Identification and quantitation of eleven sesquiterpenes in three species of *Curcuma* rhizomes by pressurized liquid extraction and gas chromatography–mass spectrometry

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Abstract

In this paper, GC–MS and pressurized liquid extraction (PLE) was developed for identification and quantitative determination/estimation 11 sesquiterpenes including germacrene D, curzerene, γ-elemene, furanodienone, curcumol, isocurcumenol, furanodiene, germacrone, curdione, curcumenol and neocurdione in *Ezhu* which are derived from three species of *Curcuma*, i.e., *Curcuma phaeocaulis*, *Curcuma wenyujin* and *Curcuma kwangsiensis* by using an analogue as standard. The results showed the methodology could quantitatively compare the quality of three species of *Curcuma*. The contents of investigated sesquiterpenes in three species of *Curcuma* were high variant. Hierarchical clustering analysis based on characteristics of 11 identified peaks in GC profiles showed that 18 samples were divided into two main clusters, *C. phaeocaulis* and *C. wenyujin*, respectively. *C. kwangsiensis* showed the characters closed to *C. phaeocaulis* or *C. wenyujin* based on its location. Five components such as furanodienone, germacrone, curdione, curcumenol and neocurdione were optimized as markers for quality control of *Ezhu*.
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Keywords: Gas chromatography–mass spectrometry; Sesquiterpenes determination; Pressurized liquid extraction; Quality control; *Curcuma*

1. Introduction

Curcuma belongs to the Family Zingiberaceae. It is a genus of about 70 species of rhizomateous herbs distributed in the world. About 20 species occur in China, of which a few are traditional Chinese medicine for a long time. Actually, it is recorded that the rhizomes of three species *Curcuma* including *Curcuma phaeocaulis*, *Curcuma kwangsiensis* and *Curcuma wenyujin* are used as *Ezhu* which is for removing blood stasis and alleviating pain [1]. The essential oil of *Ezhu* is reported possess anti-tumour [2,3] and anti-virus activities [4,5]. Several components including β-elemene, curcumol, germacrone, curdione and neocurdione are thought to be the

biological active ingredients in the essential oil [6,7]. However, the three species of *Ezhu* showed variation in their chemical compositions [8,9]. Up to date, only curcumol was usually quantitated because of the absence of chemical standards [10]. These problems, therefore, compromise the values of traditional Chinese medicine or even jeopardize the safety of the consumers. Fortunately, gas chromatography–mass spectrometry (GC–MS) offers a powerful tool for identification of chemical components in essential oil [11,12]. However, all studies of GC–MS [13–16] were focused on the identification of components and determination of their relative amount in the mixture without standards. Therefore, the results couldn't be used for evaluating the quality of different samples or batches, which obstructed the improvement of quality control for Chinese medicine. In present study, by using an analogue as chemical standard, GC–MS and pressurized liquid

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extraction (PLE) was developed for simultaneous determination/estimation of 11 sesquiterpenes including germacrene D, curzerene, γ-elemene, furanodienone, curcumol, isocurcumenol, furanodiene, germacrone, curdione, curcumenol and neocurdione in *Ezhu*. The amount of 11 sesquiterpenes in different geographical sources of *Ezhu* was also compared.

2. Materials and methods

2.1. Materials

Six batches (CW1–CW6) of *C. wenyujin* rhizomes were obtained from Yueqing, Zhejiang Province; *C. phaeocaulis* rhizomes (CP1–CP6) were separately collected from Tingjiang, Jiangyuan, Sanjiang, Zhoudu, Wangdan and Shuangliu, Sichuan Province; *C. 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.

Germacrone, curcumol and curdione were separated and purified ourselves. The structures were confirmed by comparing their EI-MS (Table 1) and NMR data (shown in Appendix) with references [17–19]. Methanol for GC was purchased from Merck (Darmstadt, Germany).

2.2. Pressurized liquid extraction (PLE)

Pressurized liquid extractions were performed on a Dionex ASE 200 (Dionex Corp., Sunnyvale, CA, USA) system. In brief, raw materials of *Ezhu* were dried at 40 °C for 6 h and was ground into powder of 0.2–0.3 mm. Powder of *Ezhu* (2.5 g) was mixed with diatomaceous earth (2.5 g) and placed into 11 ml stainless-steel extraction cell, respectively. The sample was extracted under the optimized conditions: solvent, methanol; temperature, 120 °C; particle size, 0.2–0.3 mm; static extraction time, 5 min; pressure, 1500 psi;

static cycle, 1 and 60% of the flush volume. Then, extract was transferred to a 25 ml volumetric flask, which was brought up to its volume with methanol and filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the GC–MS 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, USA). A capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. The column temperature was set at 100 °C and held for 5 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. Split injection (2 μl) was conducted with a split ratio of 10:1 and high-purity helium was used as carrier gas of 1.0 ml min^{-1} flow-rate. 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 inlet, ionization source temperature were 250 and 280 °C, respectively.

3. Result and discussion

3.1. Optimization of PLE procedure

PLE procedure was optimized. And the parameters include the type of solvent (methanol, ethanol, ethyl acetate and petroleum ether), particle size (0.15–1.99 mm), temperature (80–160 °C) and static extraction time (5–15 min) were studied by using univariate approach. Total amount of 11 investigated compounds were used as the markers for evaluation of extraction efficiency. The recovery efficiency for the PLE procedure was determined by performing consecutive pressurized liquid extractions on the same sample under the optimized PLE conditions, until no investigated compounds

Table 1
Mass data of 11 sesquiterpenes identified from *Ezhu* rhizomes

Peak no.	Compound	Rt (min)	Mass data[a]
1	Germacrene D	15.14	204($M+$, 16), 161(100), 120(20), 119(32), 105(48), 93(22), 91(42), 81(28), 79(27), 55(12), 41(17)
2	Curzerene	15.62	216($M+$, 52), 201(9), 159(16), 148(31), 145(15), 108(100), 93(19), 91(31), 79(32), 77(30), 65(24)
3	γ-Elemene	17.72	204($M+$, 21), 161(54), 136(17), 133(31), 121(100), 107(52), 105(57), 93(67), 91(42), 41(43)
4	Furanodienone	19.84	230($M+$, 43), 215(14), 162(12), 122(100), 94(33), 91(17), 77(13), 66(13), 65(16), 41(8)
5	Curcumol	20.46	236($M+$, 28), 193(24), 147(23), 136(29), 135(28), 121(100), 107(38), 93(36), 41(31)
6	Isocurcumenol	20.99	234($M+$, 7), 191(100), 105(99), 121(83), 145(60), 173(47), 91(44), 147(40), 67(40)
7	Furanodiene	24. 65	216(M+, 48), 201(12), 159(23), 148(8), 145(21), 108(100), 91(28), 77(26), 65(12), 53(16), 41(23)
8	Germacrone	24.91	218($M+$, 13), 175(27), 136(61), 135(85), 121(30), 107(100), 105(20), 91(31), 67(42)
9	Curdione	26.53	236($M+$, 17), 180(100), 167(83), 109(53), 69(52), 68(29), 67(30), 55(33), 41(31)
10	Curcumenol	27.46	234($M+$, 26), 189(53), 147(52), 145(30), 133(53), 121(39), 119(35), 105(100), 91(37), 55(18), 41(25)
11	Neocurdione	29.27	236($M+$, 17), 180(100), 167(83), 109(81), 69(90), 68(49), 67(44), 55(49), 41(42)

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

were detected by the analysis. The recovery was calculated based on the total amount of individual investigated components. Taking into account the results of optimization and recovery experiment (data not shown), the conditions of the PLE method proposed were: solvent, methanol; temperature, 120 °C; particle size, 0.2–0.3 mm; static extraction time, 5 min; pressure, 1500 psi; static cycle, 1 and 60% of the flush volume.

3.2. Identification of sesquiterpenes in Ezhu

Total ion chromatograms of PLE extracts from three species of *Curcuma* rhizomes were shown in Fig. 1. All the main components were separated completely, and 11 of them were identified according to the mass spectra. By comparing with literature [9,17,20–28] and standards, peaks 1–11 were identified as germacrene D, curzerene, γ-elemene, furanodienone, curcumol, isocurcumenol, furanodiene, germacrone, curdione, curcumenol and neocurdione, respectively (Fig. 2). The results are summarized in Table 1.

3.3. Quantitation of sesquiterpenes in Ezhu

The selected ion monitoring (SIM) method was used for the quantification of 11 sesquiterpenes. A fragment ion m/z 121 was used for curcumol, m/z 107 for germacrone and m/z 180 for curdione. The mass spectra of neocurdione and curdione are very similar (Table 1). Therefore, the content of neocurdione was estimated using the calibration curve of curdione. The contents of identified other sesquiterpenes in *Ezhu* rhizomes was estimated by using calibration curve of germacrone, which is one of the major sesquiterpenes in *Ezhu*.

The calibration curves, which were obtained from the selected ions peak area, for curcumol, germacrone and curdione were linear over the range 2.4–124.0, 12.4–82.3 and 13.0–86.7 ng absolute on column with slope of 2.38×10^5, 2.75×10^5 and 2.67×10^5, respectively. The coefficients of correlation (r) were between 0.9995 and 0.9997. The injection precision for curcumol, germacrone and curdione was determined by injecting successively standard for six times. The relative standard deviation (R.S.D.) of curcumol, germacrone and curdione was 1.94, 1.60 and 1.40%, respectively.

The short-term (12 h) repeatability as well as the long-term (24 h) repeatability of curcumol, germacrone and curdione was calculated based on six runs. The peak area of selected ions was relatively stable. The R.S.D. of short (long)-term repeatability for curcumol, germacrone and curdione was 1.93 (2.61%), 0.94 (1.55%) and 0.49% (0.87%), respectively.

The stability of curcumol, germacrone and curdione was also determined by inject freshly prepared standard solution for three times at 0, 1, 2, 4, 8, 16 and 24 h, respectively. The R.S.D. of curcumol, germacrone and curdione was 1.99, 1.89 and 2.11%, respectively. Thus, the quantitation of sesquiterpenes such as curcumol, germacrone and curdione in *Ezhu* rhizomes could be preformed within 24 h after the sample extraction.
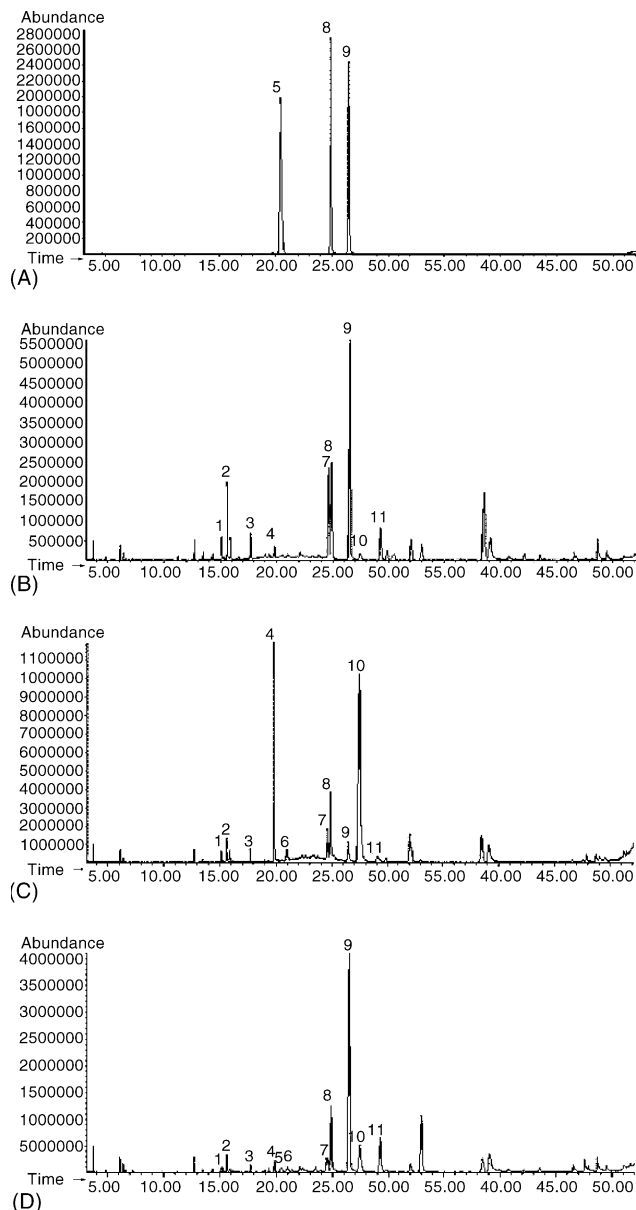

Fig. 1. GC–MS total ion chromatograms for three species of *Curcuma* used as *Ezhu*. (A) Mixture of standards, curcumol (**5**), germacrone (**8**) and curdione (**9**); (B) *C. wenyujin* derived from Yueqing, Zhejiang Province; (C) *C. phaeocaulis* derived from Tingjiang, Sichuan Province; (D) *C. kwangsiensis* derived from Guixian, Guangxi Province. (**1**) Germacrene D, (**2**) curzerene, (**3**) γ-elemene, (**4**) furanodienone, (**5**) curcumol, (**6**) isocurcumenol, (**7**) furanodiene, (**8**) germacrone, (**9**) curdione, (**10**) curcumenol, (**11**) neocurdione.

In order to validate the presented method, a known amount of curcumol, germacrone and curdione was added into the sample and extracted at specified conditions mentioned above. The extract was injected to GC–MS, and the content of the analytes was calibrated. The recovery of three tested sesquiterpenes was between 99.5 and 102.3% with R.S.D. of 1.15–2.23%, where $n = 5$.

It is difficult for GC or HPLC to identify the peaks without standards. However, it is much easer using GC–MS. The content of 11 identified sesquiterpenes in *Ezhu* rhizomes was

1. Germacrene D   2. Curzerene   3. Gamma-elemene

4. Furanodienone   5. Curcumol   6. Isocurcumenol

7. Furanodiene   8. Germacrone   9. Curdione
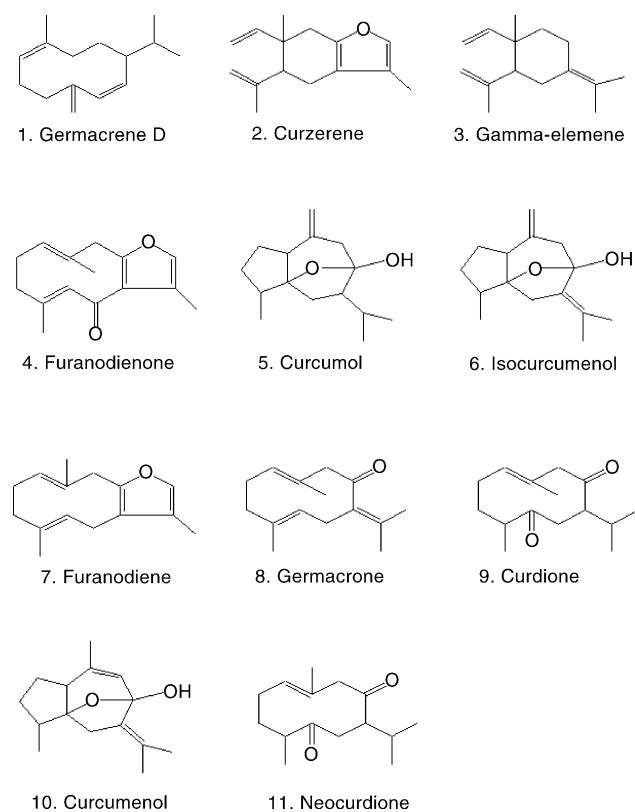
10. Curcumenol   11. Neocurdione

Fig. 2. The structures of 11 sesquiterpenes identified in *Ezhu*, three species of *Curcuma*.

determined or estimated. Table 2 shows the summary results. The data could be used for quantitatively evaluating the quality of samples, though some errors exist. The results showed the contents of 11 identified sesquiterpenes were greatly variant in different species or locations of *Curcuma* rhizomes.

3.4. Comparison of three species of Curcuma used as Ezhu

In China, *C. phaeocaulis*, *C. kwangsiensis* and *C. wenyujin* are all used as *Ezhu* [1]. However, this work showed that the chemical variation is obvious among the three species of *Curcuma*. Therefore, the exact identity is assurance of safety and efficacy of medication. In order to discriminate the three species *Curcuma*, hierarchical cluster analysis was performed based on 11 peaks' characteristics from GC profiles of different *Curcuma* samples. A method named as average linkage between groups was applied, and Squared Euclidean distance was selected as measurement. Fig. 3A shows the results on the tested 18 samples of *Curcuma*, which are divided into two main clusters, *C. phaeocaulis* and *C. wenyujin*, respectively. *C. kwangsiensis* showed the characters closed to *C. phaeocaulis* or *C. wenyujin* based on their locations. Among 11 identified sesquiterpenes, five components including furanodienone, germacrone, curdione, curcumenol and neocurdione were optimized based on cluster analysis of 11 peaks and
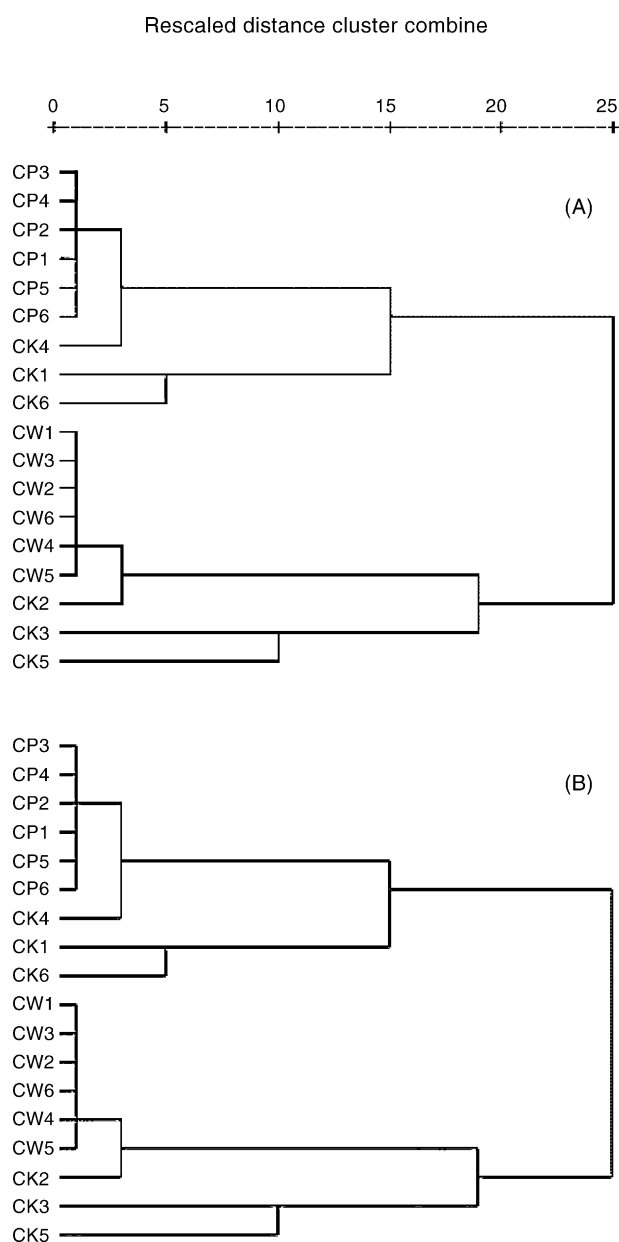

Fig. 3. Dendrograms resulting from average linkage between groups hierarchical cluster analysis. The hierarchical clustering was done by SPSS software. A method named as average linkage between groups was applied, and Squared Euclidean distance was selected as measurement. (A) Dendrogram resulting from 11 peaks, their retention times and peak area, derived from GC fingerprints of the tested 18 *Ezhu* samples. (B) Dendrogram resulting from the characteristics of five peaks, furanodienone, germacrone, curdione, curcumenol and neocurdione, derived from GC profiles of the tested 18 *Ezhu* samples. CW1–CW6 are *C. wenyujin* derived from Yueqing, Zhejiang Province, respectively. CP1–CP6 are *C. phaeocaulis* derived from Tingjiang, Jiangyuan, Sanjiang, Zhoudu, Wangdan and Shuangliu, Sichuan Province, respectively. CK1–CK6 are *C. kwangsiensis* derived from Nanning, Guixian, Wuming and Yunshan, Guangxi Province, as well as Wenshan and Malipo, Yunnan Province, respectively.

Table 2
Contents (mg/g) of 11 sesquiterpenes in three species of *Curcumae*

Samples[a]	Germacrene D[b]	Curzerene	γ-Elemene	Furanodienone	Curcumol	Isocurcumenol	Furanodiene	Germacrone	Curdione	Curcumenol	Neocurdione	Total
CW1	0.33(1.38)[c]	1.49(6.43)	0.51(2.20)	–[d]	–	–	4.36(18.82)	3.62(15.62)	11.24(48.51)	–	1.63(7.04)	23.17
CW2	0.40(1.78)	1.80(8.00)	0.59(2.62)	–	–	–	4.12(18.31)	3.56(15.82)	10.35(46.00)	0.20(0.89)	1.48(6.58)	22.50
CW3	0.26(1.08)	1.70(7.08)	0.46(1.92)	–	–	–	4.29(17.88)	3.67(15.29)	11.83(49.29)	–	1.79(7.46)	24.00
CW4	0.38(1.36)	2.28(8.13)	0.62(2.21)	–	–	–	4.60(16.40)	4.18(14.90)	13.97(49.80)	–	2.02(7.20)	28.05
CW5	0.46(1.82)	2.17(8.58)	0.67(2.65)	–	–	–	5.63(22.25)	3.92(15.49)	10.83(42.81)	–	1.62(6.40)	25.30
CW6	0.55(1.74)	2.09(6.60)	0.82(2.59)	–	–	–	5.41(17.07)	5.48(17.29)	14.94(47.14)	–	2.40(7.57)	31.69
CP1	0.10(0.80)	0.24(1.92)	0.15(1.20)	2.76(22.12)	–	0.25(2.00)	0.58(4.65)	1.26(10.10)	0.90(7.21)	6.24(50.00)	–	12.48
CP2	0.10(0.93)	0.21(1.94)	0.17(1.57)	2.87(26.57)	–	0.21(1.94)	0.50(4.63)	1.22(11.30)	–	5.52(51.11)	–	10.80
CP3	0.05(0.47)	0.11(1.03)	0.09(0.84)	2.60(24.35)	–	0.24(2.25)	0.33(3.09)	1.01(9.46)	–	6.25(58.52)	–	10.68
CP4	0.07(0.64)	0.14(1.28)	0.11(1.00)	2.70(24.64)	–	0.24(2.19)	0.40(3.65)	1.12(10.22)	–	6.18(56.39)	–	10.96
CP5	0.06(0.52)	0.12(1.03)	0.11(0.95)	2.94(25.30)	–	0.23(1.98)	0.39(3.36)	1.23(10.59)	0.58(4.99)	5.96(51.29)	–	11.62
CP6	0.07(0.59)	0.15(1.26)	0.13(1.09)	2.66(22.26)	–	0.28(2.34)	0.36(3.01)	1.27(10.63)	1.34(11.21)	5.69(47.62)	–	11.95
CK1	0.04(0.70)	0.11(1.93)	0.05(0.88)	3.70(64.80)	–	0.05(0.88)	0.31(5.43)	0.82(14.36)	0.63(11.03)	–	–	5.71
CK2	0.08(0.55)	0.35(2.40)	0.15(1.03)	0.31(2.12)	0.50(3.43)	0.15(1.03)	0.36(2.47)	2.10(14.38)	7.36(50.41)	1.60(10.96)	1.64(11.23)	14.60
CK3	0.06(4.05)	0.12(8.11)	0.09(6.08)	0.06(4.05)	0.20(13.51)	–	0.28(18.92)	0.67(45.27)	–	–	–	1.48
CK4	–	–	–	–	–	–	–	0.46(9.26)	–	4.51(90.74)	–	4.97
CK5	0.05(1.03)	0.11(2.27)	0.09(1.86)	1.64(33.88)	–	0.14(2.89)	0.14(2.89)	0.81(16.74)	1.00(20.66)	0.86(17.77)	–	4.84
CK6	0.04(1.14)	0.06(1.71)	0.07(1.99)	0.18(5.13)	–	0.08(2.28)	0.10(2.85)	1.48(42.17)	0.66(18.80)	0.77(21.94)	0.07(1.99)	3.51

[a] CW1–CW6 are *C. wenyujin* derived from Yueqing, Zhejiang Province, respectively. CP1–CP6 are *C. phaeocaulis* derived from Tingjiang, Jiangyuan, Sanjiang, Zhoudu, Wangdan and Shuangliu, Sichuan Province, respectively. CK1–CK6 are *C. kwangsiensis* derived from Nanning, Guixian, Wuming and Yunshan, Guangxi Province, as well as Wenshan and Malipo, Yunnan Province, respectively.

[b] Germacrene D, curzerene, γ-elemene furanodienone, isocurcumenol, uranodiene and curcumenol were determined as germacrone; neocurdione was determined as curdione.

[c] The data was presented as average of three replicates (R.S.D. < 3%). The percentages in 11 sesquiterpenes are shown in parenthesis.

[d] Undetected.

then principle component analysis. Using the peaks' characteristics of the five compounds, hierarchical cluster analysis was performed as before. The result was the same as the one derived from 11 peaks characteristics (Fig. 3B). Therefore, furanodienone, germacrone, curdione, curcumenol and neocurdione could be used as markers for discriminating the species and controlling the quality of *Ezhu*. It is thought that interaction of multiple chemical compounds contributes to the therapeutic effects of Chinese medicines. However, the overall clinical efficacy of these species of *Curcuma* has not been determined. Therefore, comparison of chemical components and pharmacological activities of these species of *Curcuma* is helpful to elucidate the mechanism of therapeutic effects and active components of *Ezhu*.

4. Conclusion

Absence of chemical standards is a bottleneck for quality control of Chinese medicine. Using GC–MS, it is much easer to identify the peaks of chromatograms without standards. Then, the content of identified components could be determined or estimated using an analogue as standard, which offer an approach to evaluate the quality of Chinese medicine. In this paper, the contents of 11 sesquiterpenes in three species of *Curcuma* used as *Ezhu* were identified and determined or estimated by using GC–MS coupled with PLE. The results showed that GC–MS coupled with PLE offered a simple, rapid and high sensitive method to evaluate the quality of three species of *Curcuma* rhizomes, *Ezhu*.

Acknowledgements

Appendix A. NMR data of curcumol, germacrone and curdione

A.1. Curcumol

^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 0.87(3H, d, H-15), 0.98(6H, d, H-13, 14), 1.17(1H, q, H-12), 1.50(2H, m, H-8), 1.71(2H, m, H-9), 1.75(1H, t, H-10), 1.87(1H, m, H-4), 1.90(1H, m, H-7), 2.15(2H, d, H-5), 2.54(1H, s, OH-3), 2.77(2H, d, H-2), 4.87(2H, d, H11).
^{13}C NMR (400 MHz, CDCl$_3$) δ (ppm): 144.762(1), 38.851(2), 104.521(3), 54.486(4), 34.699(5), 88.078(6), 39.378(7), 30.914(8), 28.233(9), 56.272(10), 112.787(11), 21.437(12), 28.697(13), 23.019(14), 12.326(15).

A.2. Germacrone

^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 1.44(3H, s, H-14), 1.63(3H, S, H-15), 1.73(3H, s, H-13), 1.78(3H, s, H12), 2.06–2.40(4H, m, H-2α, 2β, 3α, 3β), 2.86(1H, br d, $J = 11.2$ Hz, H-6β), 2.94(2H, m, H-6α, 9α), 3.41(1H, d, $J = 10.5$ Hz, H-9β), 4.71(1H, br d, J=8.6 Hz, H-5), 4.99(1H, brd, $J = 11.7$ Hz, H-1).
^{13}C NMR (400 MHz, CDCl$_3$) δ (ppm): 132.659 (1), 24.032 (2), 38.027 (3), 126.616 (4), 125.336 (5), 29.192 (6), 129.503 (7), 207.909 (8), 55.865 (9), 134.582 (10), 137.186(11), 22.316(12), 19.861(13), 15.548(14), 16.669(15).

A.3. Curdione, mp 61–62 °C

^{1}H NMR (400 MHz, CDCl$_3$) δ (ppm): 1.58(1H, m, H-3α), 1.66(3H, s, H-15), 1.87(1H, m, H-11), 2.09–2.14(3H, m, H-3β, 2α, 2β), 2.34(1H, m, H-4α), 2.40(1H, dd, $J = 16.6$ Hz, $J = 2.0$ Hz, H-6β), 2.69(1H, m, H-6α), 2.84(1H, d, $J = 10.9$ Hz, H-9β), 5.17(1H, br s, H-1).
^{13}C NMR (400 MHz, CDCl$_3$) δ (ppm): 131.525 (1), 26.350 (2), 33.387 (3), 46.716 (4), 211.111 (5), 44.161 (6), 53.547 (7), 214.340 (8), 55.778 (9), 129.840 (10), 29.698 (11), 21.077 (12), 19.802 (13), 18.499 (14), 16.500 (15).

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