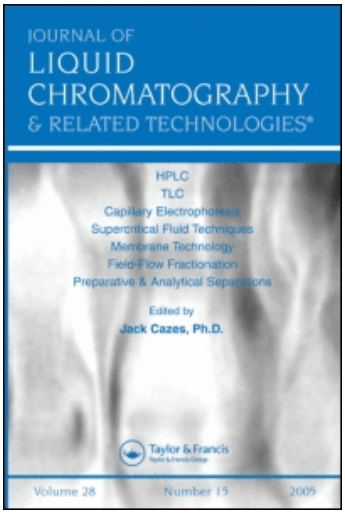

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Comparative Analysis of Allantoin, Quercetin, and 1-Methyl-1,2,3,4-Tetrahydro-β-Carboline-3-Carboxylic Acid in *Nitraria tangutorum* Bobr. Seed by HPLC-APCI-MS and CE

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Abstract: This paper describes the simultaneous determination of allantoin, quercetin, and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCCA) in *Nitraria tangutorum* Bobr seed by HPLC-APCI-MS and CE (capillary electrophoresis) methods. The final optimized chromatographic conditions were investigated in a reversed-phase Eclipse XDB-C8 column (150 × 4.6 mm, 5 μm). A seventeen-minute gradient elution, (A: aqueous acetonitrile 20% (v/v); B: aqueous acetonitrile 60% (v/v); C: pure acetonitrile 100%) at a flow rate of 1.0 mL/min was selected for the separation of three natural products with diode array detection (DAD) at 220 nm. A CE experiment was carried out in a fused silica capillary with 32 mmol/L boric acid (pH 10), 32 mmol/L SDS and acetonitrile (10.0%, v/v). The applied potential and temperature was, respectively, set at 19 kV and 25°C. After development, the validation was performed in parallel for HPLC and CE, with the same standards and

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sample to avoid differences due to the manipulation. The validation parameters of both techniques were adequate for the intended purpose.

Keywords: Allantoin, Quercetin, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, *Nitraria tangutorum* Bobr, High performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, Capillary electrophoresis

INTRODUCTION

The genus *Nitraria* (Zygophyllaceae) is a shrub that bears edible berries. It is widely distributed in the Middle East, central Asia, and the northwest region of China. Among the *Nitraria* species, only *Nitraria tangutorum* Bobr. grows in China, especially in the desert areas of Qinghai-Tibetan Plateau. A main function of the *Nitraria tangutorum* Bobr. forest is to conserve the soil and water from the wind blown sand.[1,2] Its leaves, fruits, and seeds are often used as folklore medicines such as anti-spasmodic, anti-neuropathic, and anti-arrhythmicagent[3,4] to cure weaknesses in the spleen and stomach, including indigestion, neurasthenia and cold,[5,6] and decreasing blood lipid levels and anti-oxidation.[7]

The chemical components of the *Nitraria tangutorum* seed have been studied and several kinds of alkaloids and flavones including allantoin, MTCCA, and quercetin have been separated from it. Pharmacological tests showed that allantoin was effective in treating cancer and ache.[8] Quercetin has properties such as anti-tumor,[9] anti-bacterial, and inhibition of human platelet aggregation.[10] For herb medicines, it is important to ensure the reliability and reproducibility for pharmacological and clinical research, and to improve product quality control. Therefore, there is an increasing demand for methods of rapidly identifying and characterizing constituents in herbs.

High performance liquid chromatography (HPLC) is a powerful analytical tool that has been used for several decades for analyzing traditional Chinese medicines. With the advent of soft ionization techniques, mass spectrometry has become a powerful analytical tool in phytochemistry due to its high sensitivity.[11] Although, HPLC is a rapid and precise alternative compared to other routine techniques, it has limitations, such as separation efficiency attainability with high molecular weight solutes. Improvement in the efficiency of traditional HPLC is commonly very high pressures or great amount of time consumption.[12]

Compared with HPLC, capillary electrophoresis (CE) has become a more powerful technique in separation and detection because of its minimal sample volume requirement, short analysis time, and high separation efficiency. In spite of its versatility, some limitations, such as a discontinuous mode of sample introduction, sampling bias with the electrokinetic mode, low sensitivity in terms of the concentration, and complicated sample off-line pretreatment procedures, still call for further improvements.

In this paper, we present the development, validation, and comparison between HPLC-APCI-MS and CE methods for the determination of allantoin, quercetin, and MTCCA in the *Nitraria tangutorum* seed.

EXPERIMENTAL

HPLC-APCI-MS Instrumentation and Conditions

Experiments were performed using a LC/MSD-Trap-SL liquid chromatograph/Mass Spectrometer (1100 Series LC/MSD Trap, a complete LC/MS/MS). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), and a diode array detector (DAD) (model G1315A). Ion source type, APCI (Positive/Negative model); nebulizer pressure 60 psi; dry gas temperature 450°C; dry gas flow 5.0 L/min. APCI Vap temperature 350°C; Corona Current (nA) 4000 (pos); Capillary voltage 3500 V. Samples were separated on a reversed-phase Eclipse XDB-C8 column (150 × 4.6 mm 5 μm). The flow rate was constant at 1.0 mL/min and the column temperature was set at 30°C. The HPLC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an APCI (ESI) source. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. A seventy minute gradient elution (A: 20%(v/v) acetonitrile; B: 60%(v/v) acetonitrile; C: 100% acetonitrile) was selected for the separation of three natural products. The samples were detected with a DAD at 220 nm. The mobile phase was filtered through a 0.2 μm nylon membrane filter (Alltech, Deerfiled, IL).

Capillary Electrophoresis Instrumentation and Conditions

Electrophoretic analyses were performed using a HP-3D CE system with a DAD (Agilent Technologies, USA). Data were collected on a PC computer using HP 3D ChemStation. In all experiments, 48.5 cm × 50 μm (40 cm to the detector) fused silica capillaries (Yongnian Optical Fiber Factory, Hebei, China) were used. The samples were detected at 220 nm. Before use, new capillaries were flushed with 1.0 mol/L NaOH for 1 h, and then flushed with redistilled water and background buffer for 10 min. Between runs, the capillary was rinsed under pressure with 0.1 mol/L NaOH, redistilled water, and running buffer for 2 min each. The temperature was maintained at 25°C. The other conditions are as follows: applied voltage 21 kV, samples were injected by applying a pressure of 50 mbar for 4 s.

Chemicals

Sodium hydroxide was purchased from Jining Chemical Reagent Co. (Shandong, China). HPLC grade of ethanol, and acetonitrile were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used in this study were also of analytical grade, unless otherwise stated. The three natural products (structures shown in Figure 1), allantoin, quercetin, and MTCCA were isolated from *Nitraria tangutorum* seeds. Their structures were confirmed by comparing their melting points, ^{1}H-NMR, IR, UV, and MS data, with those given in the literatures.[13–18]

Preparation of Standard Solutions

The standards of two polar components, quercetin and MTCCA, were prepared by dissolving 6.2 mg quercetin and 4.8 mg MTCCA, respectively, in 1.0 mL of 75%(v/v) ethanol, corresponding standard concentrations were 6.2 mg/mL and 4.8 mg/mL. The standard solution of allantoin (2.4 mg/mL) was prepared by dissolving 2.4 mg in 1.0 mL redistilled water. Corresponding low concentrations of solutions were obtained by diluting the stock solution with 75%(v/v) ethanol. When not in use, all reagent solutions were stored at 4°C or −20°C in a refrigerator.

Sample Preparation

Nitraria tangutorum seeds were collected from Dulan county of Qinghai province and washed successively with water and deionized water. The

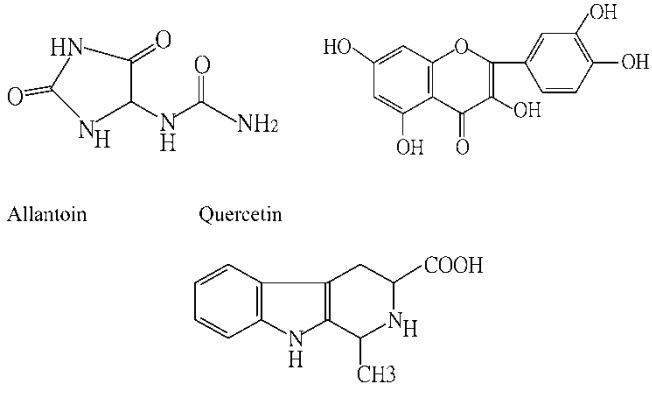

Figure 1. Structure of the three natural product test standards.

washed seeds were broken into powdered samples and dried under a stream of nitrogen. *Nitraria* seed powder (5 g) was extracted with 50 mL 75%(v/v) of ethanol at 80°C for 1.5 h. The extraction was repeated three times, the combined solution was condensed in vacuo. The condensed product was redissolved with aqueous ethanol (75%, v/v) in a 50 mL flask. The contents were then centrifuged at a speed of 4000 rpm for 15 min. The supernatant was collected and stored at 4°C in a refrigerator until analysis.

RESULTS AND DISCUSSION

Method Development

HPLC-APCI-MS

For the simultaneous separation of the three natural products, an Eclipse XDB-C8 column was used in combination with a line gradient elution. During conditioning of the column and prior to injection, the mobile phase composition was 70% A and 30% B. The percentage of mobile phase was changed as follows after injection: 0–60% (B) from 0 to 10 min (C = 0); 60–100% (B) from 10 to 11 min (C = 0); 100–0% (B) and 0–100% (C) from 11 to 12 min (A was 0%); 100-100% (C) from 12 to 17 min (A = 0, B = 0) and then kept for 5 min. Under these conditions, the three natural products were separated within 9 min (Figure 2). Although, the isocratic elution with 20% acetonitrile could separate the three natural products, the separation time was generally long (about 30 min) and the two late eluting components were broadened and tailed. To achieve the shortest analysis time and good resolution for the three natural products, the gradient elution program above was selected. The flow rate was constant at 1.0 mL/min and the column temperature was set at 30°C.

The ultraviolet absorption of the three natural products was investigated in aqueous ethanol (75%, v/v). The absorption wavelength of the three natural products was obtained with the online scanning range of 190 to 400 nm. The UV spectra of the three natural products were shown in Figure 3. To compare UV responses, it was found that they have good absorbability together from 210 to 220 nm. Absorption exhibited higher intensity in low wavelength, however, serious interferences from background electrolyte solution were also observed. The ultraviolet detection was, therefore, selected at 220 nm.

Prior to use, the instrument was checked to meet the sensitivity defined by the manufacturer. The HP1100 LC/MSD-SL was calibrated with APCI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specification and sensitivity were achieved over the entire mass range. APCI source and instrument parameters were optimized by infusing the three natural products that were

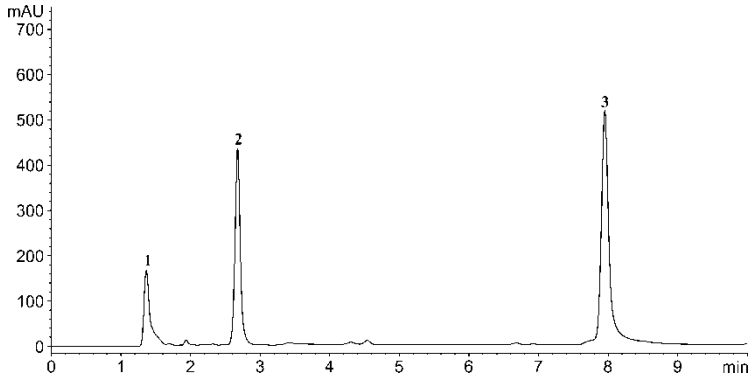

Figure 2. Typical chromatogram for the three natural products. Column temperature is set at 30°C; Column 150 × 4.6 mm Eclipse XDB-C8 (5 mm); flow rate = 1.0 mL/ min. Gradient conditions described as in experimental section. Peaks: 1. Allantoin; 2. MTCCA; 3. Quercetin.

isolated from an HPLC column with DAD detection, and into the post column on-line mass spectrometer. The ionization and fragmentation of the isolated three natural products was studied by mass spectrometry with atmospheric pressure chemical ionization detection at APCI negative-ion mode. As expected, all three natural product components produced an intense molecular ion peak at m/z $[M - H]^+$ (see Figure 4). The selected reaction monitoring was based on the m/z $[M - H]^+(157.4) \rightarrow 114.0$ and 139.9 transition for allantoin; $[M - H]^+(229.2) \rightarrow 186.0$, 116.1, and 142.0 transition for MTCCA; $[M - H]^+(301.1) \rightarrow 150.9$, 178.9, 273.0, and 229.0 transition for quercetin.

CE

The effects of the various buffer, buffer concentration, pH, and organic modifier concentration on the migration time were investigated.

Several types of buffers were tested in this study for the separation of the three natural products, including carbonate buffers, phosphate buffers, and borate buffers. The results indicated that a reasonably good separation of all three compounds was obtained with borate buffers (pH 10), at concentration ranges of 24–44 mmol/L. The resolutions of the three compounds in the CE running procedure increase progressively with increasing concentration of buffers; the migration times also increase. With high buffer concentrations of >32 mmol/L, an increased ionic strength will result in lower EOF. Taking both the shorter run time and higher zeta potential into consideration, the borate concentration was selected at 32 mmol/L. The effects of various pH on the resolution of three solutes were investigated using borate as the buffer substance in the pH range of 8.5–11.5. There was an obvious

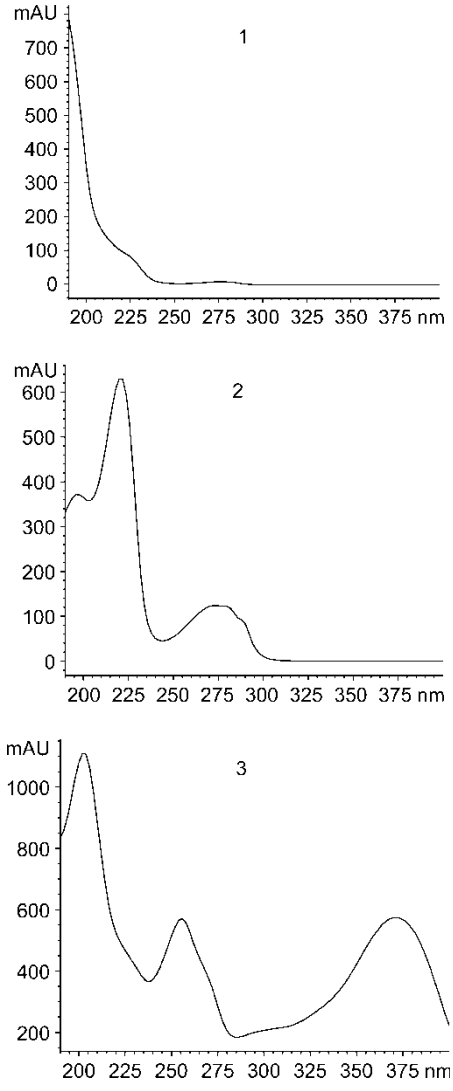

Figure 3. UV spectra of the three natural products. Peaks: 1. Allantoin; 2. MTCCA; 3. Quercetin.

improvement in efficiency with increasing the pH, but no significant change in selectivity was observed. Usually the optimum buffer capability of a solute is pKa $\pm$ 1 unit, therefore, the pKa value of borate is 9.24 $\pm$ 1. Subsequently, a 32 mmol/L of borate buffer solution at pH10.0 was used in this experiment.

An increase in the acetonitrile concentration increased the flowm rate, partially because the ionic strength was decreased and partially because a greater amount of acetonitrile increases the EOF.[19] The increase in EOF

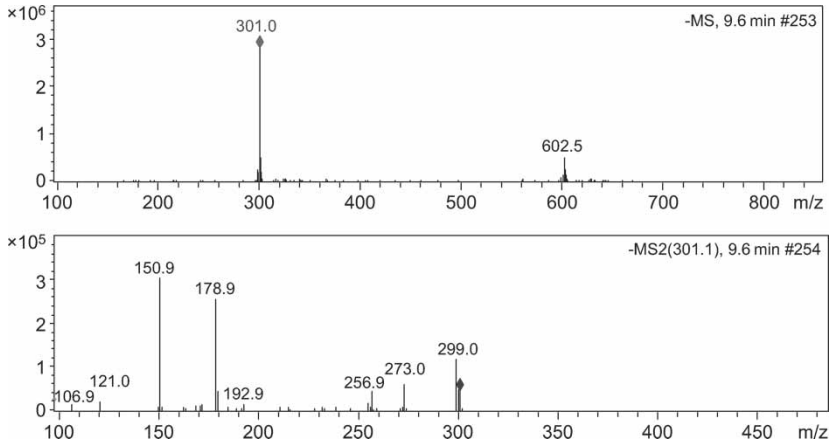

Figure 4. The profile of ion mass spectra and scanning of the isolated representative quercetin. Typical MS chromatogram of quercetin from full scanning range from 100 to 900 amu under APCI in negative-ion mode; quercetin was isolated from an Eclipse XDB-C8 column using DAD detection with on-line MS identification (top: molecular ion MS; bottom: MS/MS).

observed at the higher concentration of acetonitrile is believed to be caused by changes in the viscosity and the zeta potential. Increasing the amount of acetonitrile in the background electrolyte (BE) also made the electrolyte more polar, and this changed the partitioning equilibrium. For this experiment, 6.0–20.0%(v/v) of acetonitrile solution was investigated for the optimal separation of the three natural products. Increasing the concentration of organic solvent resulted in decreasing electroosmotic flow and, thus, a longer time for separation, but no significant change in selectivity was observed. Finally, 10.0%(v/v) of acetonitrile in the eluent was chosen. The effects of SDS concentrations on the resolution were investigated for the separation of the three natural products. For this experiment, eight CE running solutions, including 16–48 mmol/L SDS, were, respectively, prepared with the optimization pH and buffers as described above. The migration time increased with increasing SDS concentrations. When the concentration of SDS was 32 mmol/L, the optimal resolution was obtained with the shortest analysis time and good peak shapes.

The separation selectivity was affected slightly by the capillary temperature and applied voltage. Commonly, the viscosity decreased with the increasing temperature, this change would influence the distribution constants of solutes, and this would result in decreasing retention (peaks migration time were shorten). To decrease the Joul heating, the temperature for the separation of solutes was set at 25°C. The applied voltage had a larger effect than capillary temperature on the migration time. Taking both short migration time and good resolution into consideration, the applied voltage was selected at 19 kV. As a result of these

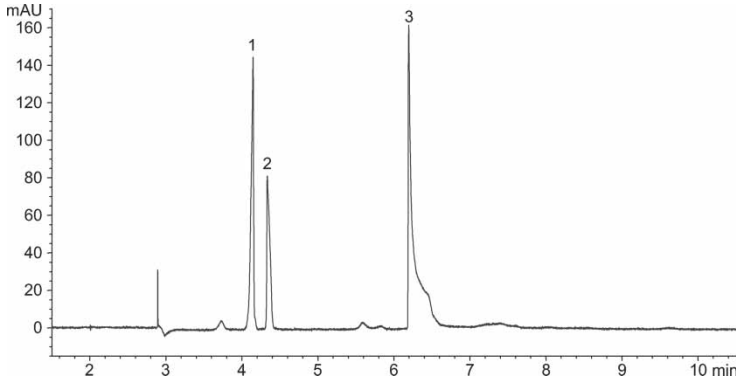

Figure 5. Electropherogram of standard solution of three polar natural products. Running buffer: 32 mmol/L broate buffer containing 32 mmol/L SDS and 10.0% acetonitrile(pH 10.0) at 25°C with 19 kV voltage, detection at 220 nm. Peaks: 1. MTCCA; 2. Allantoin.; 3. Quercetin.

experiments, the optimum separation conditions were set at 32 mmol/L SDS, 10.0%(v/v) acetonitrile containing 32 mmol/L borate buffer(pH10.0), separation voltage 19 kV, and temperature 25°C. Figure 5 shows the electropherogram of the three natural products under the proposed conditions.

Method Validation

After development, the validation was performed in parallel for HPLC and CE with the same standards and samples to avoid differences due to the manipulation. System precision was demonstrated by comparing HPLC with CE.

Preparing and measuring a standard solution examined the method repeatability by carrying out six successive injections of standard solution within one day. The relative standard deviations (RSDs) of retention times for HPLC, migration times for CE, and peak areas are listed in Table 1. As can be seen from Table 1, the RSDs of the rentenion times were 0.072%–0.242% for HPLC and 1.08–1.45% for CE. RSDs of peak areas were 1.27%–5.32% for HPLC and

Table 1. Relative standard deviation (RSD) of retention/migration time and peak area

	Allantoin	MTCCA	Quercetin
HPLC			
Retention time RSD(%)	0.242	0.072	0.148
Peak area RSD(%)	5.32	1.27	1.99
CE			
Migration time RSD(%)	1.08	1.45	1.26
Peak area RSD(%)	2.28	0.82	1.75

Table 2. Quantitative equation and related parameters

	Allantoin	MTCCA	Quercetin
HPLC			
Regression equation	$y = 0.7495x + 4.4433$	$y = 25.378x + 75.6522$	$y = 1.7271x - 59.2435$
Concentration range(ug/mL)	18.5–600	9.5–300	48.0–775
Correlation coefficient	0.99974	0.99926	0.99894
Detection limits[a] (ug/mL)	1.69	0.16	6.50
CE			
Regression equation	$y = 0.4035x - 0.4463$	$y = 0.4166x - 2.4632$	$y = 0.3541x - 1.5281$
Concentration range (ug/mL)	2.0–225	5.5–350	20.0–550
Correlation coefficient	0.99801	0.99795	0.99766
Detection limits[a] (ug/mL)	2.5	5.0	8.0

[a]$S/N = 3$.

Table 3. Recoveries and precisions obtained with the HPLC method (n = 6)

Compound	Added amount (ug/mL)	Found amount (ug/mL)	Recovery (%)	RSD (%)
Allantoin	10	9.86	98.6	1.7
MTCCA	10	9.90	99.0	1.6
Quercetin	10	9.72	97.2	2.2

0.82–2.28% for CE. Obviously, the repeatabilities of retention time using HPLC were better than those for CE. The linear calibration curve was constructed using the regression of the peak area versus concentration of the calibration standards. All of the three solutes were found to give excellent linear responses over their ranges. The correlation coefficients obtained for each individual cure was in the range of 0.9989 $\sim$ 0.9997 for HPLC, and 0.9977 $\sim$ 0.9980 for CE (see Table 2).

Detection limits are an important consideration when the components of biological matrixes are ananlyzed, particularly; they are present at low or trace concentrations. On the basis of the signal-to-noise ratio of 3, the calculated detection limits for each solute are from 0.16 $\sim$ 6.50 ug/mL for HPLC and from 2.5 $\sim$ 8.0 ug/mL for CE. The data are shown in Table 2. As can be seen from Table 2, the detection limits using the HPLC method are better than that of the CE method.

Recovery (accuracy) and precision were investigated for both methods by using a standard addition of known amounts of three standards (allantoin, quercetion, and MTCCA) into the ethanol extracts of *Nitraria* under the same conditions stated above. The mean recoveries were between 97.2% and 99.0% with acceptable RSD% values from 1.6% to 2.2% for HPLC, and between 95.2% and 97.6% with acceptable RSD% values from 1.8% to 2.9% for CE (see Table 3, Table 4).

Separation of Extracted Sample

Quantitative determination of allantoin, quercetin, and MTCCA in *Nirtraria tangutorum* seed extracts was performed by means of the two analytical

Table 4. Recoveries and precisions obtained with the CE method (n = 6)

Compound	Added amount (ug/mL)	Found amount (ug/mL)	Recovery (%)	RSD (%)
Allantoin	10	9.60	96.0	2.1
MTCCA	10	9.76	97.6	1.8
Quercetin	10	9.52	95.2	2.9

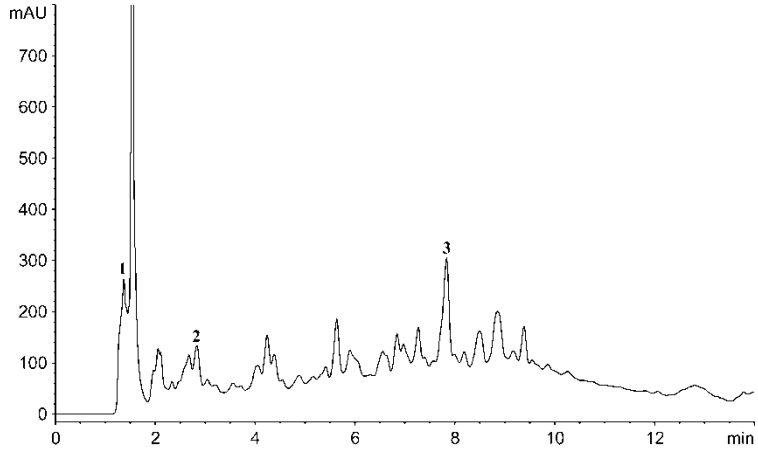

Figure 6. Chromatogram of extract of *Nitraria tangutorum* seed using ethanol as solvent. Column temperature is set at 30°C; DAD at 220 nm; Column reversed-phase Eclipse XDB-C8 (150 × 4.6 mm, 5 μm); flow rate = 1.0 mL/min. Peaks: 1. Allantoin; 2. MTCCA; 3. Quercetin.

methods. Figures 6 and 7 show the chromatogram and electropherogram of the extract of the *Nitraria tangutorum* seed using ethanol as solvent. The determined contents of allantoin, MTCCA, and quercetin in the seed are shown in Table 5.

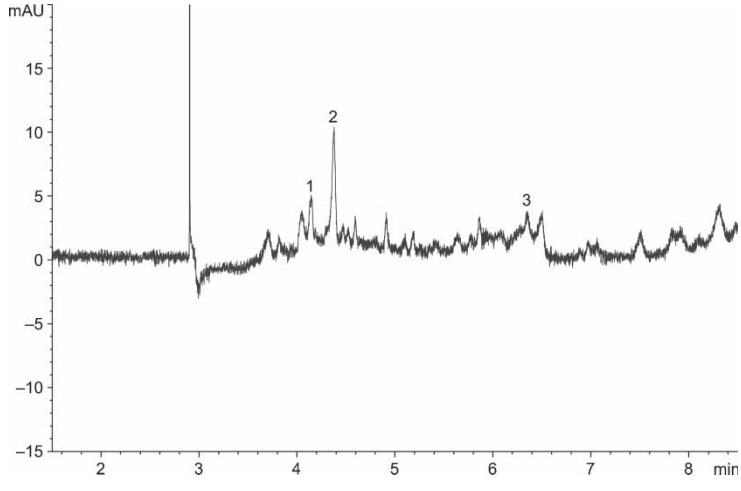

Figure 7. Electropherogram of extracts of *Rhodiola* plants using ethanol as solvent. Running buffer: 32 mmol/L broate buffer containing 32 mmol/L SDS and 10.0% acetonitrile(pH 10.0) at 25°C with 19 kV voltage, detection at 220 nm. Peaks: 1. MTCCA; 2. Allantoin; 3. Quercetin.

Table 5. Contents of allantoin, MTCCA and quercetin in *Nitraria tangutroum* seed by HPLC and CE methods

	Allantoin	MTCCA	Quercetin
HPLC			
Content(mg/g)	1.542	0.674	0.921
n	6	6	6
RSD(%)	3.03	2.17	1.63
CE			
Content(mg/g)	1.118	0.595	0.879
n	6	6	6
RSD(%)	1.95	1.30	1.42

CONCLUSION

Two methods for the quantitative determination of allantoin, quercetin, and MTCCA in *Nitraria tangutorum* seeds have been developed. Compared with HPLC, analysis time was shorter with the CE method and solvent consumption was considerably lower, which is a great economical benefit. Nevertheless, buffer preparation was a bit more difficult due to the presence of the surfactant. Both the repeatabilities of retention time and the detection limits using HPLC were better than those for CE.

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