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Metabolic Profiling Assisted Quality Control of Phorbolesters in *Jatropha curcas* Seed by High-Performance Liquid Chromatography Using a Fused-Core Column

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Supporting Information

ABSTRACT: In this work, fast and sensitive high-performance liquid chromatography (HPLC) coupled with multivariate analysis was utilized to evaluate the metabolic profiling of *Jatropha curcas* seed and screen the marker compounds of phorbolesters (PEs), which significantly contributed to the metabolic profiling for quality control of PEs in *J. curcas* seed. Thirty-two peaks were separated and detected in *J. curcas* seed on a fused-core C_{18} column between 2 and 25 min. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) of the chromatographic data demonstrated that 12 batches of *J. curcas* seed could be well-differentiated and categorized into 5 groups. Especially, one sample obtained from Lijiang Yunan was significantly different from the others. Partial least-squares discriminant analysis (PLS-DA) showed that the metabolic profiling differentiation might be explained by 9 components including *J.*factors C_1−C_5 of PEs, peak 21 (similar to *J.*factor C_1) and 3 peaks (peaks 1, 9, and 10) significantly different in retention time from the PEs. The observation that the content levels of *J.*factors C_1 and C_2 plus peak 21 (equivalent to *J.*factor C_1) were more relevant to the multivariate chromatographic data than the ones of *J.*factors C_3−C_5 was confirmed by the PLS prediction models. The results of the present study indicated not only that *J.*factors C_1 and C_2 were the more rational markers representing the comprehensive quality of PEs in *J. curcas* seed but also that peak 21 (similar to *J.*factor C_1) was a rational marker, too.

KEYWORDS: *metabolic profiling, phorbolesters, Jatropha curcas L., high-performance liquid chromatography, fused-core column, quality control*

INTRODUCTION

Jatropha curcas L. has become a famous natural resource plant for the production of biodiesel, animal feed, biopesticide, medicine, and traditional medicine.[1−5] However, the toxins presented in *J. curcas* seed, especially the phorbolesters (PEs), prevent these utilizations. Moreover, recently published papers have demonstrated the PEs display excellent bioactivity in pest control, such as against schistosomiasis,[6] and antimolluscicidal/antimicrobial activities,[7] in which PEs could be valued coproducts contributing to enhance the economic viability and sustainability of the *J. curcas* production chain. Thus, to determine whether or not to utilize the values of PEs, it is necessary to establish a fast and accurate method for the quality control of PEs in *J. curcas* seed. Six main PEs were isolated and identified as Jatropha factors C_1−C_6 belonging to 12-deoxy-16-hydroxyphorbol diesters by Hass.[8] However, due to a lack of reference standards of PEs, the content levels of PEs in *J. curcas* seed or their related products are always calculated by the total amount equivalent to phorbol 12-myristate-13-acetate (TPA) using an internal standard method.[9−13] Even though the content levels of PEs in PE-rich extracts are calculated by the total amount equivalent to the *J.*factor C_1 (a main constituent of PEs in *J. curcas* seed) in a recently published paper,[6] the accurate content levels of the PEs in *J. curcas* seed have not been investigated. Meanwhile, researchers have realized that calculating the content levels of PEs in *J. curcas* seed or their related products by the total amount equivalent to TPA significantly overestimates the content levels of PEs at 280 nm,[6] mainly because the λ_{max} of UV absorbance of TPA is at 242 nm.[11]

Metabolic profiling assisted quality control of complex samples such as herbs has exhibited much more powerful capabilities in multivariate analysis.[14,15] This work was to establish a fast and sensitive HPLC method packed with a fused-core column for evaluating the metabolic profiling of *J. curcas* seed including PEs from different regions of China. Another aim of the present work was to screen the marker compounds of PEs that significantly contribute to the metabolic profiling for quality control of PEs in *J. curcas* seed.

MATERIALS AND METHODS

Materials. Methanol and acetonitrile (HPLC grade) were purchased from Dikma Technologies (Beijing, China). Formic acid (HPLC grade) was purchased from the Chengdu Reagent Co. (Chengdu, China). Water (HPLC grade) was prepared using an

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Table 1. Content Levels of *J.*Factors C_1−C_5 in 12 Batches of *J. curcas* Seed ($n = 3$)

batch[a]	content level (μg g^{-1}, mean ± SD)								PLS-DA categorization
	*J.*factor C_1	*J.*factor C_2	*J.*factor C_3	*J.*factor C_4	*J.*factor C_5	sum[b]	sum (TPA)[c]	peak 21[d]	
A	51.3 ± 3.2	19.2 ± 0.2	6.2 ± 0.3	6.5 ± 0.2	7.2 ± 0.2	90.4 ± 3.9	1968.7 ± 75.3	9.8 ± 0.8	group 4
B	42.3 ± 0.9	15.8 ± 0.3	4.9 ± 0.3	5.4 ± 0.6	9.4 ± 6.0	77.8 ± 5.4	1732.9 ± 180.7	6.8 ± 0.2	group 5
C	44.7 ± 0.5	14.3 ± 0.2	6.4 ± 0.5	5.4 ± 1.3	5.9 ± 1.0	76.7 ± 3.3	1672.4 ± 89.1	10.0 ± 0.4	group 5
D	37.5 ± 0.3	11.6 ± 0.4	4.3 ± 0.0	3.5 ± 0.2	4.5 ± 1.0	61.4 ± 0.4	1329.5 ± 14.8	5.4 ± 0.1	group 5
E	23.3 ± 0.7	8.9 ± 0.1	2.2 ± 0.0	2.5 ± 0.1	2.7 ± 0.1	39.5 ± 0.9	871.0 ± 17.3	4.3 ± 0.1	group 3
F	52.1 ± 0.9	20.8 ± 0.8	7.2 ± 0.3	7.0 ± 0.4	5.8 ± 0.3	92.8 ± 2.3	2017.7 ± 51.9	7.9 ± 0.0	group 4
G	41.5 ± 1.4	13.5 ± 0.3	5.1 ± 0.3	3.6 ± 0.3	4.5 ± 0.3	68.1 ± 2.1	1466.6 ± 43.7	14.1 ± 0.7	group 2
H	38.4 ± 3.0	12.0 ± 0.4	4.2 ± 0.2	3.7 ± 0.4	4.9 ± 0.5	63.3 ± 4.3	1370.6 ± 89.5	6.6 ± 0.2	group 5
I	23.6 ± 2.4	8.9 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	1.9 ± 0.2	37.9 ± 3.2	820.0 ± 63.6	45.9 ± 2.4	group 1
J	48.2 ± 1.5	15.0 ± 0.4	5.9 ± 0.8	5.1 ± 0.1	5.8 ± 0.0	80.0 ± 1.3	1725.5 ± 28.4	5.9 ± 1.0	group 5
K	56.3 ± 1.0	19.7 ± 0.9	7.2 ± 0.6	6.6 ± 0.4	6.1 ± 0.3	95.9 ± 2.6	2064.1 ± 60.9	6.7 ± 0.2	group 4
L	53.6 ± 0.4	19.2 ± 0.0	7.0 ± 0.7	6.2 ± 0.2	6.4 ± 0.1	92.3 ± 0.4	1998.4 ± 13.2	7.9 ± 0.0	group 4
mean	42.7	14.9	5.2	4.8	5.4	79.0	1586.5	10.9	
SD	10.8	7.1	1.8	1.7	2.0	19.7	426.9	11.3	
variation (%)	25.3	27.9	35.1	36.4	36.1	27.0	26.9	103.4	

[a]A, from Xichang, Sichuan, in 2010; B, from Miyi, Sichuan, in 2010; C, from Yanbian, Sichuan, in 2010; D, from Panzhihua, Sichuan, in 2010; E, from Jinsha, Guizhou, in 2010; F, from Binchuan, Yunnan, in 2010; G, from Yuanmou, Yunnan, in 2010; H, from Yongren, Yunnan, in 2010; I, from Lijiang, Yunnan ,in 2010; J, from Sanya, Hainan, in 2011; K, from Haikou, Hainan, in 2011; L, from Nanning, Guangxi, in 2011. [b]Sum, total content level of *J.*factors C_1−C_5. [c]Sum(TPA), total content level of *J.*factors C_1−C_5 equivalent to TPA. [d]Peak 21, content level of peak 21 equivalent to *J.*factor C_1.

ultrapure water system (UPA, Chongqing, China). All of the other reagents used in the present study were of analytical grade. TPA (99%, CAS Registry No. 16561-29-8) was obtained from Sigma-Aldrich (Shanghai, China). *J.*factors C_1−C_5 were isolated by the authors and identified by comparison with the literature.[6,8]

Sample Preparations. Twelve batches of *J. curcas* seed identified in footnote *a* of Table 1 were cultivated from 12 different regions of China. Each batch of sample was prepared in triplicates. Fine powder of dried seed (2.50 ± 0.10 g) was placed in a 25 mL volumetric flask to which 20 mL of methanol was added. The mixture was treated by an ultrasound processor (Transsonic KH-300DB, Kunshan Hechuang Ultrasonic Instrument Co., Ltd., Kunshan, China) for 30 min at 40 °C after cold maceration for 1 h at room temperature. The sample was cooled to room temperature, and 5 mL of methanol was added for a total volume of 25 mL. Each sample was filtered through a 0.20 μm nylon syringe filter (Jinteng). One milliliter aliquots from each sample were combined in a representative pooled sample as the quality control (QC) sample.[14,15] An aliquot of 20 μL of sample was injected into the column for analysis.

HPLC Analysis for Metabolic Profiling Analysis of *J. curcas* Seed. HPLC analyses were performed on an Agilent 1200 series system (Agilent Technologies) equipped with a quaternary pump, a vacuum degasser, an autoinjector, and an ultraviolet detector. The HPLC separation was performed on a fused-core column (Halo-C_{18}, 4.6 × 150 mm, 2.7 μm, Advanced Materials Technology, Inc.) at 30 °C. The ultraviolet detector was set at 280 nm according to the λ_{max} of UV absorbance of PEs.[6,8] All of the samples were eluted at 1.00 mL min^{-1} using an isocratic elution of acetonitrile and water containing 0.2% formic acid (v/v, 72/28). Agilent Chemstation version B.04.01 was used for the system control and data acquisition. The QC sample was injected three times at the beginning of the analytical run to precondition the system and inserted every four to five samples to monitor the potential system drift of the analytical run.[15]

Data Processing and Multivariate Analysis. Data processing and multivariate analysis procedures were similar to those in our previously published paper.[15] Briefly, the chromatographic data were acquired by the Agilent Chemstation for LC systems (version B.04.01). The parameters used for peak integration were retention time (RT) of 2−25 min, slope sensitivity of 1, width of 0.02 min, minimum area of 5.0, minimum height of 0.5, advanced baseline calibration mode, and vertical shoulder peak mode. No specific peak was excluded. The resulting data set containing an arbitrarily assigned peak index along the elution time, retention time, and peak area was exported to SIMCA-P+ software 12.0 (Umetrics) for multivariate analysis. Pareto scaling was performed for pretreating the data sets. Principal component analysis (PCA) was initially used to visualize general clustering, trends, and outliers among the observations. Hierarchical cluster analysis (HCA) of the PCA scores was used to generate a dendrogram of the 12 batches of *J. curcas* seed. Thereafter, partial least-squares discrimination analysis (PLS-DA) was carried out. Variables with the higher loading values in the PLS-DA loadings plot may be regarded as the marker components that contribute significantly to the categorization of *J. curcas* seed.

Isolation and Purification of *J* Factors C_1−C_5. Dry crushed *J. curcas* seed (2.10 kg) was extracted two times at room temperature for 24 h with redistilled ethyl acetate (20 L). The extract solution was washed three times with ultrapure water (20 L). Finally, the ethyl acetate extract was concentrated using a rotary evaporator under reduced pressure (50 °C). Ethyl acetate crude extract (oily mixture, 820 mL) was divided into four equal portions. Each was subjected to open column chromatography (silica gel, 500 g). The column was washed successively with petroleum ether (8000 mL), petroleum ether/normal hexane (1:1, v/v, 8000 mL), normal hexane (8000 mL), normal hexane/ethyl acetate (3:1, v/v, 8000 mL), and normal hexane/ethyl acetate (1:1, v/v, 8000 mL). After that, the elution mixture (320.9 mg) rich in PEs was eluted with normal hexane/ethyl acetate (1:3, v/v, 8000 mL). Five major PEs were found in the elution mixture using HPLC-UV, which was consistent with the previous literature.[6,8] Purification of PEs was performed by subjecting the elution mixture to reversed phase HPLC (Welchrom-C_8, 10 × 250 mm, 5 μm) with 3 mL min^{-1} of mobile phase (acetonitrile/water containing 0.2% formic acid, v/v, 80/20) yielding compounds **1** (RT, 37.9 min), **2** (RT, 45.4 min), **3** (RT, 52.4 min), **4** (RT, 56.0 min), and **5** (RT, 63.8 min). Compounds **3**−**5** were further purified by semipreparative HPLC as above. MS spectra of these five compounds were measured in the positive ion mode ($[M + Na]^+$) using a microTOF-QII mass spectrometer (Bruker Daltonics, Germany). The molecular MS at m/z ($[M + Na]^+$) of compounds **1**−**5** are 733.3701, 733.3642, 733.3701, 733.3695, and 733.3702, respectively.

Compounds **1**−**3** are identified as *J.*factors C_1−C_3, respectively, thanks to their MS (Supporting Information, Supplementary Figure

1S) and UV spectra and comparison of the data from the literature.[6,8] Similarly, compounds **4** and **5** are deduced as *J.*factors C_4 and C_5 according to a recently published paper.[6] The purities of *J.*factors C_1–C_5 are checked as 99.957, 98.585, 97.416, 95.645, and 94.919%, respectively, by HPLC-UV using an area normalization method.

Measurement of the Content Levels of *J.*Factors C_1–C_5 in *J. curcas* Seed. HPLC analysis of *J.*factors C_1–C_5 and the sample preparation procedure were consistent with the metabolic profiling analysis. Isolated standard references of *J.*factors C_1–C_5 were accurately weighed and dissolved in methanol into mixed standard solution, which was consecutively diluted into a series of mixed working standard solutions (concentrations of 0.53–33.60, 0.32–20.16, 0.20–12.48, 0.19–12.32, and 0.20–12.72 μg/mL for *J.*factors C_1–C_5, respectively) by serial dilution method. Then, the mixed working standard solutions at seven different concentrations were injected in triplicates. The data of peak area versus the corresponding concentration were treated using linear least-squares regression analysis. The mixed working standard solution was further diluted to a certain concentration to explore the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ were determined by signal-to-noise (S/N) ratios of 3 and 10, respectively.

The intra- and interday precisions were determined by continuously injecting the mixed standard solutions at three levels for five replicates on the same day and three consecutive days, respectively. The mixed standard solutions at three levels were separately tested at 0, 2, 4, 8, 12, and 24 h for assessing stability. As for the repeatability, sample solution from identical batch samples was prepared and detected in six parallels. The recovery test for reflecting accuracy was done by the standard addition approach. Accurate amounts of mixed working standard solutions at three levels were added to identical batch sample with five parallels. The recovery was figured out according to the following formula: recovery (%) = (amount detected − original amount)/amount spiked × 100%, and RSD (%) = (standard deviation of recoveries/mean of recoveries) × 100%.

RESULTS AND DISCUSSION

HPLC Method Development. The duration of a single analytical period for the separation and determination of PEs equivalent to TPA or *J.*factor C_1 was commonly >60 min to achieve considerable separation using 1.3 mL min^{-1} of gradient elution composed of (A) 1.75 mL of 85% orthophosphoric acid in 1 L of water and (B) acetonitrile on a common C_{18} column with a 5 μm particle size.[6,10,12,13,16] To improve the HPLC separation condition of PEs, five different columns were evaluated in the present work and compared with the literature.[12,13,16] Both the sample preparation and detection conditions were identical to those from the literature.[12,13] However, the mobile phases were different among columns. Briefly, 1.3 mL min^{-1} of gradient elution from the literature[12,13] was used for column A (Ultimate XB-C_{18}, 4.6 × 250 mm, 5 μm), 1.0 mL min^{-1} of isocratic elutions with different ratios (v/v, 80:20, 72:28, 75:25, and 80:20) of solvents A (acetonitrile) and B (2.0 mL formic acid in 1 L water) were used for columns B–E (B, Welchrom-C_{18}, 4.6 × 150 mm, 5 μm; C, Halo-C_{18}, 4.6 × 150 mm, 2.7 μm; D, Welchrom-C_8, 4.6 × 150 mm, 5 μm; E, Chromstar-C_8, 4.6 × 150 mm, 3.5 μm), respectively. Figure 1 clearly shows that columns C and E provide better separation of these compounds than other types of columns.

In this work, an isocratic elution performed on a fused-core column (column C, Halo-C_{18}, 4.6 × 150 mm, 2.7 μm) was developed and optimized to guarantee the highest chromatographic resolution. The chromatograms of the 12 batches of *J. curcas* seed are illustrated in Figure 2. Thirty-two peaks are well separated and detected from 2 to 25 min (Figure 2). The present HPLC method exhibits a much more powerful separation capability compared with previously published methods.[6,10–13,16] Peaks 22, 28, 30, 31, and 32 are identified as *J.*factors C_1–C_5, respectively, by comparison with the isolated standards. By comparison with the mobile phase from the literature,[6,10,12,13,16] formic acid instead of H_3PO_4 in the aqueous solution (2.0 mL of formic acid in 1 L of water) also exhibits excellent separation capability with the best tailing factor (0.95–1.05). Under the present optimized chromatographic conditions, peaks of *J.*factors C_1–C_5 appear between 13.00 and 22.00 min. Furthermore, this HPLC method was also applied to determine the PEs equivalent to TPA using an internal standard method similar to the literature,[9–13,16] and the duration of the analytical period was reduced to 35 min (Figure 3).

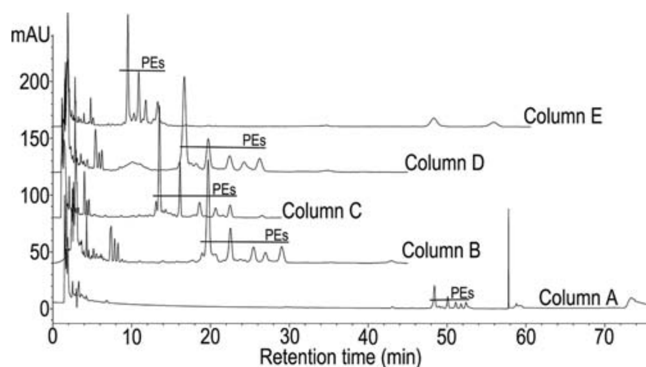

Figure 1. Typical HPLC chromatograms for separation of PEs in *J. curcas* seed with five different columns.

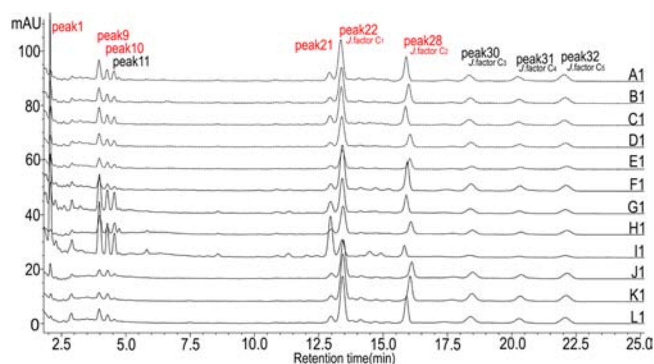

Figure 2. Typical HPLC chromatograms at 280 nm of the first samples of the triplicates of the 12 batches (A–L) of *J. curcas* seed.

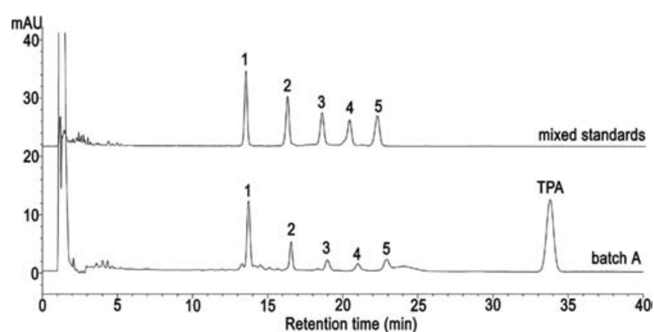

Figure 3. Typical HPLC chromatograms of the mixed standard solution including *J.*factors C_1–C_5 (4.20, 2.52, 1.56, 1.54, and 1.59 μg mL^{-1}) at 280 nm and batch A of *J. curcas* seed spiked with TPA (internal standard, 150.20 μg mL^{-1}). Peaks 1–5 represent *J.*factors C_1–C_5, respectively.

Validation of the Analytical Run. The instrumental shifts during the analytical run were monitored by a widely used pooled sample strategy.[14,15] The plot of the data set of PCA scores, including QC samples, is illustrated in Figure 4. R2X (cumulative) and Q2 (cumulative) of the PCA model are 0.955 and 0.871. All QC samples cluster well in the scores plot, indicating that the present method is robust throughout the analytical run.

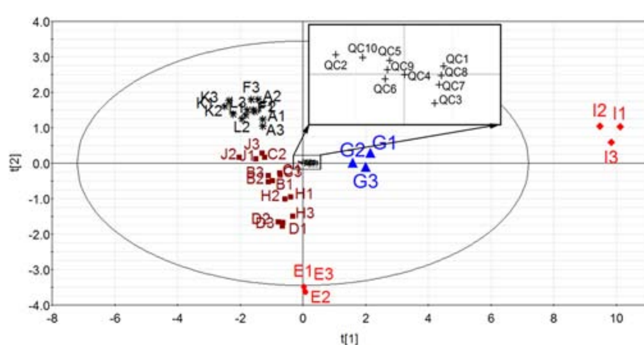

Figure 4. PCA score plot of the samples in data set including QC samples. QC1−QC10 in the plot represent the pooled quality control samples. Each batch (A−J) was prepared in triplicates with an end-label of 1−3 according to their injection sequence in the analytical run.

Metabolic Profiling of 12 Batches of *J. curcas* Seed. The plot of the data set of PCA scores without QC samples is illustrated in Figure 5a. R2X (cumulative) and Q2 (cumulative) of the PCA model are 0.955 and 0.872, respectively. Subsequently, the HCA dendrogram of the PCA scores is drawn and shown in Figure 5b, in which 12 batches of *J. curcas* seed are clearly categorized into 5 groups. Batches I, G, and E are categorized into groups 1, 2, and 3, respectively. Batches A, K, F, and L are classified into group 4. Batches B−D, H, and J are clustered into group 5. It is clearly observed from Figure 5 that the metabolic profiling of batch I from LiJiang Yunnan is significantly different from the other batches (the rescaled distance between group 1 and the other four groups is about 7, whereas the rescaled distances among other groups are all <2.5). When group 1 is excluded, it can be seen in Figure 5b that group 2 is similar to group 3 and group 4 is similar to group 5. By comparison of the categorization of 12 batches of *J. curcas* seed by HCA with the typical HPLC chromatograms at 280 nm (Figure 2), it is easily observed that the peak heights of peaks 1, 9, 10, 11, 21, and 22 in batch I are significantly different from other batches. Whether or not these peaks result in the categorization needs to be further investigated.

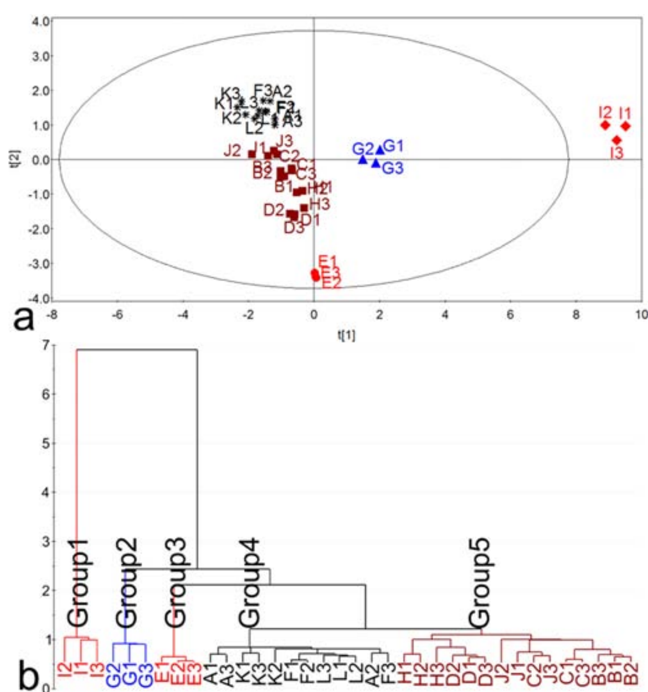

Figure 5. PCA score plot of the 12 batches of *J. curcas* seed (a) and HCA dendrogram of the PCA scores of 12 batches of *J. curcas* seed (b). Each batch (A−L) was prepared in triplicates with an end-label of 1−3 according to their injection sequence in the analytical run.

Identification of Marker Components Responsible for Metabolic Profiling Differences. PLS-DA of the HCA classification was performed to identify the characteristic components that have the most influence on the metabolic profiling of 12 batches of *J. curcas* seed. R2X (cumulative), R2Y (cumulative), and Q2 (cumulative) of the PLS-DA model were 0.934, 0.874, and 0.777, respectively. The PLS-DA scores plot demonstrates the same clustering and categorizing pattern as the PCA scores plot (Supporting Information, Supplementary Figure 2S). The PLS-DA loading plot is illustrated in Figure 6. An arbitrary loading threshold was set on the loading plot at ±0.20 for $w \times c$ [1] and at ±0.20 for $w \times c$ [2] and is highlighted in gray. The selection of the threshold was further verified by a correlation study described below and similar to our recently published paper.[15] Hence, the variables located outside the threshold region are regarded as the components that contributed most significantly to the categorization of the 12 batches of *J. curcas* seed. Nine components shown in Figure 2 including peaks 1, 9, 10, 21, and 22 (*J.*factor C_1), 28 (*J.*factor C_2), 30 (*J.*factor C_3), 31 (*J.*factor C_4), and 32 (*J.*factor C_5) were regarded as the marker components.

In combination with the HPLC chromatograms at 280 nm (Figure 2), the preliminary results above showed that the metabolic profiling differentiation might be explained by nine components including *J.*factors C_1−C_5 of PEs plus peak 21 (similar to *J.*factor C_1 in RT) and three peaks (peaks 1, 9, and 10) significantly different in RT than the PEs.

To further certify which compounds contributed most to the comprehensive quality of PEs, the correlation between the metabolic profiling and content levels of PEs in *J. curcas* seed was investigated.

Correlation between the Metabolic Profiling and Content Levels of PEs of *J. curcas* Seed. HPLC analysis of *J.*factors C_1−C_5 in *J. curcas* seed was developed and validated in this work. The typical HPLC chromatograms of the mixed standard solution including *J.*factors C_1−C_5 (4.20, 2.52, 1.56, 1.54, and 1.59 μg mL^{-1}) at 280 nm and batch A of *J. curcas* seed spiked with TPA (150.20 μg mL^{-1}) are shown in Figure 3. The resolutions of *J.*factors C_1−C_5 with other components in *J. curcas* seed are >2.0. The linear ranges of the method are 0.53−33.60, 0.32−20.16, 0.20−12.48, 0.19−12.32, and 0.20−12.72 μg mL^{-1} for *J.*factors C_1−C_5 with all correlation coefficients >0.9996. The LODs and LOQs of this method are 0.03 and 0.07 μg mL^{-1} for *J.*factor C_1; 0.02 and 0.08 μg mL^{-1} for *J.*factor C_2; and 0.02 and 0.05 μg mL^{-1} for *J.*factors C_3−$_5$, respectively. The intra- and interday precisions expressed as the RSD of three quality control levels are below ±3.0% for all analytes. The repeatabilities of samples expressed as the RSD of six

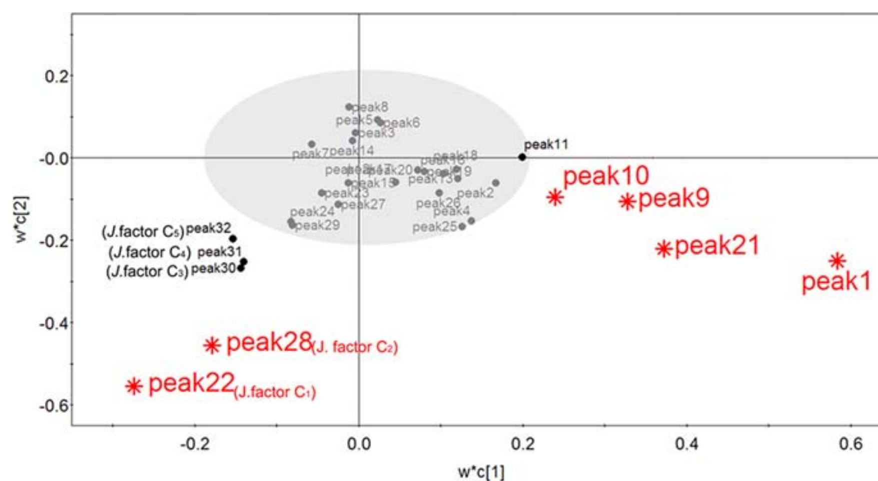

Figure 6. PLS-DA loading plot of the 12 batches of *J. curcas* seed. A loadings threshold was set at ±0.20 for $w \times c$ [1] and at ±0.20 for $w \times c$ [2] and is highlighted in gray.

parallel samples from identical batch A are below ±5.8% for all analytes. The mean recoveries of *J.*factors C_1-C_5 are between 96.86 and 100.90%. The standard solutions including *J.*factors C_1-C_5 are stable over 24 h at room temperature, with the RSDs all <4.0%.

Subsequently, the content levels of *J.*factors C_1-C_5, peak 21 (equivalent to *J.*factor C_1), and total content level of *J.*factors C_1-C_5 (equivalent to TPA) in 12 batches of *J. curcas* seed were evaluated in this work. These results are listed in Table 1.

The results show that the total content level of *J.*factors C_1-C_5 (equivalent to TPA) using the internal standard method is 20 times over the one using the external standard method, which is similar to the discovery in a recently published paper.[6] In this regard, it is readily deduced that it is unsuitable to determine the PEs in *J. curcas* seed using the internal standard method (equivalent to TPA). The results also revealed that the content level of *J.*factor C_1 accounts for 58.5% in total content level of *J.*factors C_1-C_5, which indicated that *J.*factor C_1 is the main constituent of PEs in *J. curcas* seed. Meanwhile, the content levels of peak 21 (equivalent to *J.*factor C_1) in 12 batches showed a larger variation (RSD, 103.4%) than for the other compounds.

It is easily observed that the content levels of *J.*factors C_1 and peak 21 (Table 1) seem to be related to the PLS-DA categorization of 12 batches of *J. curcas* seed. In this work, similar to our previously published paper,[15] PLS regression analysis was utilized to validate this observation. Briefly, the first two samples of the triplicates of each batch were used to build the PLS regression models, in which the 32 peak areas were set as X variables, and the content levels of *J.*factors C_1-C_5 as well as the sum of them, peak 21 (equivalent to *J.*factor C_1), were sequentially designated as the Y variable. Seven PLS prediction models were generated respectively for them. Subsequently, X variables from the third samples of the triplicates of each batch were imported into the models to predict the corresponding Y value. Thereafter, the predicted Y level was correlated to the assayed Y level. The better the correlation, the more significant the component level was relevant to the comprehensive quality of PEs in *J. curcas* seed. These correlation results are shown in Table 2.

It is clearly observed from Table 2 that peak 21 (equivalent to *J.*factor C_1), sum of *J.*factors C_1-C_5, and *J.*factors C_1 and C_2 exhibit the best correlations (r = 0.9946, 0.9931, 0.9911, and 0.9906, respectively), whereas *J.*factors C_3 and C_4 exhibit somewhat less correlation (r < 0.99) and *J.*factor C_5 exhibits poor correlation (r = 0.8793). Hence, it is concluded that among the five known PEs plus peak 21, *J.*factors C_1 and C_2 plus peak 21 are the most significant ones to represent the comprehensive content level of PEs in *J. curcas* seed. This result is consistent with the PLS-DA loading score analysis (Figure 6) showing that the loading scores of peaks 21 and 22 (*J.*factor C_1) and peak 28 (*J.*factor C_2) are higher than those of peaks 30 (*J.*factor C_3), 31 (*J.*factor C_4), and 32 (*J.*factor C_5). In this regard, the results of the present work indicate that the differences of comprehensive content level of PEs between batches of *J. curcas* seed are mainly related to the content levels of *J.*factors C_1 and C_2 plus peak 21.

This study presents an excellent strategy using metabolic profiling assisted quality control of PEs in *J. curcas* seed. A sample from Lijiang Yunnan significantly different from other samples from China is found in this work. Especially, the abnormal amount peak (peak 21) in batch I (Lijiang, Yunnan) similar to *J.*factor C_1 needs to be further investigated in a future study. The result of the present work also demonstrates that the content levels of *J.*factors C_1-C_5 in *J. curcas* seed, from different regions of China, are significantly different.

Table 2. Correlation between the PLS Predicted Levels (Y Predicted) and the Assayed Content Levels (Y Assayed) of *J* Factors C_1-C_5

analyte	calibration curve	coeff of determination (R^2)	correl coeff (r)
*J.*factor C_1	$Y = 0.9341X + 3.3742$	0.9822	0.9911
*J.*factor C_2	$Y = 0.9278X + 1.2816$	0.9813	0.9906
*J.*factor C_3	$Y = 0.9202X + 0.4739$	0.9626	0.9811
*J.*factor C_4	$Y = 0.9289X + 0.4697$	0.9756	0.9877
*J.*factor C_5	$Y = 0.8995X + 0.7065$	0.7732	0.8793
sum[a]	$Y = 0.9372X + 5.6507$	0.9863	0.9931
peak 21[b]	$Y = 1.0348X - 0.4702$	0.9892	0.9946

[a]Sum, total content level of *J.*factors C_1-C_5. [b]Peak 21, content level of peak 21 equivalent to *J.*factor C_1.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Figures 1S and 2S. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

PEs, phorbolesters; TPA, phorbol 12-myristate-13-acetate; QC, quality control; RT, retention time; PCA, principal component analysis; HCA, hierarchical cluster analysis; PLS-DA, partial least-squares discrimination analysis; LOD, limit of detection; LOQ, limit of quantification.

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