Original Article

Simultaneous determination of macaenes and macamides in maca using an HPLC method and analysis using a chemometric method (HCA) to distinguish maca origin

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ABSTRACT

Macamides and Macaenes are the bioactive marker compounds in maca (*Lepidium meyenii* Walp., Brassicaceae) tuber. To simultaneously quantify these two types of compound, HPLC method was studied. To distinguish and group the growing regions of different maca samples, Hierarchical cluster analysis, a chemometric method, was applied to analyze the HPLC data. The calibration curves obtained using the HPLC method showed satisfactory linearity with determination coefficients >0.9998. The precision and repeatability relative standard deviation values were <4%, and the accuracy relative standard deviation value was <5%. The limits of detection was <0.1 μg/ml and the limit of quantification was <0.3 μg/ml. Our HPLC method was successfully used for the separation and determination of macamides and macaenes in Maca within 45 min, *i.e.*, two macaenes (9-oxo-10*E*,12*Z*-octadecadienoic acid and 9-oxo-10*E*,12*E*-octadecadienoic acid) and five macamides (*N*-benzyl-9-oxo-10*E*,12*Z*-octadecadienamide, *N*-benzyl-9-oxo-10*E*,12*E*-octadecadienamide, *N*-benzyl-9*Z*,12*Z*,15*Z*-octadecatrienamide, *N*-benzyl-9*Z*,12*Z*-octadecadienamide and *N*-benzyl-hexadecanamide). The HPLC method was applied to analyze and quantify the seven compounds in thirty maca samples with different colors and origins. The origins of all the maca samples were distinguished and grouped using hierarchical cluster analysis of the HPLC data. Accordingly, the metabolism of macaenes and macamides in maca post-harvest processing has also been proposed. The HPLC method is efficient to simultaneously quantify the macamides and macaenes in maca. Analyzing the HPLC data using hierarchical cluster analysis can distinguish maca growing origins.

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Introduction

Maca, *Lepidium meyenii* Walp., Brassicaceae, is a plant native to the mountain areas of the Peruvian Central Andes found at an altitude of 4000 m. Maca tubers have been used as food and medicine for centuries (Gonzales et al., 2009; Campos et al., 2013). Nowadays, maca is grown and cultivated in many other countries, and used as a popular dietary supplement and functional food worldwide. Maca tubers contain abundant protein, amino acids, carbohydrates and minerals, which have been demonstrated to promote the effects of maca, such as anti-anemia, anti-motion, decline in blood sugar, fatigue and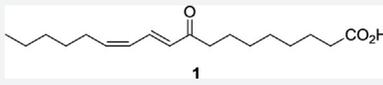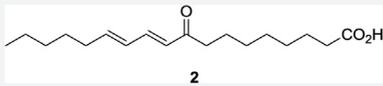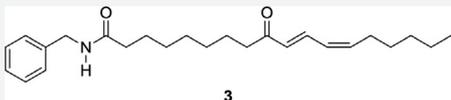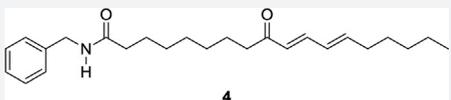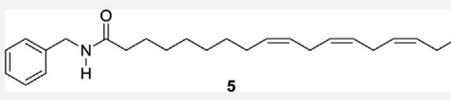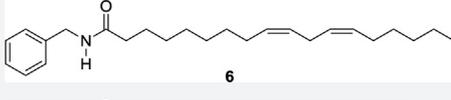 others (Piacente et al., 2002). In addition, maca is characterized by several secondary metabolites, including glucosinolates, sterols, macaenes (unsaturated long chain fatty acid derivatives), macamides (benzylamides of the long chain fatty acid derivatives) and benzyl isothiocyanate, which have been demonstrated to display many bioactive functions (Zheng et al., 2000). For example, some of the aphrodisiac activities of maca are related to the lipid fraction of maca, which mainly contains fatty acids and macamides. The proposed anti-cancer activity of maca has been attributed to the presence of alkaloids and sterols (Wang et al., 2007). Its anti-postmenopausal osteoporosis effect is related to the presence of phytosterols and other secondary metabolites (Fahey et al., 2001). Maca is used locally for the enhancement of fertility and sexual behavior in men and women, and has been generally acknowledged as a traditional remedy for menopausal symptoms (Shin et al., 2010; Lee et al., 2011). More recently, its nutritional properties and effects on sexual performance and semen quality

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Table 1
The seven compounds found in maca simultaneously analyzed using our HPLC method.

Chemical structure	Molecular weight	Chemical formula	Chemical name
1	294.2	$C_{18}H_{30}O_3$	9-Oxo-10E,12Z-octadecadienoic acid
2	294.2	$C_{18}H_{30}O_3$	9-Oxo-10E,12E-octadecadienoic acid
3	383.3	$C_{25}H_{37}NO_2$	N-Benzyl-9-oxo-10E,12Z-octadecadienamide
4	383.3	$C_{25}H_{37}NO_2$	N-Benzyl-9-oxo-10E,12E-octadecadienamide
5	367.6	$C_{25}H_{37}NO$	N-Benzyl-9Z,12Z,15Z-octadecatrienamide
6	369.6	$C_{25}H_{39}NO$	N-Benzyl-9Z,12Z-octadecadienamide
7	345.6	$C_{23}H_{39}NO$	N-Benzyl-hexadecanamide
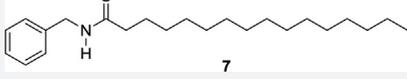
in rats and humans have been extensively reported (Poveda et al., 2013; Uchiyama et al., 2014; Melnikovova et al., 2015).

Macaenes and macamides are the most important secondary metabolites and marker compounds in maca (Zheng et al., 2000). It is believed that macamides are only found in maca. According to recent pharmacological studies, multiple bioactivities have been shown by macamides found in maca, such as sexual activity enhancement, anti-inflammatory, anti-cancer, anti-oxidative activity, neuroprotection, and vascular protection effects (Beharry and Heinrich, 2018). Macaenes are unsaturated long chain fatty acid derivatives. Some macaenes are also found in other plants, such as artemisia leaves, the calyx of eggplants and tomato fruit (Chinetti et al., 2000; Takahashi et al., 2011, 2014). It has been reported that some of these unsaturated long chain fatty acids can serve as peroxisome proliferators-activated receptor α (PPARα) agonists, which are ligand-activated transcription factors that can regulate lipid metabolism. The activation of PPARα can enhance fatty acid oxidation and decrease the levels of circulating and cellular lipids in obese diabetic patients (Kim et al., 2011). In addition, a cytotoxic fatty acid ketodiene was isolated from the calyx of eggplants, which exhibited cytotoxic activity against human ovarian cancer (HRA) cells (Zhao et al., 2015). Thus, macaenes should also contribute to the bioactivity of maca.

Maca has been transplanted to countries other than Peru and is extensively used for functional foods and supplement products. Since macamides and macaenes are two significant marker compounds in maca, an efficient scientific method is necessary to test for these compounds, in order for the authentication, identification, quantification, processing, and quality control of maca. Herein, we report an HPLC method for the simultaneous analysis of the two types of compounds (**1–7**) (Table 1) found in maca tubers. Using the developed HPLC method, these seven compounds were tested in thirty maca samples, in which twenty nine maca samples were collected from four regions in mainland China, and one maca sample originated from Peru. The HPLC analytical data was further analyzed using hierarchical cluster analysis (HCA) in order to distinguish and group maca from the different regions.

Materials and methods

Chemicals

Reference standards of compounds **1–4** were isolated from maca using column chromatography and preparative HPLC in our laboratory. Their identities were confirmed according to our previous publication (Xia et al., 2018). Macamides **5–7** have been reported in the literature (Uchiyama et al., 2014; Chain et al., 2014). The reference standards for **5–7** used in this research were synthesized and identified in our laboratory. The purity of each compound was tested by HPLC and determined to be >98%. Trifluoroacetic acid (TFA) and methanol (MeOH) were purchased from Chengdu Kelon chemical reagent factory (Chengdu, China). HPLC grade acetonitrile (ACN) was purchased from Sigma (St. Louis, MO, USA).

Sample preparation

The fresh maca tuber samples had different colors. Twenty-nine samples were harvested between 2014 and 2016 from Yunnan, Sichuan, Qinghai Provinces, and Tibet Autonomous Region of China. One Peruvian yellow dry maca powder sample was commercially purchased in a USA market in 2014. The samples were deposited in the herbarium of Institute of Agro-product Processing Science and Technology, Sichuan Academy of Agricultural Sciences. The samples number, color, growing location and altitude, harvest time and supplier code (the suppliers' names were replaced using codes) are listed in Table 2.

Fresh Chinese maca were dried naturally on an open plate at room temperature until crisp. The dried slices were milled into fine powder and stored at −18 °C prior to testing. Just before testing, the maca powder was homogenized with methanol at a ratio of 1:10

Table 2
Comprehensive information for the thirty maca samples studied.

Sample no.	Growing location	Altitude (Metre)	Color	Harvest time (Month/Year)	Supplier code
Y-1-P	Yunnan, Lijiang	3100	Purple	01/2016	SA
Y-2-Y	Yunnan, Lijiang	3300	Yellow	12/2015	SB
Y-3-Y	Yunnan, Lijiang	2950	Yellow	12/2015	SC
Y-4-Y	Yunnan, Lijiang	3150	Yellow	12/2015	SC
Y-5-Y	Yunnan, Lijiang	3350	Yellow	12/2015	SC
Y-6-Y	Yunnan, Lijiang	3200	Yellow	12/2015	SD
S-1-Y	Sichuan, Ebian	2750	Yellow	12/2015	SD
S-2-Y	Sichuan, Ebian	2750	Yellow	01/2015	SD
S-3-B	Sichuan, Ebian	2750	Black	01/2016	SD
S-4-Y	Sichuan, Liangshan	3250	Yellow	01/2016	SE
S-5-Y	Sichuan, Xichang	3200	Yellow	01/2015	SF
S-6-P	Sichuan, Xichang	3200	Purple	01/2015	SF
S-7-Y	Sichuan, Aba	3350	Yellow	01/2014	SG
S-8-Y	Sichuan, Aba	3350	Yellow	01/2015	SG
S-9-B	Sichuan, Liangshan	2900	Black	01/2015	SD
S-10-P	Sichuan, Liangshan	2900	Purple	01/2015	SD
S-11-Y	Sichuan, Ganzi	3500	Yellow	12/2016	SH
S-12-Y	Sichuan, Ganzi	3650	Yellow	12/2015	SH
S-13-Y	Sichuan, Ganzi	3650	Yellow	12/2014	SH
S-14-P	Sichuan, Ganzi	3650	Purple	12/2014	SH
S-15-Y	Sichuan, Litang	3750	Yellow	12/2015	SI
X-1-Y	Tibet, Lasa	3700	Yellow	12/2015	SJ
X-2-Y	Tibet, Lasa	3700	Yellow	12/2015	SJ
X-3-Y	Tibet, Lasa	3700	Yellow	12/2016	SJ
X-4-Y	Tibet, Lasa	3700	Yellow	12/2015	SJ
X-5-Y	Tibet, Lasa	4300	Yellow	12/2015	SJ
X-6-Y	Tibet, Lasa	4320	Yellow	12/2015	SJ
Q-1-Y	Qinghai, Xining	3800	Yellow	12/2015	SJ
Q-2-Y	Qinghai, Xining	3800	Yellow	01/2016	SK
P-2-Y	Peru, Andes	4000	Yellow	XX/2014	SL

(w/v) and extracted at 40 °C for 1 h under ultrasonication. All the testing solutions were filtered through 0.22 μm nylon membrane filters prior to HPLC analysis.

HPLC analysis

Analyses were performed using an HPLC instrument (Agilent LC 1260 series, equipped with a diode array detector (DAD), Agilent Technologies, USA). A Zorbax XDB-C18 column (250 mm × 4.6 mm, 5 μm particle size, Agilent Technologies, USA) was used. The mobile phase consisted of 0.005% trifluoroacetic acid in water (solvent A) and 0.005% trifluoroacetic acid in acetonitrile (solvent B). The gradient program was set at 0–35 min (55–95% B), 35–40 min (95–100% B), 40–45 min (100% B) using a flow rate of 1 ml/min. The column temperature was 40 °C, the injection volume was 20 μl and the detection wavelengths were 280 nm and 210 nm.

Validation of the HPLC methodology

In order to verify the applicability of present chromatographic methodology for the simultaneous analysis of the seven compounds, the analytical method was validated according to International Conference on Harmonization (ICH) (ICH, 2005) and literature processes (Sabir et al., 2017; Duong et al., 2017; Araujo-León et al., 2019). The following parameters were determined, *i.e.*, linearity, precision, accuracy, repeatability, limit of detection (LOD) and limit of quantification (LOQ).

The linearity was assessed by analyzing six different concentrations of the standard solutions of the seven compounds in triplicate. Their concentration ranges are shown in Table 3.

The repeatability test was determined by analyzing five independently prepared solutions of the same sample and expressed using the RSD of the average extraction rate. It was calculated using the same equation used for the precision.

The precision of the method was determined by the intra-day and inter-day stability test. The intra-day stability test was per-

formed using five injections of the same standard solution after it was stood for 0, 2, 4, 6, 8 h at the room temperature. The interday stability test was assessed by analyzing the standard solution continuously on three consecutive days.

The accuracy was estimated via recovery experiments, by adding a known concentration of the seven compounds standard solutions to the sample solution. After the addition of five concentration levels of the reference standard to the test samples, the samples were extracted and analyzed according to the procedure described above.

The percentage recovery was calculated using the following equation (Eq. (1)):

$$R\% = (X_1 - X_2)/X_1 \times 100\% \tag{1}$$

Where, R% is the percentage recovery of the added standard, X_1 is the concentration of each analyte in the spiked sample and X_2 is the concentration of each analyte in the non-spiked sample.

The LOD and LOQ of the seven compounds were calculated from the standard solutions of each compound on the basis of a minimal accepted value for the signal-to-noise ratio of 3 and 10, respectively.

The seven compounds of macaenes and macamides in thirty maca samples were extracted and detected using the method described in "Sample preparation". Identification of the seven compounds in the test samples was obtained by comparing the retention times of the corresponding reference standards. The contents of each compound in all the samples were calculated using the following equation (Eq. (2)):

$$C(\mu g/g) = \frac{\frac{A}{k}(\mu g/ml) \times V(ml)}{m(g)} \tag{2}$$

where, C is the content, A is the peak area, k is a constant in each equation for the seven compounds (see Table 3), V is the volume of the extraction solution and m is the quantity of powdered material.

Table 3
The calibration and validation data obtained for the quantification of the seven compounds (**n = 6**).

Compound no.	Rt (min)	Equation	Linearity		Repeatability RSD%	Precision		Limits		Accuracy	
			range (µg/ml)	R^2		Intra-day (RSD%)	Inter-day (RSD%)	LOQs (ug/ml)	LODs (ug/ml)	%R	RSD%
1	15.6	$Y = 107.52X$	0.49-62.50	0.9999	2.12	1.25	2.30	0.145	0.044	100.67	0.37
2	16.4	$Y = 106.99X$	0.28-144.55	0.9999	0.71	0.83	3.22	0.147	0.044	103.14	1.08
3	20.7	$Y = 81.487X$	0.25-73.10	0.9999	2.04	0.85	0.76	0.236	0.071	102.54	1.22
4	21.4	$Y = 83.293X$	0.25-127.06	0.9999	1.58	0.16	2.74	0.161	0.048	102.70	1.25
5	29.7	$Y = 56.774X$	0.58-118.50	0.9999	0.91	0.21	1.31	0.275	0.082	105.02	3.35
6	34.0	$Y = 39.552X$	0.40-118.50	0.9998	1.21	0.64	0.24	0.276	0.083	108.61	4.35
7	37.6	$Y = 32.199X$	0.60-220.00	0.9999	1.34	0.45	0.38	0.272	0.082	107.13	2.89

Statistical analysis

HCA was performed in order to evaluate the spectral differences of the seven compounds among the thirty samples using the HPLC data and to monitor the related clusters and sub-clusters in which the samples could be scattered. HCA was performed using SPSS 20.0 software for Windows 20.0 (SPSS, Chicago, IL). All the experiments were carried out in triplicate. Statistical analysis was performed using SPSS software. Analysis of variance (ANOVA) was used for statistical analysis. Differences were considered statistically significant when a *p*-value was <0.05.

Results and discussion

Validation of the HPLC method

The HPLC chromatograms of the seven reference standards, Tibet maca (X-4-Y) and Peruvian maca (P-1-Y) are shown in Fig. 1. The seven compounds were separated in a proceeding way and the retention time was consistent between the standards and samples. The peak areas response for the seven compounds were plotted versus their concentration at five different levels and fitted to the calibration curve, respectively. As shown in Table 3, a good linearity was exhibited by the seven compounds calibration curves with correlation coefficients (R^2) within the range of 0.9998–0.9999, which demonstrates the method is effective for the quantification of macaenes and macamindes, respectively.

In addition, the signals related to the seven compounds in maca can be appropriately identified by comparing the respective UV spectra obtained from the sample to the standard, as shown in Fig. 1. The purity of the chromatographic signals of the seven compounds in maca were assessed using the detector software and presented purity values >990 for each of the seven cases, and therefore were considered pure. Moreover, the seven compounds in maca were also matched to the standards using a HPLC library, which was established using the chromatographic signals of the seven standards.

The HPLC method was validated in terms of its linearity, precision, accuracy, repeatability, LOD and LOQ. As shown in Table 3, the repeatability RSDs of all seven compounds in the sample solution did not exceed 3%. The low RSD values suggest that the test procedure has good repeatability. The variation in the intra-day and inter-day stability of the seven compounds in the standard solutions were <4%, which indicate that the method was stable under the conditions. The recovery rates (R%) of the seven compounds all approached 100% and the accuracy RSDs did not exceed 5%. The LOQ values were <0.3 µg/ml. The LOD values were <0.1 µg/ml. The

results of the validation study demonstrated that the method was reliable when analyzing the seven compounds found in maca.

The variation in the stability was further studied using maca samples. The inter-day stability of compounds **1** and **3** in the maca samples were determined with an RSD value of 12.30 and 9.93%, respectively. The high variation should be the result of the conversion of the compounds in maca tissue catalyzed by a relevant enzyme with time because we found that the increased amount of **2** was equal to the decreased amount of **1**, and the decreased amount of **3** was similar to the increased amount of **4** in the maca samples over 30 h of the inter-day stability (Fig. 2).

Application of the method

Testing of macaenes and macamides in maca

The developed HPLC method was further applied to determine the contents of the seven macaene and macamide compounds in thirty maca samples. The contents of the compounds in the samples are presented in Table 4. The results show that compound **3** was not detected in samples Y-1-P, S-9-B, S-10-P, S-13-Y and S-14-P. Compound **1** was not found in S-9-B and S-10-P. The seven samples were detected in all the other samples. The individual and mean content data show the contents of macamides **5–7** were generally higher than other compounds found in the maca samples (except S-11-Y, S-15-Y, X-1-Y, X-2-Y, X-4-Y, X-6-Y, Q-1-Y, Q-2-Y and P-1-Y). This may be the reason why compound **5–7** were more easily identified and previously reported (Uchiyama et al., 2014; Chain et al., 2014). Macamides **3** and **4** were present in lower amounts, so they were not easy to detect and identify prior to our publication (Xia et al., 2018). However, when using the developed HPLC method, the two macamides were successfully detected. The mean contents of **3** (112.91 µg/g), **4** (272.96 µg/g) and **7** (933.65 µg/g) in maca from Xizang were higher than those found in maca from the other regions. The seven compounds in maca from Yunnan **1** (14.53 µg/g), **2** (72.37 µg/g), **3** (8.94 µg/g), **4** (50.76 µg/g), **5** (300.01 µg/g), **6** (591.50 µg/g) and **7** (760.33 µg/g), and Sichuan **1** (12.38 µg/g), **2** (66.42 µg/g), **3** (14.15 µg/g), **4** (40.67 µg/g), **5** (400.50 µg/g), **6** (649.01 µg/g) and **7** (815.04 µg/g) are found in similar amounts. Macamides **5–7** in Qinghai maca (148.72, 350.81 and 564.12 µg/g) and Peru maca (89.64, 173.47 and 247.41 µg/g) are present in much lower amounts than in Xizang, Sichuan and Yunan maca, though compounds **1–4** are found in higher and lower amounts when compared to the maca samples from other regions, respectively. This difference may be related to their germplasm sources, cultivation practices, harvest time and drying process (Beharry and Heinrich, 2018; Cui et al., 2016).

Correlation analysis of the seven compounds was performed using SPPS 20.0 software (Table 5). Both profiles for compounds
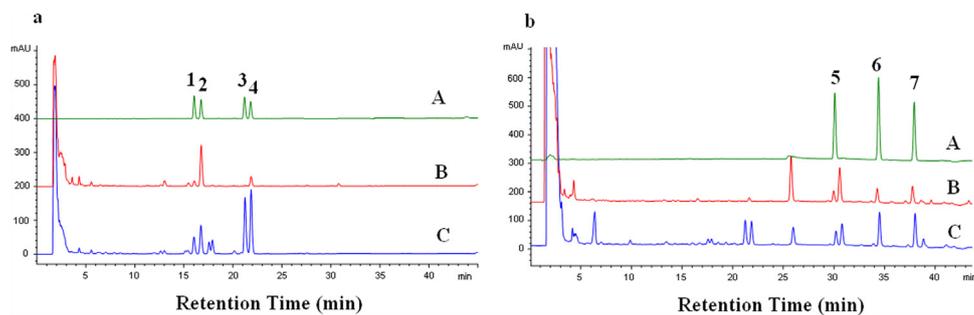
Fig. 1. HPLC chromatograms of compounds **1–7** detected at (a) 280 nm and (b) 210 nm: (A) Reference standards, (B) maca from Peru and (C) maca from Tibet.
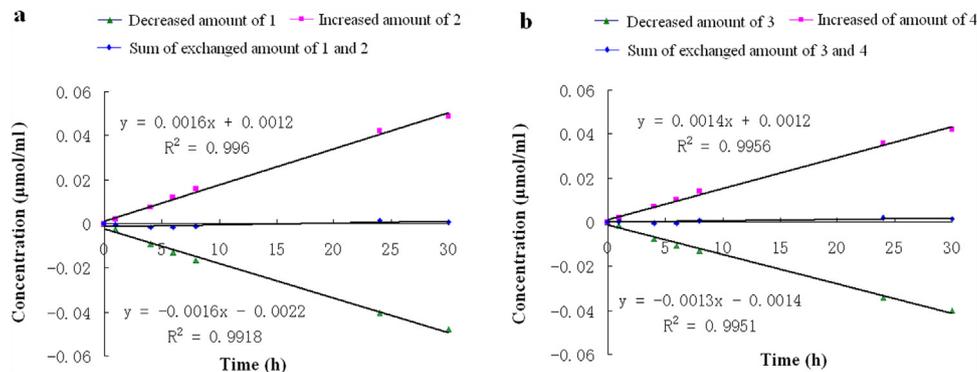

Fig. 2. The variation in compound **1–4** over 30 h. (a) The increased amount of **2** was equal to the decreased amount of **1** over 30 h. (b) The decreased amount of **3** was similar to the increased amount of **4** in the maca samples over 30 h.

Table 4
The amounts of the seven compounds in thirty maca samples (μg/g).

Sample code	Compound no.						
	1	2	3	4	5	6	4
Y-1-P	6.47 ± 0.03	31.00 ± 0.06	—	27.19 ± 0.07	410.47 ± 0.03	704.45 ± 1.07	808.80 ± 0.47
Y-2-Y	42.34 ± 0.00	48.39 ± 0.13	6.64 ± 0.00	9.35 ± 0.04	115.16 ± 0.10	170.25 ± 1.76	85.03 ± 0.01
Y-3-Y	3.33 ± 0.01	32.70 ± 0.04	2.75 ± 0.07	25.59 ± 0.19	244.00 ± 0.54	336.17 ± 1.14	582.21 ± 2.31
Y-4-Y	13.06 ± 0.03	121.33 ± 0.16	9.33 ± 10.00	76.11 ± 0.15	296.80 ± 0.85	631.35 ± 1.54	1225.07 ± 40.15
Y-5-Y	11.63 ± 0.18	120.62 ± 0.23	21.55 ± 1.53	136.91 ± 1.53	523.59 ± 0.86	1292.12 ± 2.51	1425.78 ± 17.36
Y-6-Y	10.34 ± 0.00	80.69 ± 0.18	4.43 ± 0.01	29.43 ± 0.03	210.04 ± 0.89	414.65 ± 1.75	435.06 ± 0.58
Average above	14.53	72.72	8.94	50.76	300.01	591.50	760.33
S-1-Y	19.99 ± 0.02	135.71 ± 0.19	10.45 ± 0.02	63.59 ± 0.09	154.30 ± 0.39	255.93 ± 0.33	342.10 ± 5.36
S-2-Y	4.51 ± 0.02	39.73 ± 0.07	1.65 ± 0.07	49.32 ± 0.06	481.08 ± 1.59	776.83 ± 0.40	1762.08 ± 7.13
S-3-B	5.91 ± 0.05	45.68 ± 0.08	1.27 ± 0.24	34.69 ± 0.61	345.10 ± 2.25	692.27 ± 4.01	799.15 ± 0.39
S-4-Y	13.83 ± 0.01	106.91 ± 0.25	8.86 ± 0.15	48.14 ± 0.28	715.70 ± 0.07	189.51 ± 0.66	343.63 ± 14.40
S-5-Y	10.71 ± 0.08	68.83 ± 0.24	18.32 ± 4.70	60.92 ± 56.21	573.02 ± 0.76	633.51 ± 5.10	1069.98 ± 11.67
S-6-P	8.77 ± 0.11	55.67 ± 0.14	3.64 ± 0.20	30.91 ± 0.15	410.47 ± 0.033	704.45 ± 1.15	808.80 ± 0.55
S-7-Y	14.77 ± 0.01	104.59 ± 0.03	18.32 ± 4.70	98.51 ± 3.04	273.22 ± 0.23	830.83 ± 43.70	1579.47 ± 13.19
S-8-Y	7.92 ± 0.02	55.47 ± 0.09	8.71 ± 0.02	46.37 ± 0.02	135.95 ± 0.28	413.75 ± 0.88	761.94 ± 0.48
S-9-B	—	6.25 ± 0.09	—	5.11 ± 0.10	863.10 ± 2.37	1353.69 ± 3.46	683.90 ± 2.98
S-10-P	—	6.35 ± 0.07	—	6.04 ± 0.03	794.47 ± 4.57	1287.23 ± 6.90	832.13 ± 6.25
S-11-Y	13.29 ± 0.15	130.30 ± 0.03	12.24 ± 0.08	35.29 ± 0.13	123.39 ± 0.58	406.69 ± 3.48	392.41 ± 1.89
S-12-Y	38.58 ± 0.04	136.94 ± 0.24	58.07 ± 0.04	82.23 ± 0.09	472.97 ± 0.56	681.49 ± 0.50	680.12 ± 1.48
S-13-Y	5.56 ± 0.01	16.15 ± 0.06	—	2.79 ± 0.10	297.74 ± 3.93	445.55 ± 2.08	340.98 ± 1.08
S-14-P	4.71 ± 0.02	21.29 ± 0.04	—	5.50 ± 0.02	252.65 ± 0.05	520.85 ± 2.35	388.13 ± 0.06
S-15-Y	114.42 ± 0.89	218.40 ± 1.09	72.87 ± 0.12	125.71 ± 0.37	44.07 ± 0.38	84.43 ± 1.15	190.67 ± 1.00
Average above	12.38	66.42	14.15	40.67	400.50	649.01	815.04
X-1-Y	34.17 ± 0.37	126.16 ± 0.53	190.00 ± 0.00	403.82 ± 1.38	239.76 ± 9.02	823.78 ± 8.99	847.57 ± 29.85
X-2-Y	22.68 ± 0.11	89.26 ± 0.25	147.54 ± 0.00	399.67 ± 1.13	209.63 ± 0.73	852.60 ± 12.61	1106.72 ± 18.16
X-3-Y	32.02 ± 0.18	104.23 ± 0.06	114.67 ± 0.00	209.32 ± 0.14	619.47 ± 3.75	1335.57 ± 17.06	1231.44 ± 44.02
X-4-Y	61.09 ± 0.73	117.69 ± 0.36	267.31 ± 0.00	315.66 ± 0.01	93.18 ± 0.66	324.69 ± 0.40	476.29 ± 1.68
X-5-Y	37.82 ± 0.45	132.38 ± 0.44	123.84 ± 0.00	400.62 ± 1.66	463.58 ± 2.43	852.88 ± 0.07	1703.80 ± 54.97
X-6-Y	53.33 ± 1.28	127.58 ± 1.93	133.67 ± 0.07	334.83 ± 0.14	228.11 ± 0.11	364.72 ± 0.49	864.26 ± 0.79
Average above	39.88	174.35	112.91	272.96	270.61	646.46	933.65
Q-1-Y	39.58 ± 0.38	232.48 ± 0.49	62.99 ± 0.00	201.93 ± 0.90	232.27 ± 2.05	533.88 ± 2.10	828.95 ± 30.14
Q-2-Y	74.73 ± 0.06	167.56 ± 0.36	83.88 ± 0.07	123.70 ± 0.25	65.17 ± 1.02	167.73 ± 27.61	299.29 ± 3.95
Average above	57.16	200.03	73.44	162.82	148.72	350.81	564.12
P-1-Y	20.38 ± 0.10	158.59 ± 0.05	5.30 ± 0.16	47.88 ± 0.56	89.64 ± 0.07	173.47 ± 0.54	247.41 ± 0.40

1–**4** and **5**–**7** have good correlation. Compound **5** was significantly correlated with **1** and **2**, and **6** was significantly correlated with **1**.

Macamides **3** and **4** were formed from macaenes **1** and **2** upon reaction with benzylamine to form an amide bond (Xia et al., 2018). It is possible that these compounds were generated in the post-harvest drying process of maca (Esparza et al., 2015) because they could not be detected in fresh maca tuber. As shown in Scheme 1, it is hypothesized that compound **1** can be generated from the oxi-

dization of linoleic acid (naturally existing in maca) catalyzed by lipoxygenases, then transformed into **2** via double bond isomerization from the 12Z-isomer to the 12E-isomer. Benzylamine can be generated from the metabolism of benzyl glucosinolate via benzyl isothiocyanate and benzyl isocyanate intermediates. The acids and benzylamine finally react with one another catalyzed by an enzyme to synthesize macamides **3** and **4**, respectively. Compound **3** can also transform to **4** via double bond isomerization. This hypothe-

Scheme 1. A hypothesis for the metabolism of macaenes and macamides during the post-harvest drying process of maca. 9-Oxo-10E,12Z-octadecadienoic acid (**1**), 9-oxo-10E,12E-octadecadienoic acid (**2**), N-benzyl-9-oxo-10E,12Z-octadecadienamide (**3**) and N-benzyl-9-oxo-10E,12E-octadecadienamide (**4**).

Fig. 3. HCA of the thirty maca samples.

Table 5
The correlation analysis of the seven compounds found in maca.

Compd. no.	1	2	3	4	5	6	7
1	1	0.714**	0.585**	0.458*	-0.459*	-0.412*	-0.291
2		1	0.419**	0.460**	-0.486**	-0.337	-0.104
3			1	0.893**	-0.219	0.018	0.078
4				1	-0.155	0.140	0.318
5					1	0.838**	0.481**
6						1	0.564**
7							1

sis may also explain the positive correlation of compounds **1**–**4** and the large variation in the inter-day stability testing of maca samples using HPLC methods.

Hierarchical cluster analysis (HCA)

HCA is a method of cluster analysis which seeks to build a hierarchy of clusters. In HCA, clusters and sub-clusters are visualized definitely in dendrogram graphs. When the seven characteristic peaks were used as the clustering variation, the thirty samples were categorized into two groups (Fig. 3). Among of them, twenty-one macas (Y-1-Y, Y-2-Y, Y-3-Y, Y-4-Y, Y-5-Y, Y-6-Y, S-1-Y, S-2-Y, S-3-B, S-4-Y, S-5-Y, S-6-P, S-7-Y, S-8-Y, S-9-B, S-10-P, S-11-Y, S-12-Y, S-13-Y, S-14-P and P-1-Y) were intensively grouped into cluster I, which were principally from Yunnan, Sichuan and Peru. These Chinese maca growing locations are in or nearby the Yunnan-Kweichow Plateau and located at altitudes between 2750 and 3650 m. Thus, similar environments or geographic conditions in the growing locations of Yunnan and Sichuan may be factors leading their maca products to be clustered into group I, though maca from Peru was also contained in group I. In the same squared Euclidean distance (1.1), group II was further divided into subgroup IIa (X-1-Y, X-2-Y, X-3-Y, X-4-Y, X-5-Y and X-6-Y) and IIb (Q-1-Y, Q-2-Y and S-15-Y). In group II, nine maca samples were cultivated in the Qinghai-Tibet Plateau regions in growing location at altitudes between 3700 and 4320 m. The Sichuan Litang maca sample (S-15-Y) was in group II, which can be attributed to its growing location in the plateau region at an altitude of 3750 m that has more sunshine and a larger temperature difference between day and night. These results imply that geographical origin may play an important role in the macaenes and macamides contents in maca. However, the effect of color on distinguishing samples was not found from the HCA results. So, the color of the maca tuber may not be an important factor for its quality evaluations based on macamide and macaene analysis. Similar results have also been reported in the literature (Zhou et al., 2017; Pan et al., 2016).

Conclusions

The developed HPLC method can be applied to simultaneously analyze seven compounds found in maca tubers [*i.e.*, two macaenes (**1**–**2**) and five macamides (**3**–**7**)] with successful separation and determination within 45 min. The HPLC method has been validated to have acceptable linearity, precision, accuracy, repeatability, LOD and LOQ for the analyses. It is applicable to analyze the compounds in thirty maca samples with different colors, where twenty-nine maca samples were grown in different regions of China and one sample in Peru. HCA can be applied to analyze the HPLC data with efficiency in distinguishing and grouping the maca samples from different growing regions, also indicating that the contents of macaenes and macamides in maca have a close relationship to the maca growing region. Therefore, our HPLC method and HCA analysis have great potential to be used for assessing macaenes and macamides in maca for authentication, identification, quantification, processing and quality control purposes.

Authors contributions

CX contributed to collection maca tuber samples, running laboratory works, analysis of data, and writing manuscript. JD contributed to running laboratory works, analysis of data, and writing manuscript. JC designed the studies, supervised laboratory works, reviewed data, wrote and reviewed manuscript. YZ designed and discussed in the studies. YS collected the maca tuber samples and discussed in the studies. YZ and HL carried laboratory works and discussed. CL collected the maca tuber samples and discussed.

Conflicts of interest

The authors declare no conflicts of interest.

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