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Optimization of GC–MS conditions based on resolution and stability of analytes for simultaneous determination of nine sesquiterpenoids in three species of *Curcuma* rhizomes

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Abstract

GC–MS is a powerful tool for analysis of volatile oil, and resolutions of analytes were exclusively used as marker for optimization of the conditions. However, volatile oil usually contains heat labile components which may degrade and result in wrong results during GC analysis. In present study, based on both resolutions and stabilities of 11 sesquiterpenoids, GC–MS conditions were optimized for simultaneously quantitative determination of nine compounds including β-elemene, curzerene, curcumol, isocurcumenol, germacrone, curdione, curcumenol, neocurdione and curcumenone in *Ezhu*. However, the other two compounds, i.e. furanodienone and furanodiene, were still thermal sensitive and not available for GC analysis. The results showed that both resolutions and stabilities of analytes should be considered for optimization of GC conditions because the properties of most components in volatile oil are unknown. Under optimum conditions, a capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. Pulsed splitless inlet with temperature of 190 °C was selected for sample injection (0.2 μl). The calibration curves of nine sesquiterpenoids showed good linearity ($r^2 > 0.9989$) within test ranges. The optimized method showed good repeatability for quantification of these nine components in *Ezhu* with intra- and inter-day variations of less than 1.42% and 2.79%, respectively. The validated method was successfully applied to quantify 9 sesquiterpenoids in 18 samples of 3 species of *Curcuma* used as *Ezhu*.

Keywords: Gas chromatography–mass spectrometry; Sesquiterpenoids; Resolution; Stability; Pressurized liquid extraction; *Curcuma*

1. Introduction

Curcuma belongs to the family Zingiberaceae. It is a genus of about 70 species of rhizomatous herbs distributed all over the world. Among about 20 species identified in China, a few are traditional Chinese medicine being used for a long time. It is recorded that the rhizomes of three species *Curcuma* including *C. phaeocaulis*, *C. kwangsiensis* and *C. wenyujin* are used as *Ezhu*, which is used for removing blood stasis and alleviating pain [1]. In addition, the essential oil of *Ezhu* is reported to possess anti-tumour [2,3] and antiviral activities [4,5]. Sesquiterpenoids including β-elemene, curcumol, germacrone, curdione and neocurdione are found to be the biological active ingredients in the essential oil [6,7]. Therefore, quantitative determination of these components is helpful to control the quality of *Ezhu*. It was also considered that curcumol was the major component in volatile oil of *Ezhu* [8]. However, our previous study showed that curcumol was rare in three species *Curcuma* rhizomes used as *Ezhu* [9]. In order to elucidate the contrary results, further study was performed in our lab.

Generally, GC–MS is a powerful tool for analysis of volatile oil, and resolution of analytes was exclusively used as marker for optimization of the conditions. However, volatile oil usually contains heat-sensitive components which may degrade and result in wrong results during GC analysis. For example, numerous 1,4-dienes present this property, as their skeleton rearranges thermally through a [3.3]-sigmatropic reaction (Cope rearrangement). This problem has been encountered with several sesquiterpenes such as germacrene [10,11], germacrone [12] and furanodiene [13,14]. These compounds cannot be studied by GC–MS using normal experimental conditions, as the rearrange-

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ment takes place in the injector and column [13–15]. Therefore, optimization of GC–MS conditions for analysis of volatile oil should be based on the resolution and stability of analytes while their chemical properties were unknown.

In present study, based on both resolutions and stabilities of 11 sesquiterpenoids, GC–MS conditions were optimized for simultaneously quantitative determination of nine compounds including β-elemene, curzerene, curcumol, isocurcumenol, germacrone, curdione, curcumenol, neocurdione and curcumenone in *Ezhu*. The validated method was also applied to quantify 9 sesquiterpenoids in 18 samples of three species of *Curcuma* used as *Ezhu*.

2. Materials and methods

2.1. Materials

Methanol for GC–MS was purchased from Merck (Darmstadt, Germany). β-Elemene, curzerene, furanodienone, curcumol, isocurcumenol, germacrone, furanodiene, curdione, curcumenol, neocurdione and curcumenone were separated and purified from commercial oil of *Curcuma wenyujin* using silica-gel column chromatography and recrystallization by ourselves. The purity of all compounds were >99%, which was tested by GC–MS and/or HPLC. The structures (Fig. 1) were confirmed by their UV, MS and NMR data compared with those of literatures [16–27].

Six batches (CW1–CW6) of *C. wenyujin* rhizomes were obtained from Yueqing, Zhejiang Province; *Curcuma phaeocaulis* rhizomes (CP1–CP6) were separately collected from Tingjiang, Jiangyuan, Sanjiang, Zhoudu, Wangdan and Shuangliu, Sichuan Province; *Curcuma kwangsiensis* (CK1–CK6) rhizomes were collected from Nanning, Guixian, Wuming and Yunshan, Guangxi Province, as well as Wenshan and Malipo, Yunnan Province, respectively. All the plant materials were collected in November 2003. The voucher specimens of *Curcuma* rhizomes were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China.

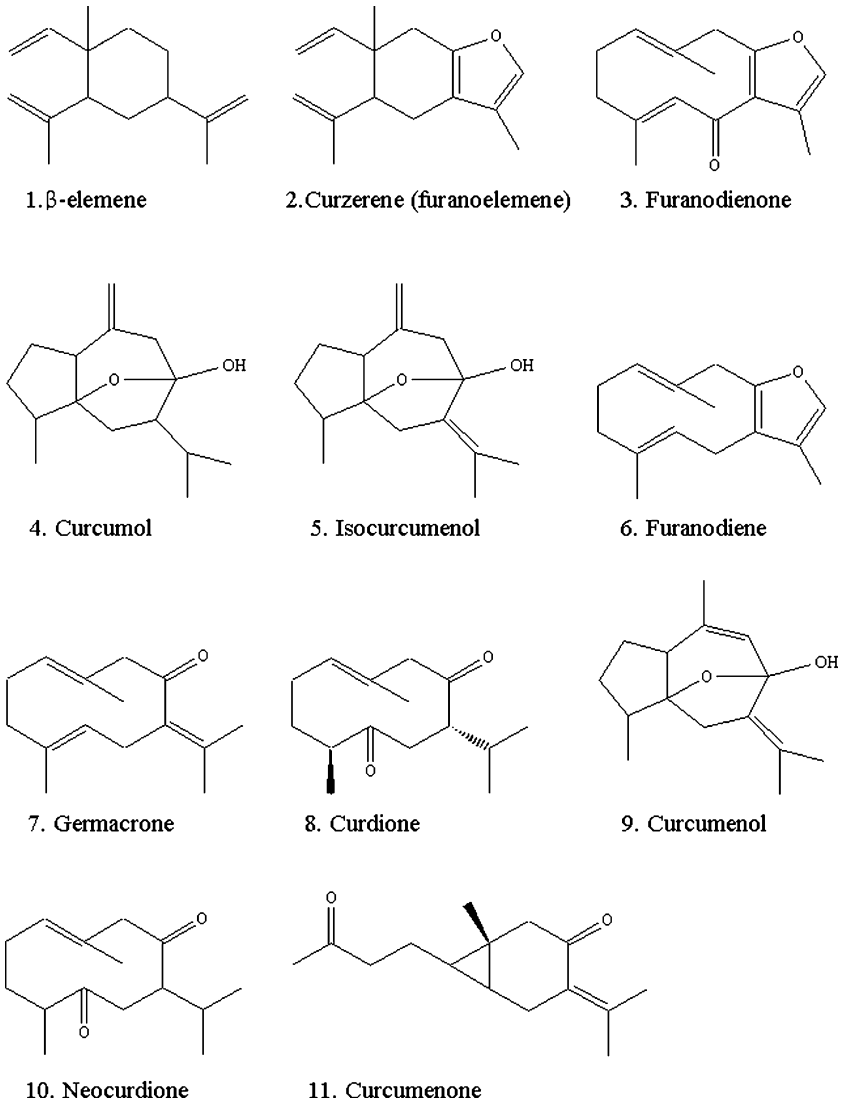

Fig. 1. Chemical structures of 11 investigated compounds.

2.2. Pressurized liquid extraction (PLE)

Pressurized liquid extractions were performed on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA) as described before [9] with minor modification. In brief, raw materials of *Ezhu* were dried at 40 °C for 6 h and were grounded into powder of 0.2–0.3 mm. Powder of *Ezhu* (0.5 g) was mixed with diatomaceous earth (0.5 g) and placed into 11-ml stainless steel extraction cell, respectively. The sample was extracted under the optimized conditions: solvent, methanol; temperature, 100 °C; particle size, 0.2–0.3 mm; static extraction time, 5 min; pressure, 1000 psi; static cycle, 1; 40% of the flush volume. Then, extract was transferred to a 25 ml volumetric flask which was made up to its volume with extraction solvent and filtered through a 0.45 μm Econofilter (Agilent Technologies, Palo Alto, CA, USA) prior to injection into the HPLC system.

2.3. GC–MS analysis

GC–MS was performed with an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and an Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). A capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. High purity helium was used as carrier gas with flow-rate at 1.0 ml min^{-1}. The other GC conditions such as inlet mode, injection temperature and separation temperature program were optimized using 11 investigated compounds as reference.

The spectrometers were operated in electron-impact (EI) mode, the scan range was 40–550 amu, the ionization energy was 70 eV and the scan rate was 0.34 s per scan. The quadrupole, ionization source temperature were 150 and 280 °C, respectively.

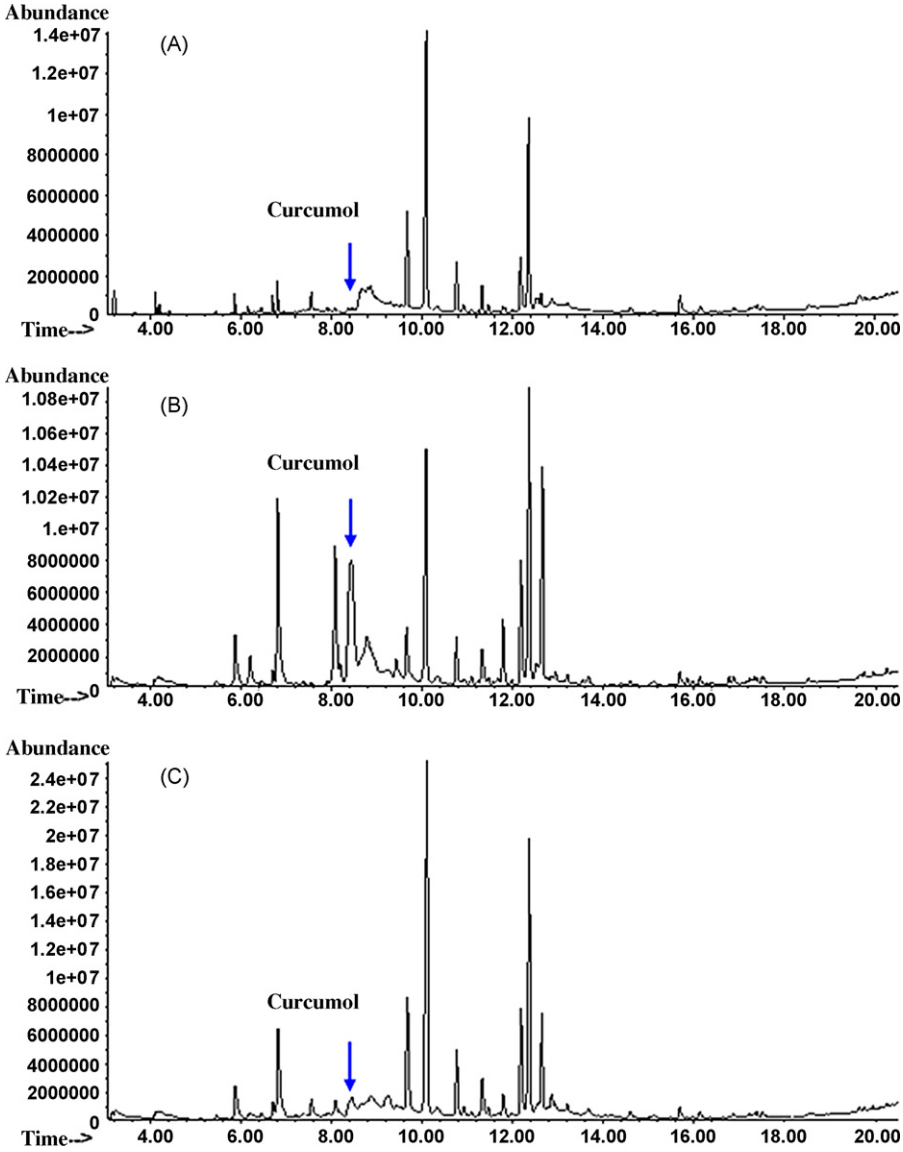

Fig. 2. Effects of inlet mode and temperature on GC–MS analysis of *Ezhu* derived from *Curcuma wenyujin* (CW6). (A) Split mode with inlet temperature of 250 °C, (B) splitless mode with inlet temperature of 250 °C, and (C) splitless mode with inlet temperature of 200 °C.

3. Result and discussion

3.1. Optimization of GC conditions

Based on the results of our previous study [9], the content of curcumol in 18 samples of *Ezhu* was rare, though it was considered that curcumol was one of the main active compounds in *Ezhu* [28]. Considering inlet mode of GC injection, the result may derive from thermal discriminative effect, which meant that more volatile compounds might be vent off easer than less volatile compounds during split injection. Therefore, split and splitless injections were investigated for GC–MS analysis of an *Ezhu* sample. The result showed that the peak of curcumol was obvious in GC–MS profile using splitless instead of split inlet mode (Fig. 2A and B). The results seemed suggest that thermal discriminative effect plays a dominant role during GC–MS analysis of curcumol. However, the peak abundance of curcumol significantly declined when splitless inlet temperature decreased from 250 to 200 °C, which showed that the inlet temperature also significantly affected GC–MS analysis of curcumol (Fig. 2C). A pulsed splitless injection with higher pressure can decrease the likelihood that highly volatile compounds will escape out the top of the injection port through the septum purge vent. In the case of thermally labile compounds, the faster they leave the hot injection port the less likely they are to degrade [29]. Therefore, pulsed splitless inlet mode was selected for avoiding thermal discriminative effect and heat degradation, which was regarded as the best way for splitless injections [30]. Fig. 3 shown the effect of splitless mode on GC–MS analysis of curdione. The result showed that curdione could change into curcumol and pulsed splitless injection can significantly decrease the formation of curcumol during GC–MS analysis. Furthermore, the inlet temperature was optimized to avoid the degradation of heat labile compounds in *Ezhu*. Six of eleven investigated components were significant degraded at high temperature during GC–MS analysis (Fig. 4). The degradation was ameliorated with inlet temperature decrease. However, the gasification of some investigated compounds such as curcumenone and curdione was not enough when the inlet temperature was below 190 °C, which still significantly induced the degradation of furanodiene and furanodienone (Fig. 5). It was noticed that furanodienone could be completely transformed into curzerenone during GC–MS analysis. And MS spectra of furanodienone and curzerenone were very similar (data not shown), which induced wrong identification of furanodienone as curzerenone without reference compound in our previous study [9]. Thus, GC–MS was not available for analysis of the two compounds, furanodiene and furanodienone, and an optimum inlet temperature (190 °C) was obtained for simultaneous determination of nine sesquiterpenoids in *Ezhu* (Fig. 6A). The pathways of thermal degradation for curcumol, curdione, furanodiene, germacrone and furanodienone were shown in Fig. 7.

As mentioned above, the optimized GC–MS conditions were as follows: column, a capillary (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane; carrier gas, high purity helium; flow-rate, 1.0 ml min^{-1}; inlet mode and temperature, pulsed splitless at 190 °C; the column temperature was set at 60 °C and held for 2 min for injection, then programmed at 5 °C min^{-1} to 145 °C and held for 25 min at the temperature of 145 °C, then at 5 °C min^{-1} to 200 °C, and finally, at 20 °C min^{-1} to 280 °C, and held for 3 min at the temperature of 280 °C.

3.2. Calibration curves

Furanodienone and furanodiene changed into curzerenone and curzerene under optimized conditions, respectively. They

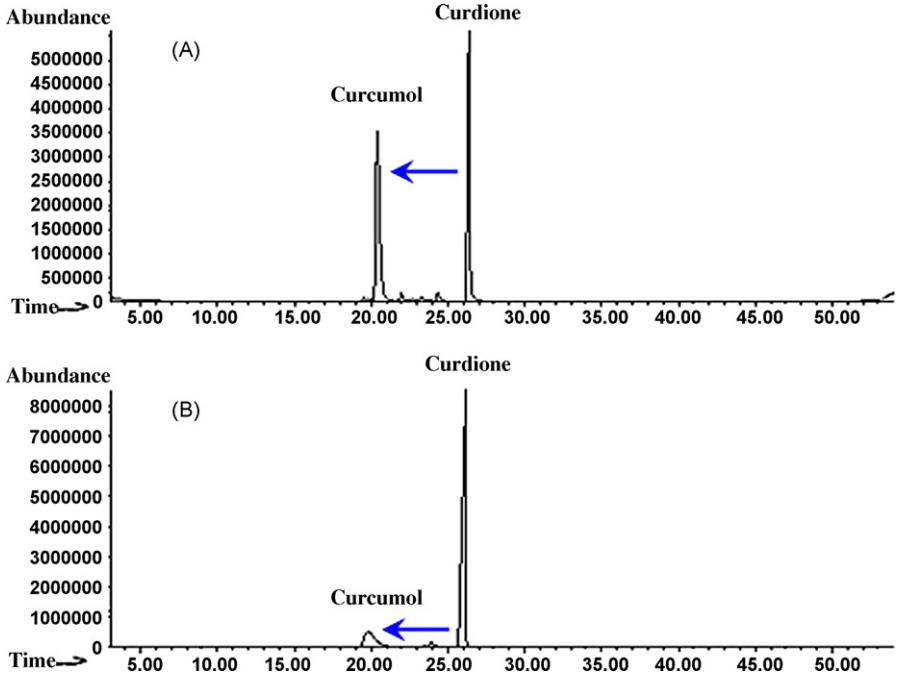

Fig. 3. Comparison of: (A) splitless inlet and (B) pulsed splitless inlet at 240 °C on GC–MS analysis of curdione (the arrows denote the degradation trend).

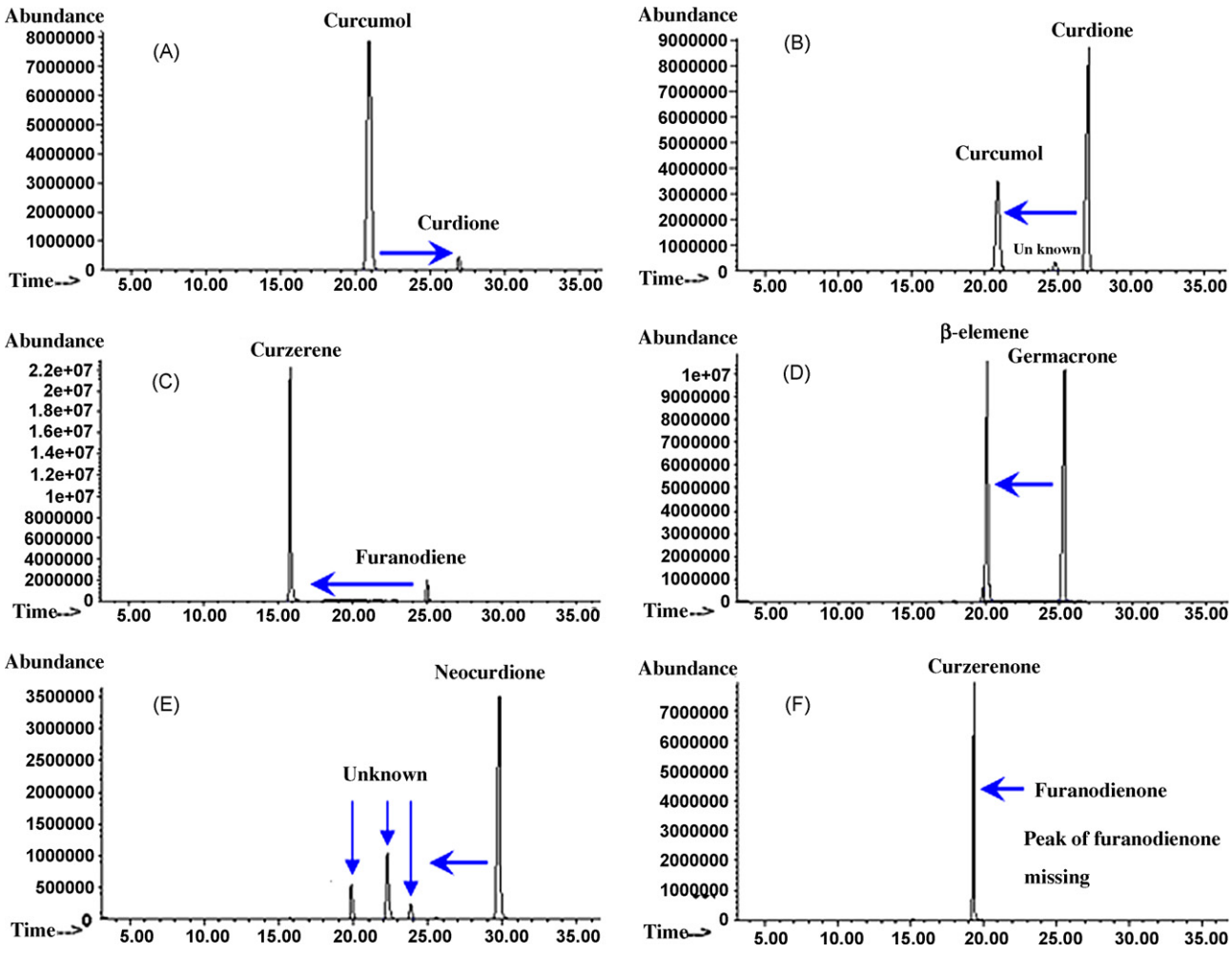

Fig. 4. Effects of splitless inlet with temperature of 250 °C on GC–MS analysis of: (A) curcumol, (B) curdione, (C) furanodiene, (D) germacrone, (E) neocurdione and (F) furanodienone (the arrows denote the degradation trend).

were not available for quantitation by GC–MS. Therefore, nine sesquiterpenoids including β-elemene, curzerene, curcumol, isocurcumenol, germacrone, curdione, curcumenol, neocurdione and curcumenone in *Ezhu* were quantified by using selected ion monitoring (SIM) method of GC–MS. The fragment ions used were *m/z* 93, 108, 121, 191, 107, 180, 105, 180 and 176, respectively (Table 1).

Methanol stock solutions containing nine analytes were prepared and diluted to appropriate concentration for the construction of calibration curves. Six concentration of the nine analytes' solution were injected in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the amount (ng) of each analyte. The results were shown in Table 2.

Table 1
Mass data of 11 investigated compounds for optimization of GC–MS conditions

Peak	Compound	Mass data[a]
1	β-Elemene	204(M^+, 4), 189(45), 147(62), 121(53), 107(70), 93(100), 91(47), 81(87), 79(56), 68(54), 67(60)
2	Curzerene	216(M^+, 17), 201(13), 159(19), 148(33), 145(13), 108(100), 93(17), 91(30), 79(32), 77(31), 65(20)
3	Furanodienone	230(M^+, 39), 215(21), 150(23), 122(100), 94(46), 91(19), 81(24), 77(18), 65(22), 41(18)
4	Curcumol	236(M^+, 36), 193(32), 147(31), 136(30), 135(31), 121(100), 107(41), 93(33), 41(30)
5	Isocurcumenol	234(M^+, 7), 191(100), 173(47), 147(40), 145(60), 121(83), 105(99), 91(44), 67(40)
6	Furanodiene	216(M^+, 52), 201(19), 159(26), 148(6), 145(27), 108(100), 91(33), 77(27), 65(14), 53(16), 41(27)
7	Germacrone	218(M^+, 13), 175(27), 136(61), 135(85), 121(30), 107(100), 105(20), 91(31), 67(42)
8	Curdione	236(M^+, 17), 180(100), 167(83), 109(53), 69(52), 68(29), 67(30), 55(33), 41(31)
9	Curcumenol	234(M^+, 28), 189(57), 147(45), 145(31), 133(57), 121(31), 119(36), 105(100), 91(40), 55(32), 41(31)
10	Neocurdione	236(M^+, 17), 180(100), 167(83), 109(81), 69(90), 68(49), 67(44), 55(49), 41(42)
11	Curcumenone	234(M^+, 26), 176(100), 163(49), 161(70), 149(72), 133(37), 107(34), 91(34), 68(67), 67(49), 43(63)

[a] *m/z*, relative intensity shown in parenthesis, and the ion of relative intensity 100 was used for the quantification.

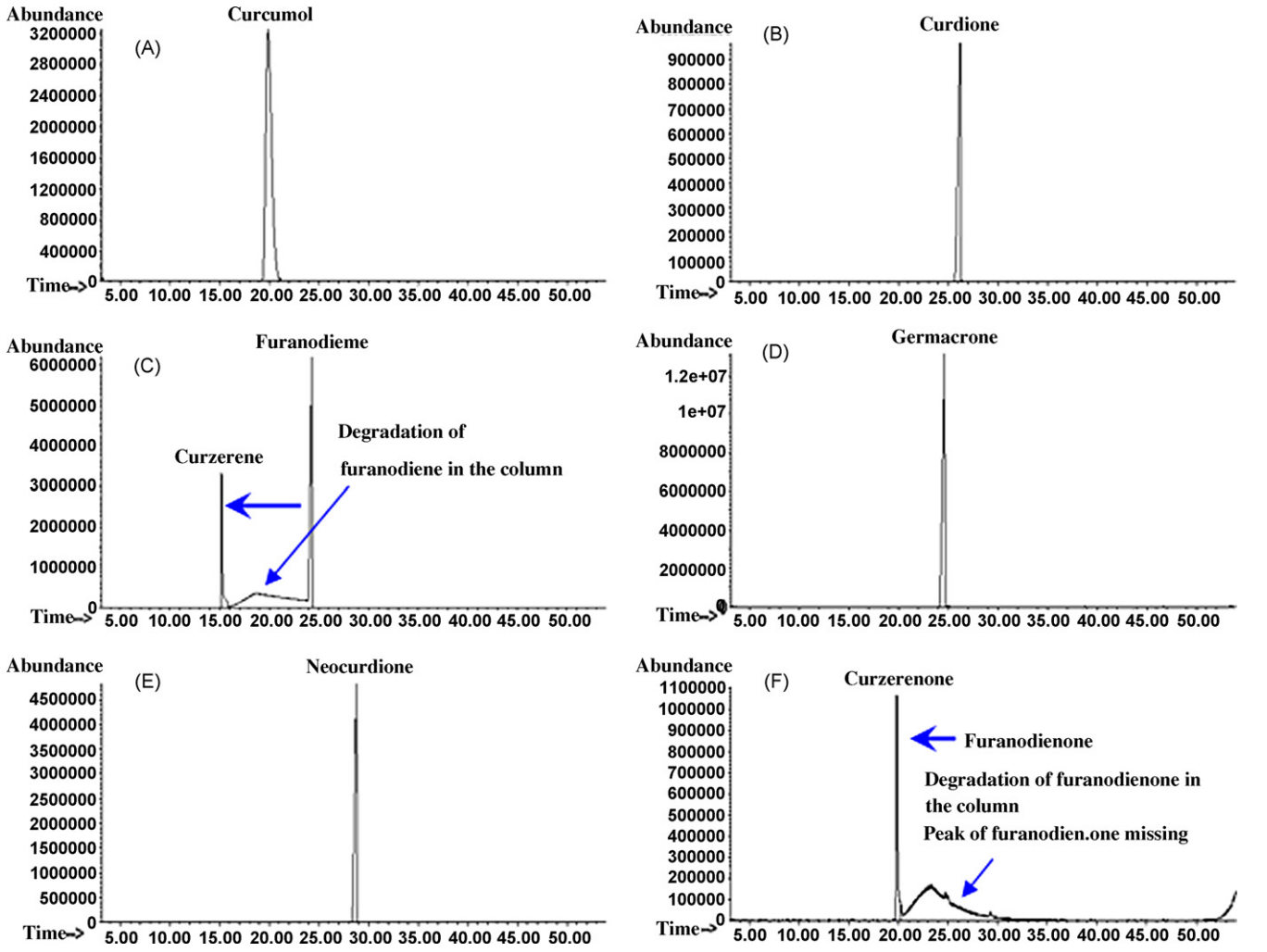

Fig. 5. Total ion chromatograms of: (A) curcumol, (B) curdione, (C) furanodiene, (D) germacrone, (E) neocurdione and (F) furanodienone under optimum GC–MS conditions (the arrows denote the degradation trend). GC–MS conditions: column, a capillary (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane; carrier gas, high purity helium; flow-rate, 1.0 ml min^{-1}; inlet mode and temperature, pulsed splitless at 190 °C; the column temperature was set at 60 °C and held for 2 min for injection, then programmed at 5 °C min^{-1} to 145 °C and held for 25 min at the temperature of 145 °C, then at 5 °C min^{-1} to 200 °C, and finally, at 20 °C min^{-1} to 280 °C, and held for 3 min at the temperature of 280 °C.

3.3. Limits of detection and quantification

Methanol stock solution containing nine reference compounds was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solutions was injected into GC–MS for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at the ratio of signal to noise

Table 2
Linear regression data, repeatability and recovery of investigated components from *Ezhu*

Analytes	SIM	Linear regression data			LOD (ng)	LOQ (ng)	Recovery* (%, $n=3$)	R.S.D. (%, $n=3$)
		Regressive equation	Linear range (ng)	r^2				
β-Elemene	93	$y=94642x-6136$	0.163–5.200	0.9993	0.025	0.032	101.53	2.27
Curzerene	108	$y=113144x+1445$	0.647–5.175	0.9996	0.027	0.041	96.58	2.03
Curcumol	121	$y=135529x-12268$	0.178–1.780	0.9996	0.146	0.161	102.11	1.89
Isocurcumenol	191	$y=11493x-965$	0.203–1.421	0.9996	0.073	0.091	100.77	2.87
Germacrone	107	$y=148624x-22521$	0.575–8.050	0.9993	0.059	0.072	99.05	1.07
Curdione	180	$y=103694x-33012$	0.107–30.924	0.9994	0.054	0.083	99.48	0.56
Curcumenol	105	$y=97915x-65935$	0.790–31.600	0.9993	0.298	0.392	101.97	1.29
Neocurdione	180	$y=102923x-30295$	0.391–14.076	0.9994	0.159	0.185	102.19	1.17
Curcumenone	176	$y=45780x-42740$	2.475–24.750	0.9993	0.743	0.921	98.50	0.57

r^2, squares of correlation coefficients for the standard curves.
* Average of three tests.

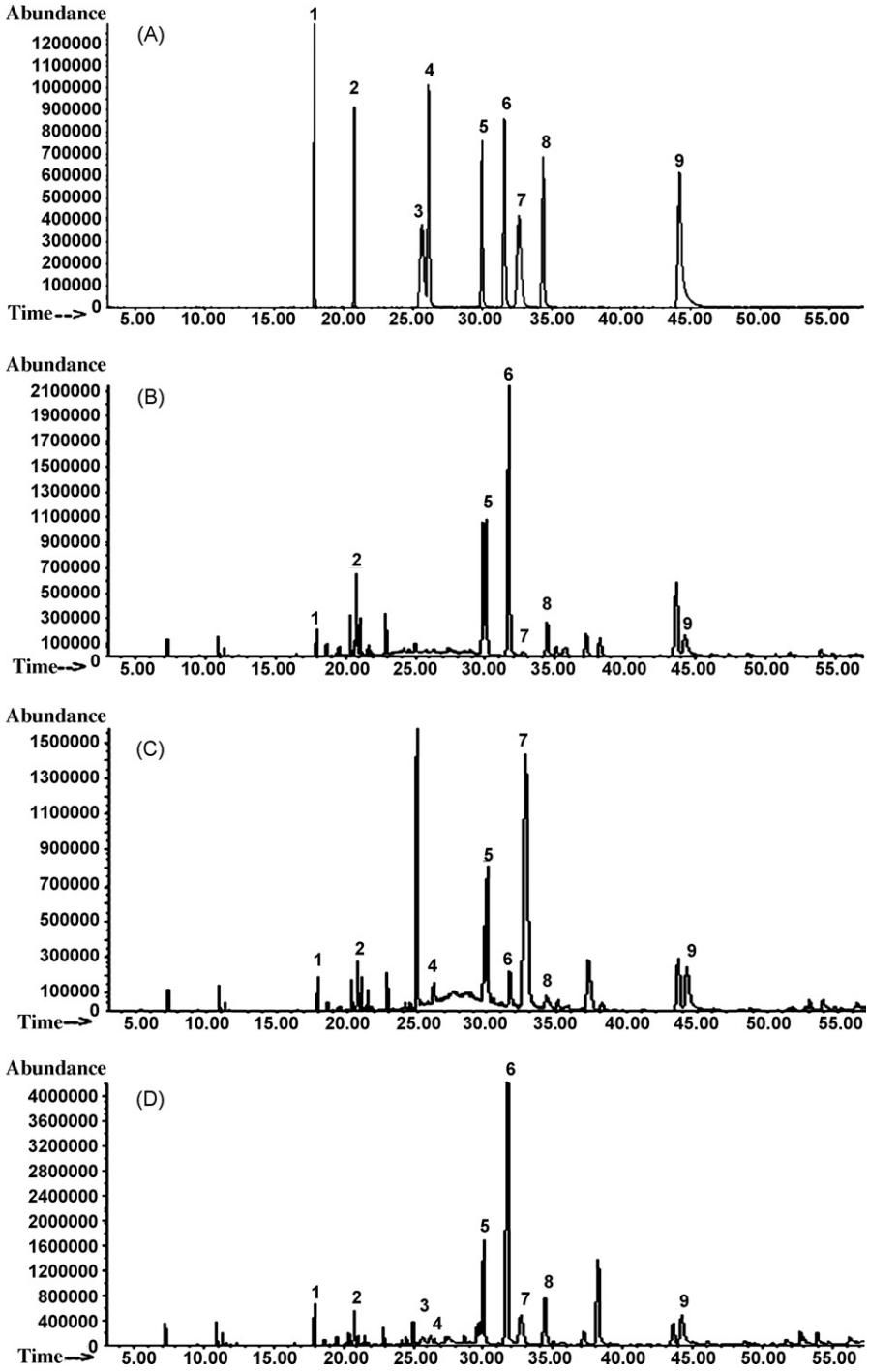

Fig. 6. Total ion chromatograms of: (A) mixed standards, (B) *C. wenyujin*, (C) *Curcuma phaeocaulis* and (D) *Curcuma kwangsiensis* analyzed by GC–MS. GC–MS conditions were described as Fig. 5. *C. wenyujin* derived from Yueqing, Zhejiang Province; *C. phaeocaulis* derived from Tingjiang, Sichuan Province; *C. kwangsiensis* derived from Guixian, Guangxi Province. (1) β-Elemene, (2) curzerene, (3) curcumol, (4) isocurcumenol, (5) germacrone, (6) curdione, (7) curcumenol, (8) neocurdione, and (9) curcumenone.

(S/N) equal to 3 and 10, respectively. Table 2 showed the data of LOD and LOQ for each investigated compound.

3.4. Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. A certain concentration solution of nine reference compounds was tested. For intra-day variability, the samples were analyzed in triplicate for three times within 1 day, while for inter-day variability, the samples were examined in triplicate for consecutive 3 days. Variations were expressed by the relative standard deviations (R.S.D.), which were less than 1.42% and 2.79%, respectively.

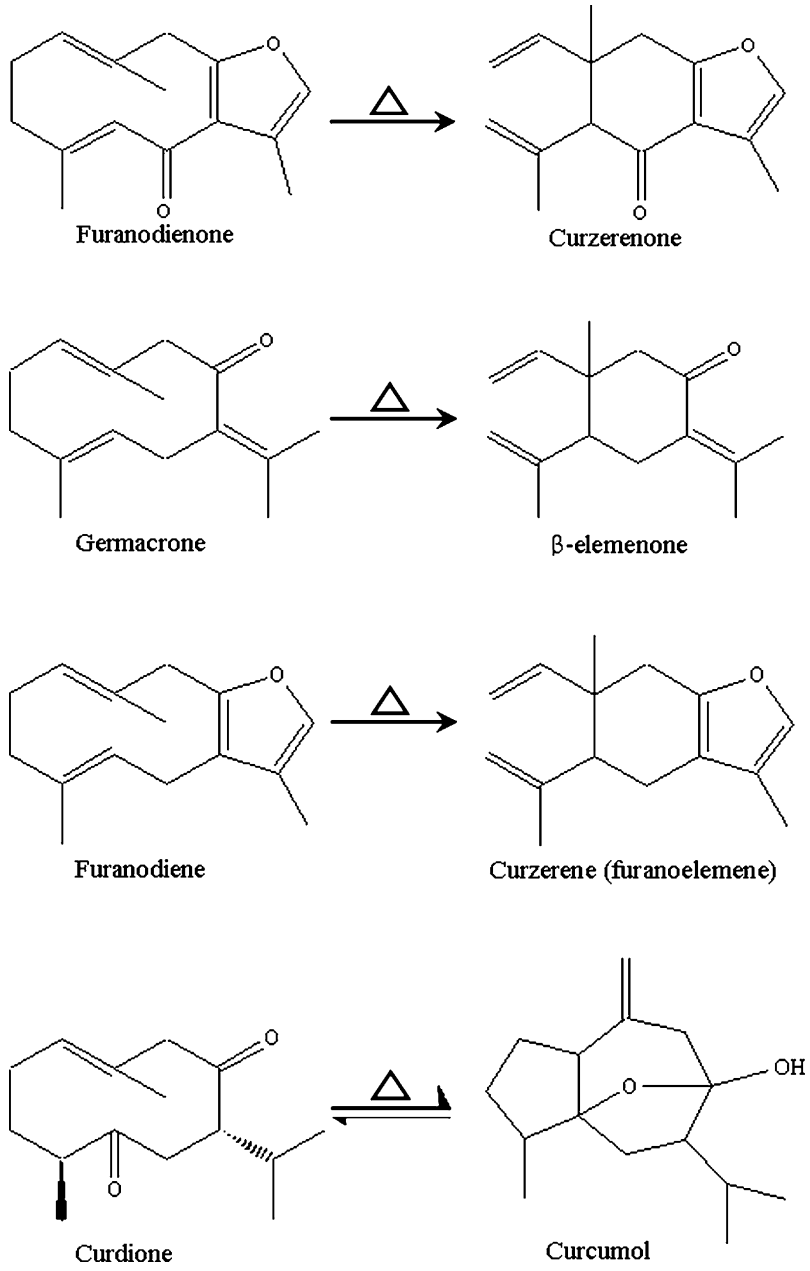

Fig. 7. The pathway of thermal degradation for curcumol, curdione, furanodiene, germacrone and furanodienone.

Recovery test was used to evaluate the accuracy of this method. Accurate amounts of nine investigated compounds were added to approximate 0.25 g of *Ezhu*, and then extracted and analyzed as described above:

$$\text{recovery}\,(\%) = 100 \times \frac{\text{amount found} - \text{original amount}}{\text{amount spiked}}$$

The results were shown in Table 2.

3.5. Quantification of nine investigated components in Ezhu

Total ion chromatograms of PLE extracts from three species of *Curcuma* rhizomes were shown in Fig. 6. All the main components were separated completely. The identification of investigated components was carried out by comparison of their retention time and mass spectra with those obtained injecting standards in the same conditions.

By using the calibration curve of each investigated compound, 18 *Ezhu* samples from three species of *Curcuma* were analyzed. Table 3 shown the summary results. The results showed the contents of nine investigated sesquiterpenoids were greatly variant in different species or locations of *Curcuma* rhizomes, which were in accordance with our previous report [9]. Especially, the content of curzerene was derived from both its original amount and the furanodiene degradation in raw material. Moreover, curcumol was only detected in one sample of *C. kwangsiensis* (CK2) produced from Guixian, Guangxi Province.

Table 3
Contents (mg/g) of nine sesquiterpenoids in three species of *Curcuma* used as *Ezhu*

Samples[a]	β-Elemene	Curzerene	Curcumol	Isocurcumenol	Germacrone	Curdione	Curcumenol	Neocurdione	Curcumenone	Total
CW1	0.368[b]	2.423[c]	–[d]	–	4.031	16.661	0.513	2.166	4.605	30.767
CW2	0.415	2.939	–	–	3.892	15.459	0.559	2.045	7.007	32.316
CW3	0.280	1.657	–	–	3.835	16.044	0.422	2.180	3.969	28.387
CW4	0.364	1.800	–	–	4.051	17.902	0.345	2.283	3.349	30.094
CW5	0.373	2.601	–	–	4.331	15.746	0.564	2.167	5.419	31.201
CW6	0.567	3.898	–	–	5.670	20.677	0.883	2.922	8.004	42.621
CP1	0.177	0.476	–	0.253	1.110	0.515	7.490	–	2.984	13.005
CP2	0.217	0.468	–	0.283	1.290	0.165	8.280	–	3.092	13.795
CP3	0.154	0.272	–	0.219	0.918	0.107	7.142	–	2.533	11.345
CP4	0.158	0.312	–	0.221	1.069	0.111	7.748	–	2.782	12.401
CP5	0.175	0.417	–	0.215	1.135	0.184	7.213	–	2.526	11.865
CP6	0.205	0.295	–	0.250	1.159	1.361	6.054	0.229	2.821	12.374
CK1	0.095	0.204	–	–	0.682	0.173	0.391	–	–	1.545
CK2	0.415	0.676	0.410	0.191	2.291	11.684	2.430	1.850	4.561	24.508
CK3	0.104	0.195	–	–	0.431	0.150	0.186	–	–	1.066
CK4	0.198	0.084	–	–	1.606	0.252	1.200	0.150	0.986	4.476
CK5	0.150	0.160	–	0.081	0.193	–	1.859	–	0.644	3.087
CK6	0.174	0.188	–	0.130	0.709	0.709	1.494	0.112	1.511	5.027

[a] CW1–CW6 are *Curcuma wenyujin* derived from Yueqing, Zhejiang Province, respectively. CP1–CP6 are *Curcuma phaeocaulis* derived from Tingjiang, Jiangyuan, Sanjiang, Zhoudu, Wangdan and Shuangliu, Sichuan Province, respectively. CK1–CK6 are *Curcuma kwangsiensis* derived from Nanning, Guixian, Wuming and Yunshan, Guangxi Province, as well as Wenshan and Malipo, Yunnan Province, respectively.
[b] The data was presented as average of three replicates (R.S.D. < 3%).
[c] The amount included the degradation of furanodiene.
[d] Undetected.

4. Conclusion

Optimization of GC–MS conditions should be based on resolutions and stabilities of analytes because volatile oil usually contains heat-sensitive components which may degrade and result in wrong results during GC analysis. However, chemical properties of the components in volatile oil were unknown in most cases and the pure compounds were difficult to be obtained for the related study. Therefore, it should be developed that a method for optimization of GC–MS conditions based on both resolutions and stabilities of analytes even if the reference compounds are not available.

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