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Short communication

Qualitative and quantitative analysis of four species of *Curcuma* rhizomes using twice development thin layer chromatography

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ABSTRACT

The rhizomes of *Curcuma phaeocaulis*, *Curcuma kwangsiensis*, *Curcuma wenyujin* and *Curcuma longa* are used as *Ezhu* or *Jianghuang* in traditional Chinese medicine for a long time. Due to their similar morphological characters, it is difficult to distinguish their origins of raw materials used in clinic. In this study, a simple, rapid and reliable twice development TLC method was developed for qualitative and quantitative analysis of the four species of *Curcuma* rhizomes. The chromatography was performed on silica gel $60F_{254}$ plate with chloroform–methanol–formic acid (80:4:0.8, v/v/v) and petroleum ether–ethyl acetate (90:10, v/v) as mobile phase for twice development. The TLC markers were colorized with 1% vanillin–H_2SO_4 solution. The four species of *Curcuma* were easily discriminated based on their characteristic TLC profiles, and simultaneous quantification of eight compounds, including bisdemethoxycurcumin, demethoxycurcumin, curcumine, curcumenol, curcumol, curdione, furanodienone and curzerene, in *Curcuma* were also performed densitometrically at $\lambda_{scan} = 518$ nm and $\lambda_{reference} = 800$ nm. The investigated compounds had good linearity ($r^2 > 0.9905$) within test ranges. Therefore, the developed TLC method can be used for quality control of *Curcuma* rhizomes.

1. Introduction

Curcuma belongs to the family Zingiberaceae. It is about 20 species distributed in China and some of which are used as traditional Chinese medicine (TCM) for a long time. Generally, the rhizomes of *Curcuma phaeocaulis*, *Curcuma kwangsiensis*, *Curcuma wenyujin* and *Curcuma longa* are used as *Ezhu* or *Jianghuang* according to Chinese Pharmacopoeia (2005 edition) [1]. However, it is difficult to distinguish their origins of raw materials in clinic because of their similar morphological characters, though their pharmacological activities [2–9] and chemical characteristics [10–13] are obviously different. Therefore, qualitative and quantitative analysis of these materials is very important for ensuring the safety and efficacy of the herbs. Up to date, GC–MS [10,11,13], HPLC [12], LC-MS [14,15], LC-MS/MS [16] and microemulsion electrokinetic chromatography (MEEKC) [17] have been used for the determination of sesquiterpenoids or curcuminoids which are usually considered as the biological active ingredients in *Ezhu* and *Jianghuang* [10,14]. Unfortunately, simultaneous determination of these compounds in *Ezhu* or *Jianghuang* has not been achieved because of the obvious difference of their volatile ability, polarity and/or poor UV absorbability [12,14]. Actually, thin layer chromatography (TLC) is an approach with low cost, easy maintenance and good selectivity of detection. In addition, TLC can analyze several parallel samples in a run. It also facilitates repeated detection (scanning) of the chromatogram with the same or different parameters. Therefore, simultaneous assay of several components in a multicomponent formulation is possible. Especially, multi-step TLC technique allows for simultaneous separation of complex mixtures [18]. In this paper, a simple twice development TLC method was developed for discrimination of the rhizomes from four species of *Curcuma*, and quantitative determination of three curcuminoids (bisdemethoxycurcumin, demethoxycurcumin and curcumine) and five sesquiterpenoids (curcumenol, curcumol, curdione, furanodienone and curzerene) was also performed.

2. Experimental

2.1. Plant materials

C. wenyujin were obtained from Yueqing (W1, W4 and W5), Rui'an (W2 and W3), and Yongjia (W6), Zhejiang Province; *C. phaeocaulis* were separately collected from Chongzhou (P1, P2 and P3),

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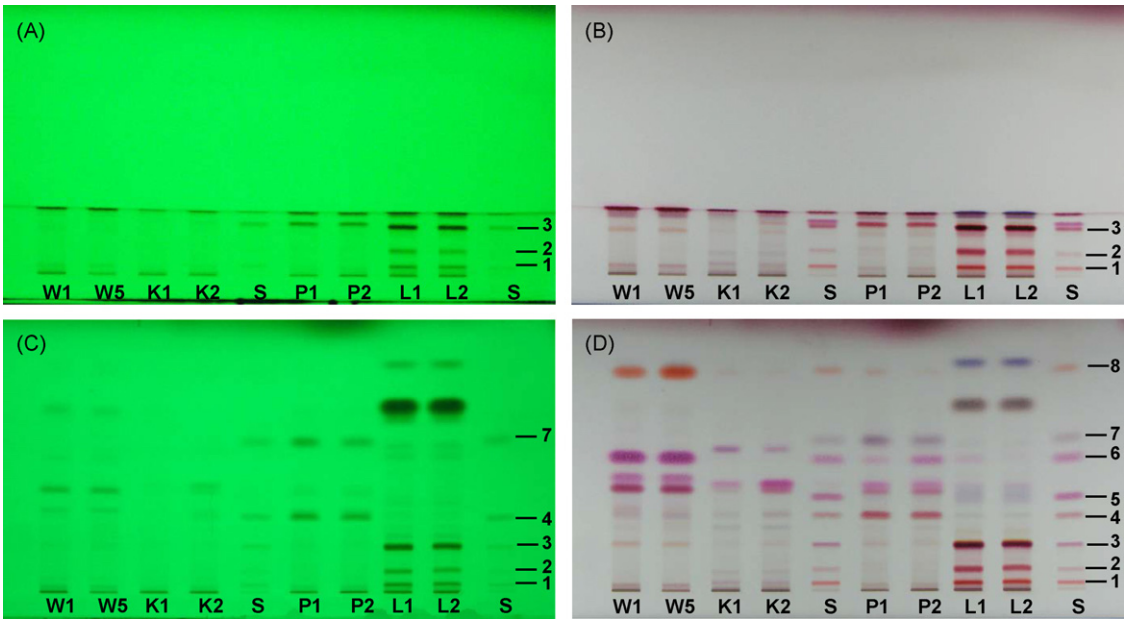

Fig. 1. Separation of mixed standards and methanol extracts of *Curcuma* using twice development on silica gel 60 F_{254} TLC plate (10 cm × 20 cm). First development with chloroform–methanol–formic acid (80:4:0.8, v/v/v): (A) viewed at λ = 254 nm without coloration and (B) colorized with 1% vanillin–H_2SO_4 solution. Second development with petroleum ether–ethyl acetate (90:10, v/v): (C) viewed at λ = 254 nm without coloration and (D) colorized with 1% vanillin–H_2SO_4 solution. W1 and W5 are *C. wenyujin* derived fromYueqing, Zhejiang Province. K1 and K2 are *C. kwangsiensis* derived from Qingzhou and Wuming of Guangxi Province, respectively. P1 and P2 are *C. phaeocaulis*, and L1 and L2 are *C. longa* derived from Chongzhou, Sichuan Province. **S**, mixed standards; **1**, bisdemethoxycurcumin; **2**, demethoxycurcumin; **3**, curcumine; **4**, curcumenol; **5**, curcumol; **6**, curdione; **7**, furanodienone; **8**, curzerene.

Zhoudu (P4), Wangdan (P5) and Shuangliu (P6), as well as *C. longa* L. rhizomes were obtained from Chongzhou (L1, L2, L4 and L5), Shuangliu (L3 and L6), Sichuan Province; *C. kwangsiensis* were collected from Qingzhou (K1 and K5), Wuming (K2, K3 and K4), and Lipu (K6) of Guangxi Province. The botanical origin of materials was identified by corresponding author and the voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China. The material was carefully cleaned and cut into slices, then dried to constant weight at 40 °C. Dried slices were powdered and then passed through a 40–120 mesh sieve.

2.2. Chemicals

All solvents and reagents (analytical grade) were obtained from UNI-CHEM d.o.o. (Belgrade, Serbia and Montenegro). Deionized water was prepared by Millipore Milli Q-Plus system (Millipore, Billerica, MA, USA). Bisdemethoxycurcumin (98%, HPLC) and demethoxycurcumin (98%, HPLC) were purchased from Chengdu Must Biological Technology Limited Company (Sichuan, China). Curcumine (99%, HPLC) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Curcumenol, curcumol, curdione, furanodienone and

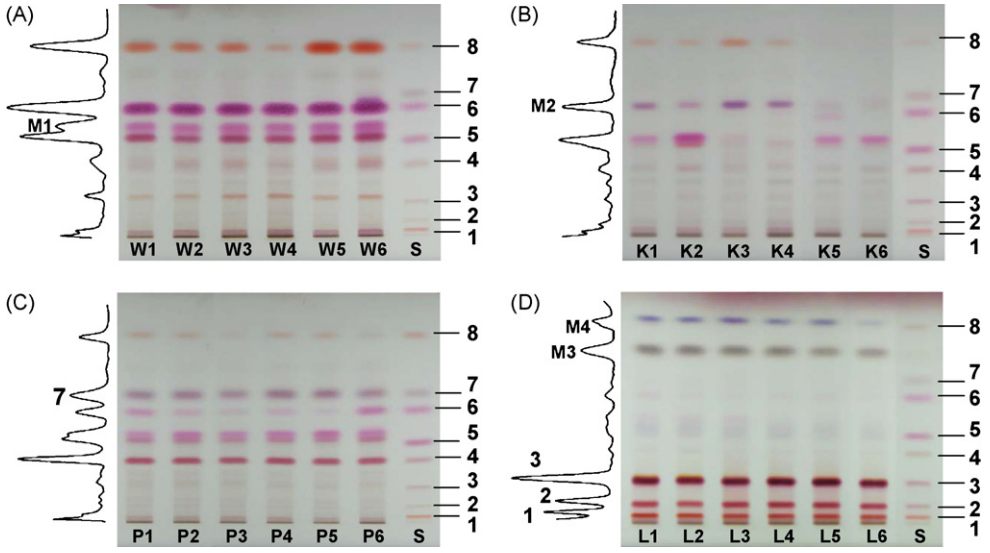

Fig. 2. TLC photographs of four species of *Curcuma* and their specific markers (M1–M4) besides the eight investigated compounds: (A) *C. wenyujin* from Yueqing (W1, W4 and W5), Rui'an (W2 and W3) and Yongjia (W6), Zhejiang Province; (B) *C. kwangsiensis* from Qingzhou (K1 and K5), Wuming (K2, K3 and K4) and Lipu (K6), Guangxi Province; (C) *C. phaeocaulis* from Chongzhou (P1, P2 and P3), Zhoudu (P4), Wangdan (P5) and Shuanliu (P6), Sichuan Province; (D) *C. longa* from Chongzhou (L1, L2, L4 and L5) and Shuanliu (L3 and L6), Sichuan Province. **S**, mixed standards. **1–8**, eight compounds same as in Fig. 1.

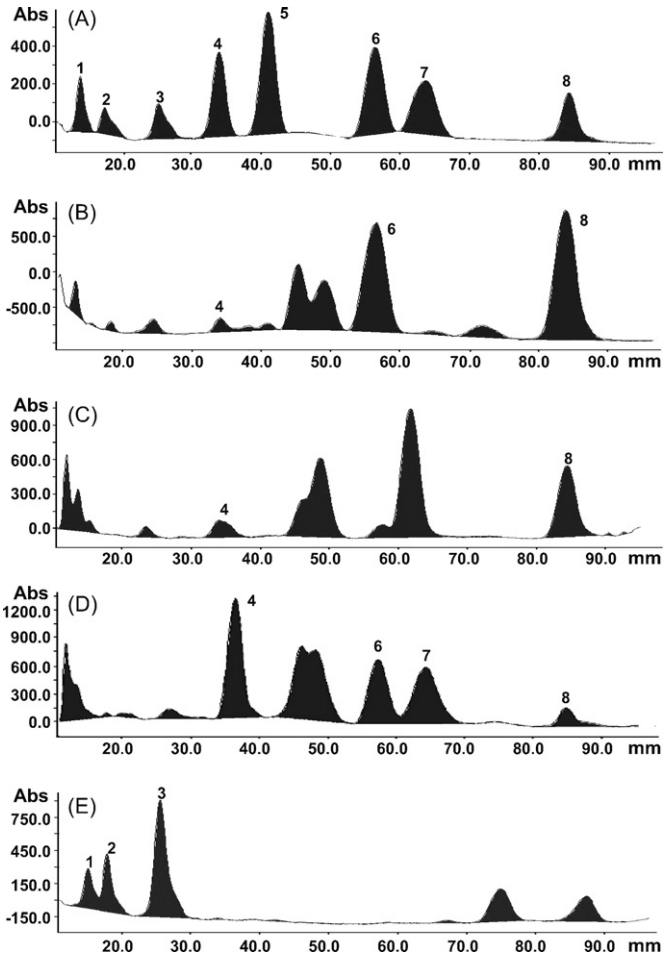

Fig. 3. Typical TLC densitograms of mixed standards (A), methanol extracts of *C. wenyujin* (B), *C. kwangsiensis* (C), *C. phaeocaulis* (D) and *C. longa* (E).

curzerene were separated from commercial oil of *C. wenyujin* in our lab, and the structures were confirmed by their UV, MS and NMR data, purity of all compounds were >99% measured by HPLC and/or GC [11].

2.3. Standard and sample preparation

Mixed standards solution was obtained by mixing equal volume of the stock solutions of bisdemethoxycurcumin (740 μg/mL), demethoxycurcumin (1020 μg/mL), curcumine (760 μg/mL), curcumenol (1955 μg/mL), curcumol (1236 μg/mL), curdione (835 μg/mL), furanodienone (2620 μg/mL) and curzerene (3840 μg/mL) in methanol.

Approximately 500 mg dried rhizome powder, accurately weighed, was mixed with 5 mL methanol in a sealed tube. The solution was treated in an ultrasonic clean bath (881 w, 43 kHz, Bransonic, Danbury, CT) for 60 min, at room temperature (25 ± 2 °C). Then methanol was added to compensate for the lost weight during the extraction. After centrifugation in an Allegra X-15R refrigerated centrifuge (Beckman Coulter, Fullerton, CA) for 10 min (at 4500 rpm), the supernatant was collected for analysis.

2.4. TLC

Chromatography was performed on silica gel $60F_{254}$ TLC plates (Merck, Darmstadt, Germany), and a HPTLC system (Desaga GmbH, Germany) including AS30 HPTLC Applicator, CD 60 HPTLC densitometer with Pro Quant Windows software. Mixed standards (5 μL) and samples (8 μL) of four species of *Curcuma* were spotted in duplicate on the plate as bands 9 mm wide, 7 mm apart and 10 mm from the bottom edge, respectively. First, the plate was developed to a distance of 30 mm with chloroform–methanol–formic acid (80:4:0.8, v/v/v) in a Desaga 20 cm × 20 cm glass flat-bottom chamber after equilibration with mobile phase vapor for 30 min. The developed plate was dried in an aerator at room temperature, and then further developed to the distance of 90 mm with petroleum ether–ethyl acetate (90:10, v/v) in another chamber after equilibration with the same solution vapor. The developed plate was viewed sequentially under UV 254 and 365 nm and then colorized with vanillin–H_2SO_4 solution (1% vanillin dissolved in 100 mL 70% H_2SO_4) and heated at 105 °C on a YOKO-XR plate heater (Wuhan YOKO technology Ltd., China) to make spots colored clearly. The treated plate covered with a transparent glass and scanned at λ_s (scan wavelength) = 518 nm and λ_r (reference wavelength) = 800 nm in reflectance–extinction mode by use of the densitometer. The source of radiation was a tungsten/deuterium lamp. The slit dimensions were 6.00 nm × 0.02 nm.

3. Results and discussion

3.1. Optimization of method

Both mobile phases and developing mode were optimized to obtain good separation. Chloroform–methanol–formic acid (80:4:0.8 or 96:4:0.1, v/v/v), petroleum ether–ethyl acetate (90:10 or 15:2, v/v) [1,19,20], methanol–chloroform–acetone (5:4:1, v/v/v), petroleum ether–ethyl acetate (8:2, v/v) [21] and toluene–ethyl acetate–glacial acetic acid (66:32:2, v/v/v) [22] were not available for simultaneous separation of low- and high-polarity compounds in *Curcuma* with good resolution in one run. Thus, twice development was employed. The optimum development reagents are: chloroform–methanol–formic acid (80:4:0.8, v/v/v) for the first development and petroleum ether–ethyl acetate (90:10, v/v) for the second development. As the results, the high-polarity compounds such as bisdemethoxycurcumin (**1**), demethoxycurcumin (**2**) and curcumine (**3**) were separated, while the low-polarity compounds were developed as one main band in the first run because of the high polarity of mobile phase. Then the low-polarity components, including curcumenol (**4**), curcumol (**5**), curdione (**6**), furanodienone (**7**), curzerene (**8**), were further resolved using low-polarity mobile phase in the second run, while the high-polarity compounds could not be driven. Finally, eight investigated compounds and the components in four species of *Curcuma* rhizomes were well separated in twice development (Fig. 1).

The detection wavelengths for quantification of the investigated compounds were selected based on their absorption spectra. The maximum absorption of compounds **4**–**7** were nearby 518 nm, and that of compound **8** was at 508 nm, while compounds **1**–**3** showed constant absorption between 500 and 600 nm. Considering the sensitivity for all investigated compounds, 518 nm was selected for detection.

3.2. Validation of the method

Mixed standards solutions at ten amounts (0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6, 6.4, 7.2 and 8.0 μL) were applied for linearity test. Calibration curves were constructed by plotting the peak areas versus amount of each analyte. The investigated compounds had good linearity ($r^2 > 0.9905$) within test ranges.

The limits of detection (LOD) and limits of quantification (LOQ) were defined as the amounts of which the signal-to-noise ratios

Table 1
The contents (mg/g) of eight compounds in four species of *Curcuma*

Samples[a]	Analytes [mean (RD%[b], $n = 2$)]							
	Bisdemethoxycurcumin	Demethoxycurcumin	Curcumine	Curcumenol	Curcumol	Curdione	Furanodienone	Curzerene
W1	ND[c]	ND	ND	0.29 (1.86)	ND	7.02 (2.88)	ND	21.08 (2.45)
W2	ND	ND	ND	0.75 (1.31)	ND	7.90 (0.42)	ND	19.01 (1.60)
W3	ND	ND	ND	0.80 (3.67)	ND	8.77 (1.34)	ND	20.61 (3.70)
W4	ND	ND	ND	0.69 (0.71)	ND	8.26 (2.09)	ND	6.43 (1.56)
W5	ND	ND	ND	1.03 (1.88)	ND	11.45 (0.93)	ND	85.28 (2.63)
W6	ND	ND	ND	1.95 (0.16)	ND	13.89 (1.19)	ND	79.08 (0.62)
P1	ND	ND	ND	4.53 (2.12)	ND	0.58 (2.26)	3.88 (4.23)	2.74 (0.26)
P2	ND	ND	ND	4.43 (0.17)	ND	0.33 (0.94)	4.36 (1.08)	2.61 (0.76)
P3	ND	ND	ND	4.44 (2.86)	ND	0.19 (0.69)	3.71 (0.32)	1.73 (0.43)
P4	ND	ND	ND	5.69 (2.22)	ND	0.29 (2.96)	5.39 (1.39)	2.49 (2.33)
P5	ND	ND	ND	4.53 (0.02)	ND	0.17 (2.54)	5.84 (2.25)	2.65 (0.72)
P6	ND	ND	ND	4.73 (0.76)	ND	1.51 (0.18)	5.49 (0.94)	2.09 (0.84)
K1	ND	ND	ND	0.27 (0.19)	ND	ND	ND	3.16 (2.13)
K2	ND	ND	ND	0.72 (1.96)	ND	ND	ND	2.70 (3.35)
K3	ND	ND	ND	+[d]	ND	ND	ND	3.44 (0.59)
K4	ND	ND	ND	ND	ND	ND	ND	3.62 (3.07)
K5	ND	ND	ND	4.37 (0.12)	ND	1.30 (0.03)	ND	+
K6	ND	ND	ND	0.58 (3.79)	ND	ND	ND	+
L1	4.85 (0.06)	8.83 (0.08)	14.34 (0.59)	ND	ND	+	ND	ND
L2	5.48 (0.04)	10.93 (0.17)	14.62 (0.29)	ND	ND	+	ND	ND
L3	4.05 (0.54)	11.32 (0.32)	14.41 (0.42)	ND	ND	+	ND	ND
L4	4.50 (0.15)	14.02 (0.88)	18.31 (0.73)	ND	ND	+	ND	ND
L5	2.61 (0.06)	8.10 (0.05)	18.14 (1.24)	ND	ND	+	ND	ND
L6	4.86 (0.09)	12.72 (0.19)	15.74 (0.69)	ND	ND	+	ND	ND

[a] W1–W6, *C. wenyujin* derived from Yueqing (W1, W4 and W5), Rui'an (W2 and W3), Yongjia (W6), Zhejiang Province; P1–P6, *C. phaeocaulis* derived from Chongzhou (P1, P2 and P3), Zhoudu (P4), Wangdan (P5) and Shuangliu (P6), Sichuan Province; K1–K6, *C. kwangsiensis* derived from Qingzhou (K1 and K5), Wuming (K2, K3 and K4) and Lipu (K6), Guangxi Province; L1–L6, *C. longa* derived from Chongzhou (L1, L2, L4 and L5) and Shuangliu (L3 and L6), Sichuan Province.
[b] RD, relative deviation (%) = (|measured value − mean|/mean) × 100.
[c] Not detected.
[d] Under the limit of quantification.

(S/N, noise was defined as the peak area corresponding to the solution containing none of the constituents) were about 3:1 and 10:1, respectively. The LOD and LOQ values of eight investigated compounds were between 5–746 and 21–1144 ng, respectively.

The precision was expressed as instrumental precision, identical and different plate precision. Instrumental precision was checked by scanning the same spot of the investigated compound in mixed standards solution (5 μL) six times, and the overall RSDs were less than 2.67% ($n = 6$). Identical plate precision was determined by analyzing six spots of the mixed standards solution (5 μL) on one plate, while different plate precision was tested by determining one spot of the mixed standards solution (5 μL) per plate on six TLC plates. The overall RSDs of identical and different plate were less than 4.19% ($n = 6$) and 8.73% ($n = 6$), respectively.

The repeatability was evaluated by preparing and analyzing six solutions of the same sample (500 mg each). *C. wenyujin* (for curzerene), *C. phaeocaulis* (for curcumenol, curdione and furanodienone) and *C. longa* (for bisdemethoxycurcumin, demethoxycurcumin and curcumine) were used because no sample contained all investigated compounds. One spot of each solution was analyzed on the same plate, and RSDs of the investigated compounds were calculated, which were between 2.08 and 4.84%.

The accuracy of the method was evaluated as extraction recovery and detection recovery. The extraction recovery was determined by performing consecutive extractions of the same sample for three times and the investigated compounds were detected by TLC analysis. The recovery was calculated based on the total amount of individual investigated components, and was 86.75–99.01% with RSD of 0.16–3.71% ($n = 6$).

The detection recovery was determined at three levels of mixed standard solutions, i.e., 80% (low), 100% (middle) and 120% (high) of the middle amount of the linear range of each investigated compound. The recovery (R, %) was calculated as follows: R (%) = $[A_m/A_s] \times 100\%$, where A_m is the measured amount and A_s is the sampling amount. The overall recoveries were 83.04–116.46% with RSD of 0.24–3.90% ($n = 3$).

3.3. Discrimination of four species of Curcuma

Six samples of each species of *Curcuma* collected from different places were investigated, and their TLC profiles were shown in Fig. 2. It is obvious that *Jianghuang* is different from the three species, *C. wenyujin*, *C. kwangsiensis* and *C. phaeocaulis*, used as *Ezhu* based on the bands of bisdemethoxycurcumin (**1**), demethoxycurcumin (**2**), curcumine (**3**), M3 and M4 (Fig. 1D, Fig. 2). Among three species of *Curcuma* used as *Ezhu*, each has its individual characters, such as an orchid band at R_F 0.48 (M1) in *C. wenyujin*, a purple band at R_F 0.62 (M2) in *C. kwangsiensis*, and a reddish blue-violet band at R_F 0.66 (furanodienone, **7**) in *C. phaeocaulis*. Therefore, TLC could rapidly and easily discriminate the rhizomes of *C. wenyujin*, *C. kwangsiensis*, *C. phaeocaulis* and *C. longa*.

3.4. Determination of eight investigated compounds in Curcuma

The contents of eight investigated compounds in four species of *Curcuma* were determined using TLC scanning (TLCs). Their TLCs profiles were shown in Fig. 3. Table 1 shows the summary results. Generally, curcumol is rare in all of four species of *Curcuma* rhizomes though it is usually considered as the active component in volatile oil of *Ezhu* [23], which is in accordance with the previous report in our lab [10–12,24]. In addition, curcumenol and curzerene were found in three species of *Curcuma* used as *Ezhu* but not in *C. longa*. Three curcuminoids are mainly contained in *C. longa*. The highest content of curcumenol was determined in rhizomes of *C. phaeocaulis*, while highest content of curzerene existed in the samples of *C. wenyujin* [24].

4. Conclusion

A simple and reliable TLC method using twice development was developed for rapid qualitative and quantitative analysis of four species of *Curcuma* rhizomes. Eight compounds, including five sesquiterpenoids and three curcuminoids, in *Curcuma* were simultaneously determined. The developed TLC method can be used as an economical alternative method for routine quality control of *Curcuma* rhizomes.

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