formamide, *N*-methylformamide (NMF), *N*, *N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO) and mixtures of methanol and ACN commonly used in NACE. Electrophoretic medium containing a mixture of methanol and ACN was found not only to be particularly advantageous for achieving high selectivity, but also posses good dissolving powers for the three analytes since the three analytes don't dissolve in both neat methanol and ACN. So this type of mixture was chosen for examination here.

Electrolyte is also an important factor influencing the separation and sensitivity in this system. In order to select a appropriate electrolyte, the separation of the three analytes was first tried with buffer containing 50 mM ammonium acetate,

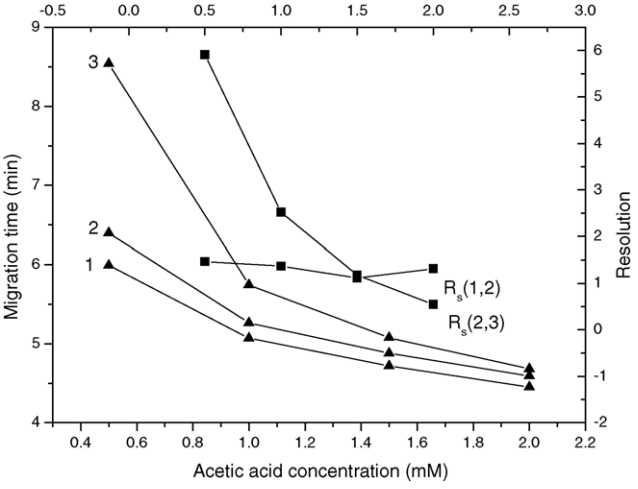

Fig. 3. Effect of acetic acid concentration on the migration time and resolution of **1, 2** and **3**. Buffer: 0.5–2.0% acetic acid, 50 mM SC and 20% ACN. 1-Hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D-glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**). Capillary: 40.0 cm (32.4 cm to detector) × 75 μm i.d. Applied voltage: 25 kV; cartridge temperature: 21.0 ± 0.5 °C; detection: 254 nm. (▲) Migration time; (■) resolution.

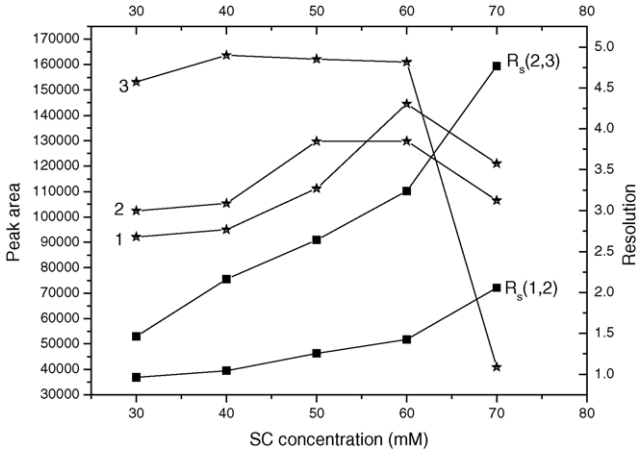

Fig. 4. Effect of SC concentration on the peak area and resolution of **1, 2** and **3**. Buffer: 1.0% acetic acid, 30–70 mM SC and 20% ACN. The experiment conditions are same as Fig. 3. (★) Peak area; (■) resolution. 1-Hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D-glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**).

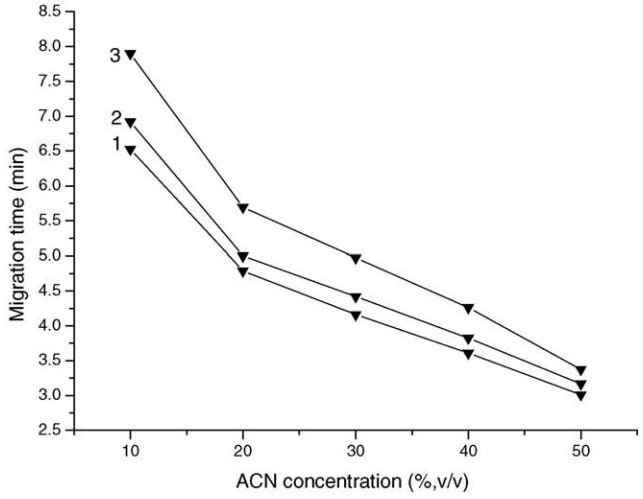

Fig. 5. Effect of ACN concentration on the migration time and resolution of **1, 2** and **3**. Buffer: 1.0% acetic acid, 50 mM SC and 10–50% ACN. Other conditions are same as Fig. 3. (▲) Migration time; (▲) resolution. 1-Hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D-glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**).

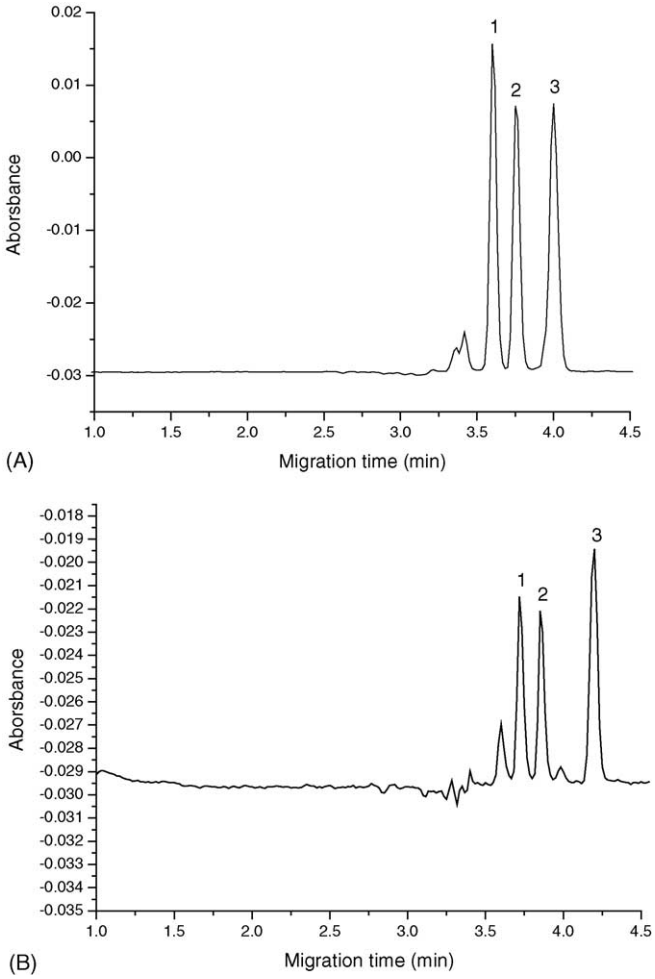

Fig. 6. The electropherograms of the standards mixture solution and the real samples. (A) The standards mixture. (B) Extract of the *Xanthophytum attopvensis pierre*. Buffer: 1.0% acetic acid, 50 mM SC and 40% ACN. Run current: 102 μA. Other conditions are same as Fig. 3. 1-Hydroxy-2-methoxy-3-hydroxymethyl-9, 10-anthraquinone-1-*O*-β-D -glucoside (**1**), rubiadin- 1-methylether (**2**) and 1-methoxy-2-formyl-3-hydroxy-9, 10-anthraquinone (**3**).

0.5% acetic acid and 20% ACN, but an effective separation was not achieved (Fig. 2). This result may be explained that **1** and **2** were nearly electroneutral. To separate **1** and **2**, a surfactant should be introduced. Because SC is not only a biological surfactant, but also a good electrolyte; which is easily dissolved in methanol, the separation was attempted in a buffer containing 50 mM SC, 0.5% acetic acid and 20% ACN. In this case, the three analytes were effectively separated (Fig. 2), so a buffer containing SC, acetic acid and ACN was selected as the electrolyte for this system.

Table 2
Contents of the analytes in *Xanthophytum attopvensis pierre* ($n = 5$)

Sample	**1** ($mg\,g^{-1}$)	R.S.D. (%)	**2** ($mg\,g^{-1}$)	R.S.D. (%)	**3** ($mg\,g^{-1}$)	R.S.D. (%)
Total grass	0.49	2.43	0.44	2.15	0.52	2.28

3.2. Effect of concentration acetic acid

The acidity of the buffer plays an important role in improving selectivity in NACE. In this paper, the acidity of buffer was adjusted using acetic acid. To verify the effect of acetic acid concentration (0.5–2.0%, v/v; apparent pH 7.11–6.42) on the resolution and migration time of the analytes, experiments were performed with 50 mM SC and 20% (v/v) ACN as the electrophoretic medium (Fig. 3). As shown in Fig. 3, the resolution of **1** and **2** did not change significantly, while that of **2** and **3** decreased markedly in the concentration range of 0.5–2.0%. This effect may be explained by the fact that **1** and **2** were nearly electroneutral and have been affected by acetic acid slightly, but **3** with an active aldehyde group was ionizable and its extent of ionization decreased with increasing the acetic acid concentration. Considering the total analysis time and resolution, 1.0% acetic acid concentration was chosen as the optimum.

3.3. Effect of SC concentration

Buffer concentration also has a significant effect on the separation performance through its influence on the ionic strength and the viscosity of electrolyte, adsorption of capillary wall for the analytes and the current produced in the capillary. Electrophoretic media containing 20% (v/v) acetonitrile, 1.0% acetic acid and various concentrations of SC, ranging from 30 to 70 mM, to evaluate the influence on the separation of electrolyte ionic strength. As shown in Fig. 4, it was found the resolution increased with the SC concentration increasing, and when the SC concentration was more

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

Analyte	Regression equation $Y = a + bx$[a]	Correlation coefficient	Linear range ($mg\,L^{-1}$)	Detection limit ($mg\,L^{-1}$)[b]
1	$Y = -47.24 + 327.4x$	0.99999	0.78–250	0.24
2	$Y = 349.5 + 252.4x$	0.99995	1.56–250	0.37
3	$Y = -280.7 + 573.2x$	0.99997	0.78–250	0.19

[a] Y and x stand for the peak area and the concentration ($mg\,L^{-1}$) of the analytes, respectively.
[b] The detection limit was defined as the concentration where the signal–noise ratio is 3.

Table 3
Recovery of the three analytes ($n = 5$)

Sample	**1**		**2**		**3**	
	Added ($mg\,L^{-1}$)	Recovery (%)	Added ($mg\,L^{-1}$)	Recovery (%)	Added ($mg\,L^{-1}$)	Recovery (%)
Total grass	125	94.9	125	101	125	102
	50	98.2	50	96.1	50	106
	25	103	25	105	25	97.3
R.S.D.		4.13		4.43		4.27

than 50 mM, the three analytes were separated completely. Moreover, the electric current increased from 54 to 115 μA producing joule heating, evaporation and the detection sensitivity decreasing problems. In order to obtain a higher separation and detection sensitivity, 50 mM SC was chosen as the appropriate concentration of buffer.

3.4. Effect of concentration of ACN (v/v)

In previous studies concerning the application of NACE to the analysis of pharmaceutical drugs, it was demonstrated that the organic solvent composition has a critical effect on resolution, efficiency and migration time [37,38]. Pure acetonitrile was not selected as nonaqueous solvent due to the very low solubility of SC in this solvent. Thus, the effect of ACN concentration was tested from 10 to 50%. The migration time of the analytes against ACN concentration (v/v) is shown in Fig. 5. It can be seen that the migration time decreased with increasing ACN concentration. In addition, the higher ACN percentages give a greater likehood for bubble formation and a lower solubility of SC, resulting in current breakdown during the experimental work. Considering the total analysis time and solubility of SC, 40% ACN was chosen as the optimum.

3.5. Effect of applied voltage

High voltage was required in CE to reduce the analysis time. Therefore, attempt was made to optimize the separation conditions by using different applied voltages ranging from 15 to 27.5 kV. It was observed that the separation voltage determines the migration time directly. But a voltage higher than 25 kV may cause bubble formation, a result that can cause current breakdown during the experiment. Based on the experiment, 25 kV was selected as the optimum voltage for the best compromise.

According to the factors mentioned above, the best resolution was obtained with an electrolyte containing 50 mM SC, 1.0% acetic acid and 40% ACN in methanol medium and 25 kV applied voltage. A typical electropherogram for a standard mixture analyzed using the optimum conditions is shown in Fig. 6A. All three analytes were well separated within 5 min.

3.6. Linearity, repeatability and detection limit

Linear relationships between the concentration of the three analytes and the corresponding peak areas are listed in Table 1. The calibration curves exhibit excellent linear behavior over the concentration range (0.78–250 $mg\,L^{-1}$ for **1**, 1.56–250 $mg\,L^{-1}$ for **2** and 0.78–250 $mg\,L^{-1}$ for **3**). The detection limits are also given in Table 1.

The repeatability of the migration time and peak area of **1**, **2** and **3** in the experiment were determined at three concentration levels ($n = 5$) in the ranges given above, respectively. The relative standard deviations (RSD) of thes migration time and peak area of each peak were 0.45–0.62% and 0.15–3.4% (intra-day), and 0.93–1.0% and 3.2–6.3% (inter-day), respectively.

3.7. Application and recovery

The optimum conditions were applied to the separation and determination of **1**, **2** and **3** in the powdered total grass of (1.0 g) of *X. attopvensis pierre*. A typical electropherogram obtained is shown in Fig. 6B. The peaks were identified by comparison with previously obtained migration times and standard addition.

The contents of the analytes found in *X. attopvensis pierre* **1**, **2** and **3** together with their relative standard deviations (RSD) are given in Table 2. It was found that this plant was the most effective for anthraquiones preparations.

Recovery studies: **1**, **2** and **3** were added to the samples in known concentrations, and were then analyzed according to the proposed procedure. The results are listed in Table 3. From Table 3, it was found that the recovery of the proposed method was consistent with the request of quantitative analysis (R.S.D.$\leq \pm 5\%$) [39].

4. Conclusion

These results demonstrate that the NACE method proposed in this study was useful, simple and repeatable for identification and determination of analytes **1–3** in *X. attopvensis pierre* using direct on-column UV detection. The proposed NACE method is a good alternative for

simultaneous analysis of these bioactive components in *X. attopvensis pierre*. As an efficient technique it may be used for the investigation of other anthraquinones in Chinese traditional herbs.

Acknowledgements

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References

[1] J.W. Jorgenson, K.D. Lukacs, Science 222 (1983) 266–272.
[2] C.J. Shaw, N.A. Guzman, Applications of capillary electrophoresis technology in the pharmaceutical industry, in: Handbook of Pharmaceutical Analysis, Series Drugs and the Pharmaceutical Sciences, vol. 117, Marcel Dekker, New York, 2001.
[3] M. Blanco, I. Valverde, Electrophoresis 23 (2002) 578–584.
[4] M.A. Raggi, V. Pucci, F. Bugamelli, V. Volterra, J. AOAC Int. 84 (2001) 361–368.
[5] M.A. Raggi, G. Casamenti, R. Mandrioli, G. Izzo, E. Kenndler, J. Pharm. Biomed. Anal. 23 (2000) 973–980.
[6] D.L. Baker, Capillary Electrophoresis, Wiley, New York, 1995.
[7] S.F. Wang, J.Q. Dai, X.G. Chen, Z.D. Hu, Plant Med. 68 (2002) 1–5.
[8] S.H. Liu, X. Tian, X.G. Chen, Z.D. Hu, J. Chromatogr. A 928 (2001) 109–115.
[9] H.T. Liu, K.T. Wang, Y. Zhao, X.G. Chen, Z.D. Hu, J. High Resol. Chromatogr. 23 (2000) 697–698.
[10] S.G. Zhao, X.G. Chen, Z.D. Hu, Chromatographia 57 (2003) 593–598.
[11] S. Cherkaoui, L. Mateus, P. Christen, J.-L. Venthey, Chromatographia 46 (1997) 351–357.
[12] A.T. Balchunas, M.J. Sepaniak, Anal. Chem. 60 (1988) 617–621.
[13] S. Fuj wara, S. Honda, Anal. Chem. 59 (1987) 487–490.
[14] Y. Walborehl, J.W. Jorgenson, Anal. Chem. 58 (1986) 479–481.
[15] C. Reichardt, Solvent and Solvent Effects in Organic Chemistry, VCH, Weinheim, 1990.
[16] S.P. Porras, M.-L. Riekkola, E. Kenndler, J. Chromatogr. A 924 (2001) 31–42.
[17] I. Bjornsdottir, J. Tjornelund, S.H. Hansen, Electrophoresis 19 (1998) 2179–2186.
[18] Y.Q. Li, S.Y. Cui, Y.Q. Chen, X.G. Chen, Z.D. Hu, Anal. Chem. Acta., in press.
[19] J. Tjornelund, S.H. Hansen, J. Biochem. Biophys. Methods 38 (1999) 139–153.
[20] M.L. Riekkola, Electrophoresis 23 (2002) 3865–3883.
[21] China-English Manual of Commom-used in Traditional Chinese Medicine, Publisher of Guangdong Science and Technology, 1992, p. 514.
[22] G.C. Yen, P.D. Duh, D.Y. Chuang, Food Chemi. 70 (2000) 437–441.
[23] J. Koyama, I. Morita, K. Tagahara, M. Ogata, T. Mukainaka, H. Tokuda, H. Nishino, Cancer Lett. 170 (2001) 15–18.
[24] D.L. Barnard, D.W. Fairbairn, K.L. O'Neill, T.L. Gage, R.W. Sidwell, Antiviral Res. 28 (1995) 317–329.
[25] The Editorial Committee of Chinese Science Plants Records, Illustrated Handbooks of Chinese High Plant, fourth ed., Science Press, Beijing, 1980, p. 210.
[26] E. Varga, K. Szendrei, Z. Hajdú, L. HornoK, G. Csaki, Herba Hung. 25 (1986) 115–133.
[27] D. Louden, A. Handley, R. Lafont, S. Taylor, I. Sinclair, E. Lenz, Anal. Chem. 74 (2002) 288–294.
[28] M. Bathori, I. Mathe, A. Guttman, Chromatographia 48 (1998) 145–148.
[29] C.H. Kuo, S.W. Sun, Anal. Chim. Acta 482 (2003) 47–58.
[30] X.Y. Shang, Z.B. Yuan, J. Pharm. Biomedi. Anal. 31 (2003) 75–81.
[31] X.Y. Shang, Z.B. Yuan, Bioorg. Med. Chemi. Lett. 13 (2003) 617–622.
[32] X.Y. Shang, Z.B. Yuan, Anal. Chim. Acta 456 (2002) 183–188.
[33] X.Y. Shang, Z.B. Yuan, Anal. Chim. Acta 309 (1995) 361–367.
[34] M. Puchalska, M. Orlińska, M.A. Ackacha, K. Poleć-Pawlak, M. Jarosz, J. Mass Spectrom. 38 (2003) 1252–1258.
[35] W.C. Weng, S.J. Sheu, J. High Resol. Chromatogr. 23 (2000) 147–148.
[36] Y. Li, H.W. Liu, X.H. Ji, J.L. Li, Electrophoresis 21 (2000) 3109–3115.
[37] S. Cherkaoui, E. Varesio, P. Christen, J.-L. Venthey, Electrophoresis 19 (1998) 2900–2906.
[38] S. Cherkaoui, J.-L. Venthey, Analusis 127 (1999) 765–771.
[39] The Experimental Tutorial of Quantitative Analysis Chemistry, Publisher of Fudan University, 1990. p. 62.