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Article

Analysis of Volatile Components in *Curcuma* Rhizome by Microemulsion Electrokinetic Chromatography

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Volatile chemicals are a group of very important compounds in natural products. *Curcuma* rhizome, which contains many bioactive volatile compounds, is a traditional Chinese medicine that has long been used for the treatment of several diseases. In the present study, a microemulsion electrokinetic chromatography (MEEKC) method was developed for the analysis of four volatile components in *Curcuma* rhizome, including germacrone, furanodiene, curcumenol and curdione. Experimental parameters, including the pH, type and concentrations of background electrolyte, and microemulsion compositions (type and concentrations of surfactant, co-surfactant and oil phase) were intensively investigated. Finally, the primary compounds in the methanol extract of *Curcuma* rhizome were separated within 30 min using a running buffer composed of 2.31% w/v (80 mmol/L) sodium dodecyl sulfate (SDS), 0.91% w/v (80 mmol/L) 1-octane, 6.95% w/v (937.5 mmol/L) 1-butanol and 1.88% w/v (312.5 mmol/L) propanol in a 5-mM borate buffer (pH 8.1). The contents of the four investigated compounds were determined in the rhizome from *C. phaeocaulis*. The results showed that the developed MEEKC method provided an alternative tool for the analysis of volatile components, especially those of heat-sensitive compounds from natural products.

Introduction

Essential oils are among the most valuable natural products, with multiple pharmacological activities. Among the Chinese medicines (CMs) recorded in the *Chinese Pharmacopoeia* (2010 edition) (1), approximately 20% of herbs contain essential oils, which are usually considered to be bioactive fractions. Therefore, analyses of volatile components in those CMs are very important for their quality control. To date, colorimetry, thin-layer chromatography (TLC), gas chromatography (GC) with flame ionization detection or mass detection, and high-performance liquid chromatography (HPLC) have been used for the quality assessment of essential oils in CMs (2). However, the poor selectivity of colorimetry and the relative low separation efficiency of TLC have hindered their applications. Furthermore, although GC–mass spectrometry (MS) is a powerful tool for the analysis of volatile compounds, some heat sensitive compounds, such as germacrone, furanodiene and furanodinone, may be degraded and lead to incorrect results during the GC analysis (3). Therefore, an analytical method without elevated temperature is necessary for the analysis of these compounds. In addition, HPLC is difficult for the analysis of extremely low polarity compounds, which may pollute a column packed with reverse-phase materials. Microemulsion electrokinetic capillary chromatography (MEEKC) is an electrodriven separation technique that offers the possibility of highly efficient separations of both charged and neutral solutes covering a wide range of compounds. MEEKC has been intensively used for the analysis of highly hydrophobic substances such as β-diketones, polyaromatic hydrocarbons, steroids, polymer additives, fatty acids and lipid-soluble vitamins (4, 5).

Ezhu, one of commonly used traditional CMs, is derived from the rhizomes of three species of *Curcuma*, including *C. phaeocaulis*, *C. kwangsiensis* and *C. wenyujin*, according to the record in *Chinese Pharmacopoeia* (1). At present, the essential oil of Ezhu is considered to possess anti-tumour (6, 7) and antiviral activities (8, 9). Sesquiterpenoids, including β-elemene, curcumol, germacrone, curdione and neocurdione, are thought to be the biological active ingredients in the essential oil (3). Therefore, determination of the sesquiterpenoids is very important for pharmacological study and quality control of Ezhu.

In the present study, an MEEKC method was developed for the analysis of four volatile compounds in *Curcuma* rhizome, including germacrone, furanodiene, curcumenol and curdione. The parameters for MEEKC analysis were systematically investigated, including the pH, type and concentrations of background electrolyte, and microemulsion compositions (type and concentrations of surfactant, co-surfactant and oil phase).

Experimental

Instruments and chemicals

A self-assembled capillary electrophoresis (CE) system equipped with a 0–30 kV adjustable DC power supply, an absorption detector of variable wavelengths in 190–900 nm and a photomultiplier tube detector (Beijing Zolix Instrument Co., China) were employed in the experiment. Instrumental controlling and data acquisition and analysis were performed on an HW-2003 chromatographic workstation (Nanjing Qianpu Software Co., China). The uncoated fused-silica capillary (Hebei Yongnian Ruifeng Chromatographic Implements) was of 75 μm i.d. and had a total length of 58 cm (49.7 cm effective length). All analyses in the present study were performed on the same capillary. The AS 3120A ultrasonic cleaner (Tianjin Automatic Science Instrument Co., China) was applied for preparation of microemulsion and sample extraction. A Delta 320 pH meter (Mettler-Toledo Instruments, Shanghai) was used for the measurement of pH of running media.

Tween-80, hexane, octane and ethyl acetate, acetone, petroleum ether, isopropanol, ethanol and methanol were purchased from Chongqing Chuandong Chemical Co. (Chongqing, China).

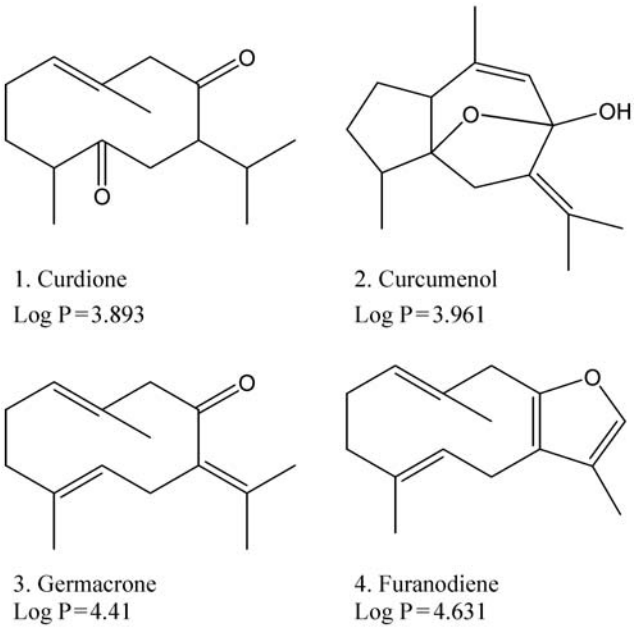

Figure 1. Chemical structures of the four investigated compounds.

Dimethyl sulfoxide (DMSO), butanol, *n*-heptane, propanol and sodium dodecyl sulfate (SDS) were obtained from Chengdu Kelong Chemical Works (Chengdu, China). All reagents were of analytical grade, and distilled water was used throughout the study.

Germacrone, furanodiene, curcumenol and curdione (Figure 1) were separated in our lab. In brief, 240 g commercial oil of *C. wenyujin* was mixed with silica gel (77–147 μm), and applied onto a pre-filled silica gel column (10–77 μm). Petroleum ether and solvents of different polarity(petroleum ether–ethyl acetate = 95:5, 70:30, 50:50, chloroform, ethyl acetate and methanol) were used for step gradient elution. The collected fraction size was approximately 500 mL, and the solvent was recycled at 40°C using a spin evaporator (Büchi, Flawil, Switzerland). TLC was used for monitoring the status of elution. Fractions 1–23 were kept in a refrigerator (−20°C) for crystallization. The crystal was re-crystallized twice with methanol as solvent, the compound furanodiene was obtained. Fractions 33–50 were crystallized in a refrigerator (0°C), then re-crystallized twice with methanol as solvent, and germacrone was obtained. Fractions 61–98 were applied onto a silica gel column for further separation and eluted by the mixed solvent of petroleum ether and ethyl acetate (90:10), and the crystal of curdione was obtained under refrigeration (0°C). Furthermore, 5 kg of dry raw materials (*C. phaeocaulis*) were sliced and milled; the powder was extracted by ultrasonication with ethanol for 30 min and filtered, and the filtrate was collected. The procedure was repeated eight times. The ethanol extract was condensed, mixed with silica gel and applied to a silica gel column for separation, and eluted with a mixed solvent of petroleum ether–ethyl acetate (90:10). Fractions 50–72 were kept in a refrigerator (−20°C) for crystallization, re-crystallized twice with methanol, and the compound curcumenol was obtained. The purity of all compounds was >99%, which was tested by GC–MS and/or HPLC.Preparative GC, which provides an efficient approach to obtain pure volatile compounds from natural products, has been used for the separation of components from the essential oil of *Curcuma* rhizome (10). The rhizome of *C. phaeocaulis* sample was purchased from a local drug store.

Microemulsion electrolyte and sample preparation

The microemulsion electrolyte was prepared by using the following procedure: a desired amount of surfactant (SDS or Tween-80) was dissolved in borate buffer solution. The oil phase (hexane, heptane, octane or ethyl acetate) and a small portion of co-surfactant (butanol, propanol, isopropanol, ethanol or methanol) were added sequentially and mixed by ultrasonication for approximately 5 min. Finally, another portion of co-surfactant was slowly and quantitatively added into the buffer solution with ultrasonic mixing until the solution became clear. The freshly prepared microemulsion electrolyte was allowed to stand for at least 30 min at ambient temperature before use. All microemulsion electrolytes were prepared by the same procedure, and the measurement of the surfactant, co-surfactant and oil phase was weighted by analytical balance (AY120, Shimadzu, Koyoto, Japan). Furthermore, acetone was used as electroosmotic flow (EOF) marker.

For the preparation of sample solution, 2.0 g of *Curcuma* dry powder was accurately weighted into a 10-mL flask and 10 mL methanol was added. The flask was put into an ultrasonic tank and extracted for 20 min. The extract was filtered and the filtrate was completely dried in a water bath. The dried extract then was dissolved by the running buffer (10 mL).

CE procedures

CE was performed with a separation voltage of 15 kV at ambient temperature. The sample was injected hydrostatically (18 cm height) for 5 s. The ultraviolet (UV) detection was set at 214 nm for all the analytes. Before the first use, the new capillary was conditioned by flushing with 0.1 mol/L NaOH for 30 min, followed by water for 10 min, and finally with microemulsion electrolyte for 10 min. Before the run each day, the capillary was flushed with 0.1 mol/L NaOH for 5 min, water and microemulsion electrolyte for 10 min, sequentially. Between the electrophoretic runs, the capillary was flushed with microemulsion electrolyte for 5 min.

Calibration curves

Stock solutions containing reference compounds were prepared and diluted to appropriate concentrations with the microemulsion electrolyte for the construction of calibration curves. At least six concentrations of the solution were analyzed in duplicate, and then the calibration curves were constructed by plotting the peak area of the individual standard versus the concentration of each analyte.

Limits of Detection and Quantification

The stock solutions of reference compounds mentioned previously were diluted to a series of appropriate concentrations with microemulsion electrolyte to determine the limit of

detection (LOD) and limit of quantification (LOQ), where LOD and LOQ were defined as 3 and 10 times the signal-to-noise ratio (S/N), respectively.

Precision and accuracy

Intra-day and inter-day variations were chosen to determine the precision of the developed assay. The known concentrations of germacrone, furanodiene, curcumenol and curdione reference standards were tested. For the intra-day variability test, the mixed standard solutions were analyzed for six replicates within one day, while for the inter-day variability test, the solutions were examined in duplicate for three days consecutively.

The recovery was used to evaluate the accuracy of the method. Known amounts of individual standards were added to a certain amount (1.0 g) of *Curcuma* sample. The mixture was extracted and analyzed using the previously described method. Three replicates were performed for the test.

Results and Discussion

Optimization of MEEKC conditions

Type of surfactants

MEEKC separations are significantly affected by the choice of microemulsion surfactant, which affects droplet charge and size, level and direction of EOF, and the degree of ion-pairing with solutes. Different surfactants, including SDS (an anionic surfactant) and Tween-80 (a neutral surfactant), were investigated for the separation of volatile compounds in Ezhu. The results (Figure 2) indicated that the compounds can be separated by using SDS as surfactant (Figure 2A). However, no separation trend was observed when using Tween-80 as surfactant (Figure 2B), which cannot be used for separation of neutral solutes. Therefore, SDS was chosen as surfactant for further study.

Type of oil phase

Although hexane, heptane and octane have been shown to give similar selectivity and migration times, octane has been reported to give more repeatable microemulsions and superior peak resolution, efficiency and precision. Therefore, octane is usually used as the microemulsion oil. In the present study, hexane, heptane, octane and ethyl acetate were compared [60 mmol/L SDS, 80 mmol/L of the previously mentioned oil phases, 850 mmol/L *n*-butanol in 5 mmol/L borate buffer (pH 7.6)]. The results indicated that the oil types have no significant effects on the separation of compounds in Ezhu. Therefore, octane was selected for its satisfactory repeatability.

Type of co-surfactant

The co-surfactant is the most influential of the microemulsion constituents on separation selectivity (11, 12), because it can partition into the oil droplet, modify the chromatographic properties of the microemulsion oil phase, and therefore have quite an influence upon its properties (11). Different alcohol molecules, including butanol, propanol, isopropanol, ethanol and methanol, were investigated in the present study [60 mmol/L SDS, 80 mmol/L octane, 850 mmol/L of the previously mentioned co-surfactants in 5 mmol/L borate buffer (pH 7.6)]. The results indicated that butanol, propanol and methanol showed separating potential, while butanol had a shorter analysis time. Furthermore, the preparation of the microemulsion is the most stable when using butanol as co-surfactant. When the carbon number of surfactant is equal to the total carbon number of co-surfactant and oil phase, the molecules are properly ranked, and the microemulsion will be more stable, and therefore better separation efficiency and repeatability can be achieved. When using SDS, butanol and octane as surfactant, co-surfactant and oil phase fulfilled the pattern mentioned previously.

Concentration of SDS

A series of concentrations of SDS were compared (30, 45, 60, 80 and 100 mmol/L). The results are shown in Figure 3. As the

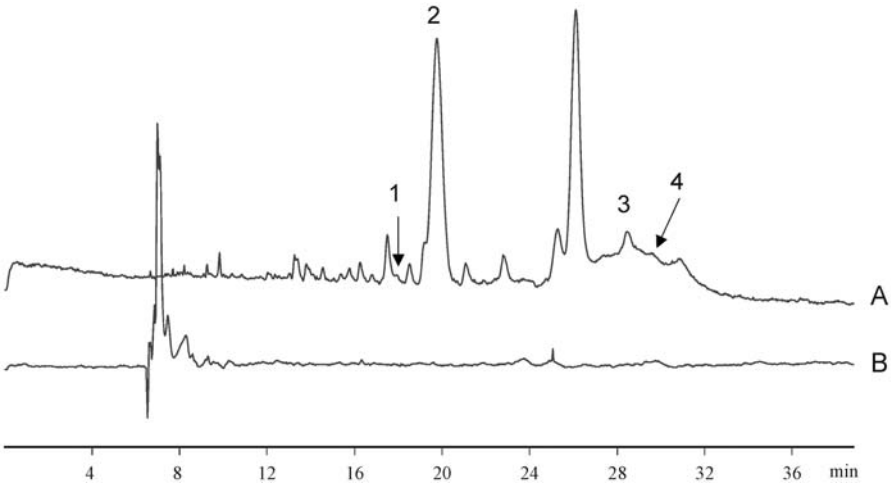

Figure 2. Effects of different types of surfactants on the separation of investigated compounds in methanol extract of *C. phaeocaulis* by MEEKC: SDS (A); Tween-80 (B). 1, curdione; 2, curcumenol; 3, germacrone; 4, furanodiene Running buffer: 45 mmol/L surfactant (SDS or Tween-80), 80 mmol/L *n*-heptane, 850 mmol/L *n*-butanol in 5 mmol/L borate buffer (pH 7.6). Operating conditions: capillary, 75 μm, 58 cm in total with 49.7 cm effective length; injection, 18 cm × 5 s; applied voltage, 15 kV; detection, 214 nm.

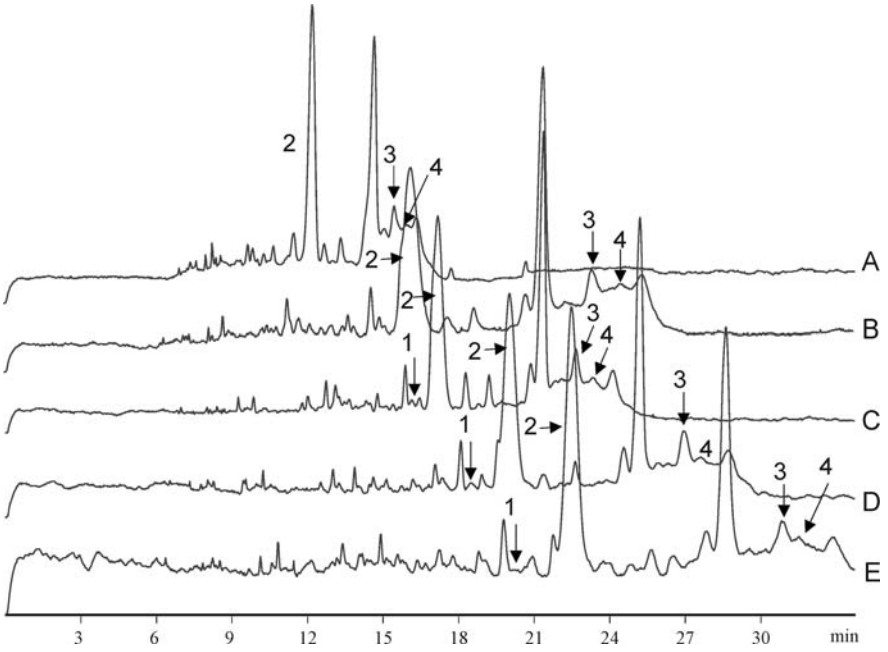

Figure 3. Effects of different concentrations of SDS on the separation of investigated compounds in methanol extract of *C. phaeocaulis* by MEEKC. Running buffer: 30 (A); 45 (B); 60 (C); 80 (D); 100 (E) mmol/L SDS, 80 mmol/L octane, 850 mmol/L *n*-butanol in 5 mmol/L borate buffer (pH 7.6). Operating conditions: capillary, 75 μm, 58 cm in total with 49.7 cm effective length; injection, 18 cm × 5 s; applied voltage, 15 kV; detection, 214 nm.

concentration of SDS increases, the surface tension of the oil phase and water decrease, and the microemulsion will be more stable and the separation efficiency will be better. However, too much SDS will significantly increase the current, which will cause the baseline to be unstable and prolong the analysis time. Therefore, 80 mmol/L of SDS was selected.

Concentration of oil phase

The effect of the concentration of the oil phase on the separation was also investigated. The results indicated that the range of 40 to 120 mmol/L of octane had similar separation efficiency, so 80 mmol/L of octane, which can result in a more stable microemulsion, was used for further study.

Concentration of co-surfactant

When the co-surfactant concentration increased, the solution viscosity and EOF rate changed and the microemulsion droplet increased in size, reducing its ability to oppose the EOF (13). In the present study, different concentrations of butanol were investigated, including 650, 850, 950, 1,050, 1,150 and 1,250 mmol/L. The results showed that the separation of the primary volatile components in Ezhu is slightly improved when the concentration of co-surfactant is higher. Therefore, 1,250 mmol/L of butanol was selected.

Ratio of co-surfactant

Based on the investigation of different types of co-surfactants, a shorter analysis time with poorer separation efficiency was obtained when using butanol as co-surfactant; however, while using propanol as co-surfactant, the resolutions of the peaks was better, but the analysis time was prolonged. Therefore, to obtain a good separation efficiency with a shorter analysis time, a mixture of butanol and propanol was used as co-surfactant (1,250 mmol/L in total concentration). Different ratios of butanol to propanol were compared (1:0, 3:1, 2:1, 1:1 and 1:2). The results indicated that the resolutions of the peaks increase when the proportion of propanol increases, while the analysis time is prolonged. Furthermore, it was difficult to form a stable microemulsion when the proportion of propanol was too high. Therefore, a mixture of 937.5 mmol/L butanol and 312.5 mmol/L propanol (3:1) was used.

Table I
Linear Regression Data, LOD and LOQ of Investigated Compounds

Analytes	Linear regression data			LOQ (mg/mL)	LOD (mg/mL)
	Regressive Equation	Test range (mg/mL)	r		
Curdione	$y = 240853x - 1850$	1.9670–0.1229	0.9992	0.1229	0.0594
Curcumenol	$y = 509610x - 83$	1.5730–0.0983	0.9996	0.0875	0.0438
Germacrone	$y = 1E + 06x - 31671$	1.4750–0.0922	0.9989	0.0563	0.0281
Furanodiene	$y = 809333x - 23906$	0.5900–0.0738	0.9976	0.0738	0.0369

Table II
Intra-Day and Inter-Day Precision and Recovery for the Investigated Compounds

Analytes	Intra-day ($n = 6$, RSD, %)	Inter-day ($n = 6$, RSD, %)	Recovery ($n = 3$, %)
Curdione	2.7	1.9	101.6
Curcumenol	4.8	2.4	107.8
Germacrone	3.8	2.8	102.3
Furanodiene	2.6	2.9	97.6

Effect of pH

The pH value, which affects the value of EOF and the ionization of solutes, has a significant effect on the resolution of the

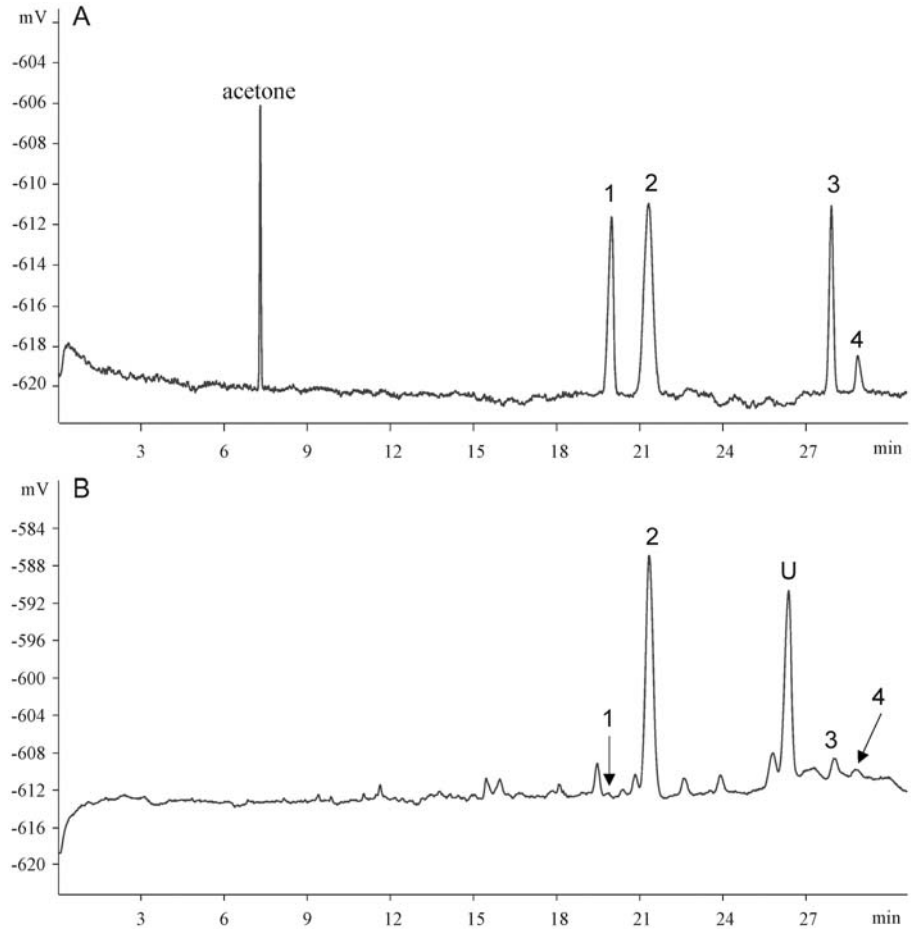

Figure 4. MEEKC electrochromatogram of: mixture of chemical standards (A); methanol extract from *C. phaeocaulis* (B). 1, curdione; 2, curcumenol; 3, germacrone; 4, furanodiene; U, furanodienone. Running buffer: 80 mmol/L SDS, 80 mmol/L 1-octane, 937.5 mmol/L 1-butanol and 312.5 mmol/L propanol in 5 mmol/L borate buffer (pH 8.1). Operating conditions: capillary, 75 μm, 58 cm in total with 49.7 cm effective length; injection, 18 cm × 5 s; applied voltage, 15 kV; detection, 214 nm.

peaks. Generally, high pH buffers are used for MEEKC because they generate a higher EOF towards the cathode when the voltage is applied across the capillary. In the present study, different pHs were compared, including 7.4, 7.6, 7.8, 8.1, 8.5 and 8.8. Finally, pH 8.1 was selected.

Method validations

Linear regression data, LOD, LOQ, intra-day and inter-day precision and recovery for the investigated compounds are shown in Table I and II, respectively. The results indicated that the developed method can be used for the quantification of these four compounds in Ezhu: curdione, curcumenol, germacrone and furanodiene.

Determination of four investigated compounds in Ezhu

Under the optimum conditions, the methanol extract of Ezhu was analyzed. The electrochromatogram is shown in Figure 4. The resolution values of curdione, curcumenol, germacrone and furanodienone to their adjacent peaks were 1.72, 1.41, 1.46 and 2.17, respectively. The identification of compounds was conducted by adding the individual standard to the sample. By using the calibration curve of each standard, the contents of four primary components in the Ezhu sample were determined. The curdione was below the LOQ, but the contents of curcumenol, germacrone and furanodiene were 4.59, 0.30 and 0.23 mg/g, respectively. Furthermore, the unknown peak (U) may be furanodienone, according to previous reports.

Conclusions

An MEEKC method was developed in the present study for the analysis of volatile components in Ezhu. The results showed that MEEKC is an alternative approach for the analysis of volatile components with highly hydrophobic property. Furthermore, MEEKC may be a good choice for the analysis of essential oils contain heat-sensitive compounds.

Acknowledgment

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