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Simultaneous determination of α-, β- and γ-asarone in *Acorus tatarinowii* by microemulsion electrokinetic chromatography with [BMIM]PF_6 as oil phase

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ABSTRACT

In the present study, a rapid and repeatable microemulsion electrokinetic chromatography (MEEKC) method was developed for the simultaneous determination of three isomers (α-, β- and γ-asarone) in *Acorus tatarinowii* by using ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) as oil phase. Experimental parameters including the microemulsion compositions (concentrations of surfactant, co-surfactant and oil phase), pH, concentration of borate buffer, capillary temperature and voltage were intensively investigated. Finally, the main compounds in the methanol extract of *A. tatarinowii* were well separated within 11 min using a running buffer composed of 40 mmol/L sodium dodecyl sulfonate (SDS), 2.0 mol/L n-propanol, 8 mmol/L [BMIM]PF_6 in 10 mmol/L borate buffer of pH 9.5. The developed method was applied to determine the contents of α-, β- and γ-asarone in *A. tatarinowii* from five different producing areas in China (Anhui, Hebei, Sichuan, Zhejiang and Chongqing). The results indicated that the contents of three asarones are quite different in the investigated *A. tatarinowii* samples. On the other hand, the MEEKC with ionic liquid as oil phase should be a promising method for the analysis of volatile components especially isomers in medicinal herbs.

1. Introduction

The rhizome of *Acorus tatarinowii* Schott, which belongs to the *Araceae* family, has been widely used in China and other places of Asia [1]. Pharmacological studies indicate that *A. tatarinowii* possesses sedative, digestive, analgesic, diuretic, and antifungal activities [2,3]. Furthermore, *A. tatarinowii* contains about 0.11%–0.42% of volatile oil which is mainly composed of asarone components [4], including three isomeric forms, namely α-, β- and γ-asarone (Fig. 1) [5]. The α- and β-asarone have various biological activities. For example, they had been reported to be of anthelmintic and pesticidal activities and neuroprotective action against the excitotoxicity induced by N-methyl-D-aspartate (NMDA) or glutamate (Glu) in cultured rat cortical cells [6–8]. And the β-asarone was found to exhibit toxic and sterilizing effects [9]. However, both α- and β-asarone are known carcinogenic compounds [10,11]. γ-Asarone, was a rare phenylpropanoid first isolated from *Caesulia axillaries* [12] and later was proved as an antimicrobial [13], antiallergic [14], fungitoxic [15] and nontoxic [16] constituent in various essential oils. Therefore, analysis of those three asarone isomers in *A. tatarinowii* is beneficial for its quality control.

To date, asarones were analyzed mostly by high performance liquid chromatography (HPLC) [17–20], gas chromatography–mass spectrometry (GC–MS) [18–25] and high performance thin layer chromatography (HPTLC) [26,27]. However, HPTLC is often insufficient to resolve closely related substances and isomers [28]. Although GC–MS is a powerful tool for the analysis of volatile compounds, there are some heat sensitive compounds such as germacrone, furanodiene and furanodinone etc, which may be degraded and lead to wrong results during the GC analysis [29]. HPLC needs large amount of organic solvents and usually needs special stationary phase or modifiers for the separation of isomers [30–33]. In addition, only the contents of α- and/or β-asarone w orts [17–19,21,25,26].

Microemulsion electrokinetic chromatography (MEEKC) has been widely used for resolving isomers [34,35] and hydrophobic substances [36,37] due to its unique properties of microemulsion. Generally, *n*-octane and *n*-heptane were used as oil phase in most cases. Actually, ionic liquids are molten salts and have been testified that they are more environmentally attractive than organic solvents. They are receiving more and more attentions because of their special physical and chemical properties [38,39], such as strong dissolution ability for most organic and inorganic compounds, very low volatility and high thermal stability. To date, there are some reports have used ionic liquids as the constituents (surfactant, co-surfactant or oil phase) of microemulsion [40–45].

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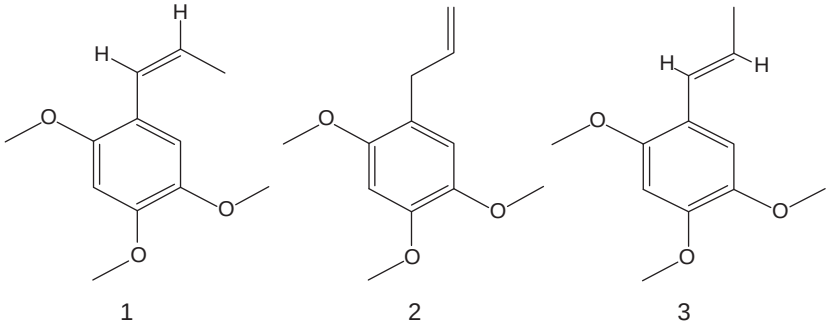

Fig. 1. Chemical structures of three asarone isomers. 1, β-asarone; 2, γ-asarone and 3, α-asarone.

Therefore, in the present study, a MEEKC method with ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) as oil phase was developed for the simultaneous determination of α-, β- and γ-asarone in *A. tatarinowii*. The contents of those three investigated compounds in *A. tatarinowii* from different areas of China were also compared.

2. Experimental

2.1. Instruments and Chemicals

All CE separations were performed on an Agilent 7100 3D CE system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD and an Agilent ChemStation software. The uncoated fused-silica capillary (Hebei Yongnian Ruifeng Chromatographic Implements) was of 50 μm i.d and had a total length of 55 cm (47 cm effective length). All the analyses in the present study were performed on the same capillary. The KQ-100B ultrasonic cleaner (Kunshan ultrasonic instruments Co., Ltd.) was applied for the preparation of microemulsion and samples. The operation conditions for the ultrasonic cleaner were: frequency 40 KHz, power 100 W, bath dimensions $300 \times 150 \times 100\ mm^3$. A Delta 320 pH meter (Mettler-Toledo Instruments, Shanghai) was used for the measurement of pH of running buffer.

[BMIM]PF_6 was purchased from Lanzhou Institute of Chemical Physics (Lanzhou, China). Sodium dodecyl sulfonate (SDS), octane and methanol were purchased from Chongqing Chuandong Chemical (Group) Co., Ltd. (Chongqing, China). Acetone, n-propanol, n-butanol, sodium hydroxide and boric acid were obtained from Chengdu Kelong Chemical Works (Chengdu, China). All reagents above were of analytical grade, and reverse osmosis (RO) water used throughout was prepared by an AKWL-IV-16 water purification system (Chengdu Tang's Kangning Science and Technology Development Co., Ltd., Chengdu, China).

The rhizomes of *A. tatarinowii* were purchased from Anhui, Hebei, Sichuan, Zhejiang and Chongqing of China. The botanical origins of all the materials were identified morphologically and microscopically by Dr. Guo-Yue Zhong (Chongqing Academy of Chinese Materia Medica, Chongqing, China). The voucher specimens of *A. tatarinowii* was deposited at the Department of Pharmaceutics, College of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China. β- and γ-asarone were prepared by using a preparative GC instrument described before [46]. α-asarone was purchased from Chengdu Food and Drug Control Center (Chengdu, China).

2.2. Procedures

The running buffer was prepared by adding 40 mmol/L SDS, 2 mol/L n-propanol and 8 mmol/L [BMIM]PF_6 to 10 mmol/L borate buffer of pH 9.5 (adjusted by 1 mol/L NaOH solution). The mixture was then ultrasonicated for 20 min. Prior to be used, the freshly prepared microemulsion running buffer was stood for at least one hour at ambient temperature.

The standard stock solutions of α-asarone (10.2 mg/mL), β-asarone (11.3 mg/mL) and γ-asarone (12.5 mg/mL) were prepared by accurately weighted of each standard and dissolved in running buffer (2 mL). A desired amount (1.0 g) of *A. tatarinowii* powder was extracted with 10 mL absolute methanol in a 50 mL flask by ultrasonication for 30 min. After extraction, the extract was cooled down to the room temperature (about 25 °C), and made up the lost weight with methanol, then centrifuged for 5 min at $1.36 \times 10^4\ g$, and the supernatant was filtered through a 0.45 μm nylon membrane (Auto science instrument Co., Ltd., Tianjing, China), then concentrated until to dryness. Finally, the residue was dissolved in 4 mL running buffer.

The capillary was flushed daily with 0.1 mol/L sodium hydroxide for 15 min, followed by RO water for 5 min and finally with running buffer for 15 min. Between consecutive analyses, the capillary was rinsed with 0.1 mol/L sodium hydroxide (2 min), then with RO water (2 min), and finally with the running buffer (5 min). The CE operating conditions were: injection pressure 50 mbar; injection time 5 s and detection wavelength 275 nm.

3. Results and discussion

3.1. Optimization of separation conditions

3.1.1. Effect of SDS concentration

Sample of *Acorus tatarinowii* (from Chongqing) prepared by the above mentioned method was used for the optimization of separation conditions. As the main constituent in the microemulsion, the concentration of SDS could affect the selectivity and the total migration time of the analysis [47], as the surfactant could affect the oil droplet charge and size, the level and direction of the EOF, and the level of any ion-pairing with solutes [48]. By using the resolution (*Rs*) of β- vs. γ-asarone (*Rs1*), α-asarone vs. unknown adjacent peak U (*Rs2*) and retention time of α-asarone (*RTa*) as markers, different concentrations (30, 40, 60, 80 and 100 mmol/L) of SDS were investigated (the other conditions were: 2.75 mol/L n-propanol, 20 mmol/L [BMIM]PF_6, 50 mmol/L borate buffer, pH 9.5, 25 kV of applied voltage and 25 °C of capillary temperature). As shown in the Fig. 2A, the higher concentration of SDS, the longer migration time and higher resolutions. With the increase of the concentration of SDS, the EOF decreased by the increase of the buffer ionic strength, and the baseline was deteriorated correspondingly when the concentration of SDS higher than 100 mmol/L. However, when the concentration of SDS was lower than 40 mmol/L, the baseline separation of investigated compounds could not be achieved ($Rs1 < 1.19$ and

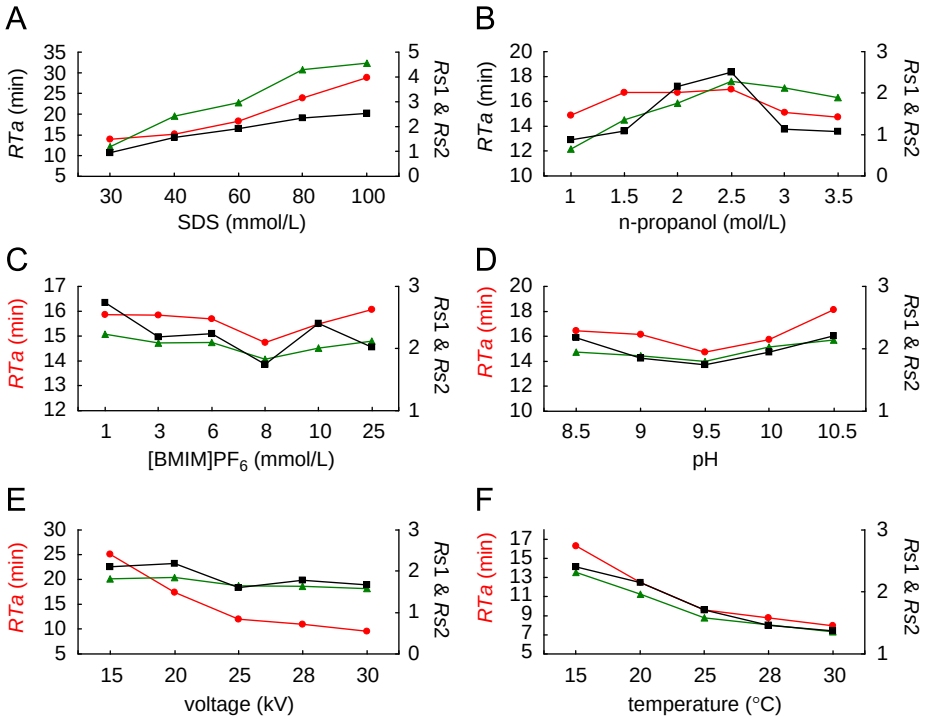

Fig. 2. Effects of SDS concentration (A), n-propanol concentration (B), [BMIM]PF_6 concentration (C), buffer pH (D), applied voltage (E) and capillary temperature (F) on the *RS1* (▲), *RS2* (■) and *RTa* (●).

Rs2 < 0.95). Therefore, in order to achieve better separation efficiency within shorter analysis time, 40 mmol/L of SDS was used in the subsequent experiments.

3.1.2. Effect of co-surfactant concentration

The co-surfactant was found to be one of the most noticeable factors impacting on the separation selectivity in MEEKC [47,49]. In MEEKC, the microemulsion system became more stable when some short chain alcohols such as n-butanol, n-propanol etc. were added as co-surfactant [50]. Furthermore, the concentration of co-surfactant could change the viscosity of the solution, and therefore affected the EOF. In present study, the n-butanol and n-propanol were compared when the other conditions were: 40 mmol/L SDS, 20 mmol/L [BMIM]PF_6, 50 mmol/L borate buffer, pH 9.5, 25 kV of applied voltage and 25 °C of capillary temperature. The results showed that the system contained n-propanol was more stable than that of n-butanol. Therefore, n-propanol was used. Furthermore, the effects on the separation of investigated compounds by different concentrations of n-propanol include 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mol/L were investigated. The results in Fig. 2B demonstrated that with the concentration of n-propanol increased from 1.0 to 2.5 mol/L, both the migration time and the resolutions of compounds increased and reached to the highest at 2.5 mol/L. However, further increased the concentration of n-propanol from 2.5 to 3.5 mol/L, the migration time of analytes was shortened despite the lowering of EOF and the peak sequence of α-asarone with the adjacent substance was changed. Maybe once n-propanol exceeded 2.5 mol/L, the excess n-propanol in the aqueous phase might be adsorbed and shielded the negative-charged micelle, consequently increased the viscosity of micelle and shortened the migration time [51]. Finally, 2.0 mol/L of n-propanol was chosen for its shorter analysis time.

3.1.3. Effect of ionic liquid concentration

Different oil phases including octane and [BMIM]PF_6 were tested when the other conditions were: 40 mmol/L SDS, 2.0 mol/L n-propanol, 50 mmol/L borate buffer, pH 9.5, 25 kV of applied voltage and 25 °C of capillary temperature. The results indicated that [BMIM]PF_6 made the microemulsion solution more stable and showed better repeatability when using n-propanol as co-surfactant. Furthermore, the amount of [BMIM]PF_6 from 1 to 25 mmol/L were investigated. The results in Fig. 2C showed that the migration time shortened as the concentration of [BMIM]PF_6 increased from 1 to 8 mmol/L but prolonged if continuously increased the concentration of [BMIM]PF_6 from 8 to 25 mmol/L. Therefore, 8 mmol/L of [BMIM]PF_6 was chosen to achieve acceptable resolutions (*Rs1* > 1.83, *Rs2* > 1.75) within the shortest analysis time (14.7 min).

3.1.4. Effect of pH, buffer concentration, voltage and temperature

The pH of the buffer would affect on the value of EOF and the ionization of solutes. In present study, pH 8.5–10.5 was investigated (the other conditions were: 40 mmol/L SDS, 2.0 mol/L n-propanol, 8 mmol/L [BMIM]PF_6, 50 mmol/L borate buffer, 25 kV of applied voltage and 25 °C of capillary temperature). The experimental results in Fig. 2D revealed that all the studied pH provided acceptable resolutions (*Rs1* > 1.8, *Rs2* > 1.75) of peaks. Therefore, pH 9.5 was selected as it could provide the shortest analysis time.

On the other hand, using low-ionic-strength (5–10 mmol/L) borate or phosphate buffers generated relatively low currents and high EOF value [44]. In present study, different concentrations (5–50 mmol/L) of borate buffer were investigated (the other conditions were: 40 mmol/L SDS, 2.0 mol/L n-propanol, 8 mmol/L [BMIM]PF_6, pH 9.5, 25 kV of applied voltage and 25 °C of capillary temperature). As the results indicated that the analytes could be separated with acceptable resolutions (*Rs1* > 1.67,

$Rs2 > 1.5$) within the tested concentrations of buffer. Finally, 10 mmol/L of borate buffer was used for its shortest analysis time and more stable system.

The effects of applied voltage and temperature were shown in Fig. 2E and F. Finally, 30 kV and 25 °C were selected as the optimum applied voltage and temperature of the capillary.

Based on the optimization, the best conditions for the analysis were: running buffer compose of 40 mmol/L SDS, 2.0 mol/L n-propanol, 8 mmol/L [BMIM]PF_6 in 10 mmol/L borate buffer of pH 9.5. Applied voltage was 30 kV and capillary temperature was 25 °C.

3.2. Method validation

3.2.1. Linearity, LOD, and LOQ

Stock solution containing three standard compounds (α-, β- and γ-asarone) was prepared and diluted to appropriate concentrations for the construction of calibration curves. At least six concentrations of the solution were analyzed in triplicates, and then the calibration curves were constructed by plotting the peak area of individual standard vs. the concentration of each analyte. The limits of detection (LOD) and quantification (LOQ) under the optimum conditions were determined based on the signal-to-noise (S/N) ratio of 3 and 10, respectively. Table 1 shows the linear regression data, LOD and LOQ. As compared to the published methods such as HPLC [17–19], GC–MS [21] and HPTLC [25,26] for the determination of α- and β-asarone, the linearity range of present study is larger than that of HPLC and HPTLC, and similar to that of GC–MS. Furthermore, the LODs and LOQs for α- and β-asarone of the present study is similar to that of HPLC, but ten times higher than that of GC–MS, which may be due to the higher sensitivity of MS detector.

3.2.2. Precision, repeatability and recovery

For intra-day variability test, the mixed standards' solutions were analyzed in three different concentrations (high, median and low) for six replicates within one day. And for inter-day variability test, the solutions were examined in three different concentrations for duplicates in consecutive 3 days. Table 2 summarizes the results of intra- and inter-day variations test for each compound.

Table 1
Linear regression data, LOD and LOQ of α-, β- and γ-asarone.

Analytes	Calibration curves	Linear range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)
α-asarone	$y=142.35x+3.35$	26.60–3400.00	0.9996	8.15	26.60
β-asarone	$y=157.78x+5.85$	14.70–3770.00	0.9988	6.65	14.70
γ-asarone	$y=115.09x+4.04$	32.60–4170.00	0.9990	2.76	32.60

Table 2
Short term and long term repeatability of the investigated compounds ($n=6$).

Analytes	Concentration (μg/mL)	Intra-day (RSD %)	Inter-day (RSD %)
α-asarone	3400.00	1.93	4.95
	425.00	2.70	2.63
	26.60	3.89	4.44
β-asarone	3770.00	1.84	4.89
	471.25	2.14	3.21
	29.50	3.97	2.56
γ-asarone	4170.00	1.30	4.85
	521.25	2.68	3.64
	32.60	4.00	4.62

Table 3
Precision of the peak areas of α-, β- and γ-asarone in real sample.

Analytes	Weight of crude materials (g)	Mean peak areas ($n=3$)	RSD %
α-asarone	0.8	5.5	3.15
	1.0	7.3	4.20
	1.2	8.0	3.31
β-asarone	0.8	246.9	3.81
	1.0	286.9	3.52
	1.2	347.7	2.37
γ-asarone	0.8	124.3	1.53
	1.0	141.4	0.62
	1.2	172.1	4.45

Table 4
Recoveries for the assay of α-, β- and γ-asarone in *Acorus tatarinowii*.

Analytes	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	RSD %
α-asarone	–*	1.49	1.45	97.3	5.70
β-asarone	1.95	0.51	2.47	102.0	6.09
γ-asarone	1.40	0.65	2.04	98.5	6.97

* under the limit of quantitation.

The repeatability of real sample analysis was examined in three levels (0.8, 1.0 and 1.2 g). The samples were prepared and analyzed for triplicates as the method mentioned above. Table 3 shows the repeatability of the peak areas of each analyte.

The recovery was performed by adding a known amount of individual standards into a certain amount (0.5 g) of *A. tatarinowii* (obtained from Chongqing). The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test. Table 4 shows the summarized results.

3.3. Determination of three asarone isomers in Acorus tatarinowii

Under the optimum conditions, the methanol extracts of *A. tatarinowii* from different locations of China were analyzed. The electrochromatograms are shown in Fig. 3. The identifications of compounds were done by comparing the retention time to those of standards, as well as adding the individual standard to the sample. By using the calibration curve of each standard, the contents of three main asarone isomers in *A. tatarinowii* sample were determined. The quantification results were shown in Table 5. Except for α-asarone in the samples from Sichuan and Chongqing lower than LOQ, all the other analytes were successfully determined. The results indicated that the contents of three asarone isomers existed difference in *Acorus* samples from different areas of China. For example, the content of γ-asarone in samples from Sichuan and Chongqing obvious higher than the others, while the sample from Zhejiang contains the highest amount of α-asarone. Therefore, further comparing pharmacological studies should be done for those asarone isomers to provide evidence for evaluating the quality of *A. tatarinowii* from different producing areas.

4. Conclusion

In present study, an MEEKC method was developed for the simultaneous determination of α-, β- and γ-asarone in *Acorus tatarinowii* by using ionic liquid [BMIM]PF_6 as oil phase. The results show that MEEKC with ionic liquid as oil phase is an

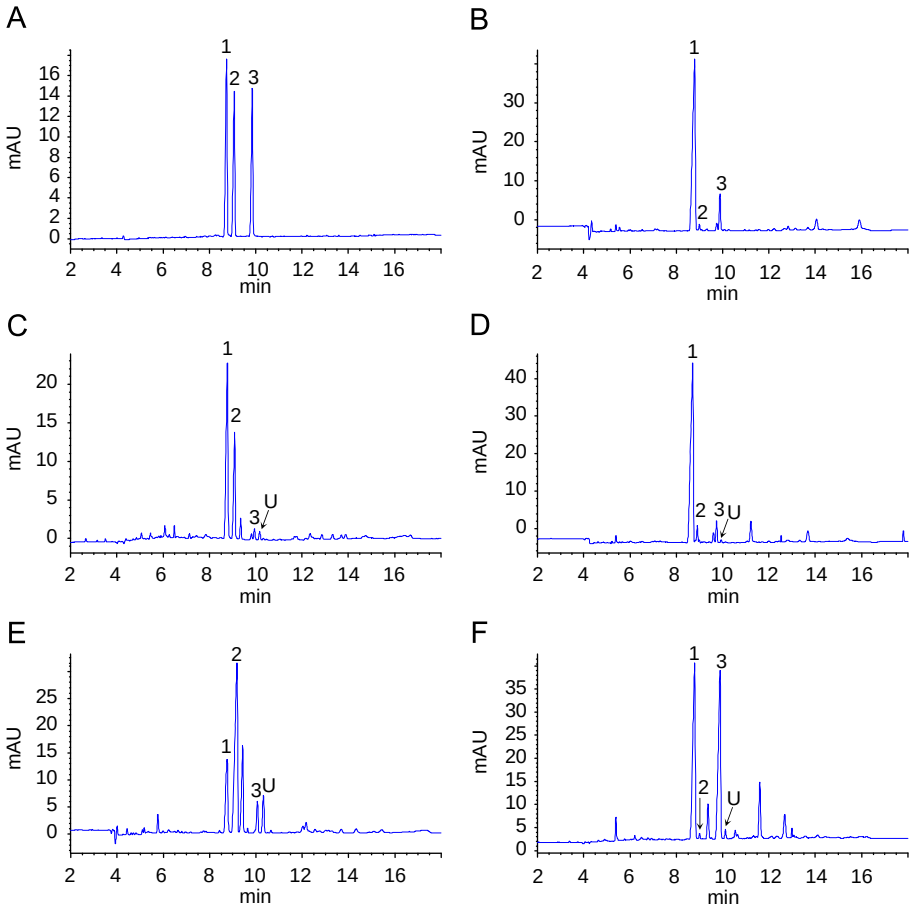

Fig. 3. Electrochromatograms for the mixture of standards (A), and *Acorus tatarinowii* samples from Anhui (B), Chongqing (C), Hebei (D), Sichuan (E) and Zhejiang (F). 1, β-asarone; 2, γ-asarone; 3, α-asarone; U, unknown.

Table 5
Contents (mg/g) of α-, β- and γ-asarone in *Acorus tatarinowii* from different locations of China.

Analytes	Anhui	Hebei	Sichuan	Zhejiang	Chongqing
α-asarone	0.11	0.22	–*	7.31	–
β-asarone	9.67	12.80	2.46	7.02	3.38
γ-asarone	0.05	0.47	7.25	0.05	2.23

* under the limit of quantitation.

alternative approach for the analysis of volatile isomers with highly hydrophobic property.

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