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Identification and quantification of flavonoid aglycones in rape bee pollen from Qinghai-Tibetan Plateau by HPLC-DAD-APCI/MS

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ABSTRACT

For identification and quantification of flavonoid aglycones in rape bee pollen (RBP) collected from the Qinghai-Tibetan Plateau, a high-performance liquid chromatography (HPLC) separation method with diode array detector (DAD) and atmospheric pressure chemical ionization/mass spectrometric (APCI/MS) detection and four extraction methods (i.e. microwave-assisted extraction, Soxhlet extraction, cold-soaked extraction, and heat reflux extraction) were developed in this study. The identification of flavonoid aglycones was based on retention time and mass spectra by comparison with standards. Results demonstrated that this method showed excellent reproducibility and correlation coefficient, and offered the detection limits of 0.77–15.50 pmol at signal-to-noise ratio of 3. Quercetin and kaempferol were presented in RBP, and microwave-assisted extraction (MAE) was superior to the other three methods in terms of efficiency, convenience and high content of quercetin (1.37 ± 0.059 mg/g) and kaempferol (23.44 ± 0.544 mg/g). Our work indicated that: 1) the proposed HPLC-DAD-APCI/MS was an accurate and precise analysis method to identify and quantify the flavonoid aglycones in RBP; and 2) MAE was efficient to extract flavonoids from RBP with short extraction time, low solvent consumption, and homogeneous extraction conditions.

1. Introduction

Rape (*Brassica campestris* L.), widely distributed in the Qinghai-Tibetan Plateau, is one of the main oilseed crops with economic significance. Its products, such as rape oil and rape bee pollen (RBP), are extensively consumed by the indigenous populations of this region (Wu and Lou, 2007). RBP has been used as a nutritional food and traditional medicine with the effect of anti-inflammatory, antioxidant, anti-allergic and cardioprotective activity (Wagenlehner et al., 2009; LeBlanc et al., 2009; Medeiros et al., 2008).

RBP contains significant amounts of polyphenol compounds, mainly flavonoids. Previous studies indicated that flavonoids are the principal bioactive compounds in RBP. Thus, it was used for establishing the quality standards related to its nutritional-physiological properties and for quality control of commercially pollen preparations (Yang et al., 2007; Kroyer and Hegedus, 2001; Cartea et al., 2011; Paramás et al., 2006; Almeida-Muradian et al., 2005). Recently, increasing evidence has proven that the flavonoids in RBP have potential therapeutic benefits via free radical scavenging activities and inhibition of lipid peroxidation (Saric et al., 2009; Leja et al., 2007; Silva et al., 2006; Almaraz-Abarca et al., 2007). Research has shown that the antioxidant activity of flavonoid aglycones is better than that of flavonoid glycosides, and flavonoids are absorbed by human in the form of flavonoid aglycones (Nuutila et al., 2002; Merken and Beecher, 2000). Up to present, few reports are available on the identification and quantification of flavonoid aglycones in RBP collected from the Qinghai-Tibetan Plateau. Therefore, it is necessary to set up an appropriate method for qualitative and quantitative analysis of flavonoid aglycones in RBP from the plateau.

HPLC is one of the most popular and reliable chromatographic techniques for separation of flavonoids and their derivatives. Factors, such as the detection wavelength, the ratio of mobile phase and the elution program are all crucial for HPLC separation. Thus, we further optimized the conditions of HPLC separation by adjusting the above-mentioned factors. As flavonoids compounds contain several hydroxyl groups and can form stable negative oxygen ion (Seo et al., 2012), we adopted APCI in negative mode to identify the flavonoids. Negative ion mode usually produces parent ion as $[M-H]^-$ in a single form, thus the flavonoids are easy to

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identify. The preparation of crude extracts is also a key aspect in the qualitative and quantitative analysis of flavonoid chemical constituents present in RBP.

Microwave-assisted extraction, Soxhlet extraction, cold-soaked extraction, and heat reflux extraction are four common extraction methods widely used in the extraction of natural products. Generally, these methods were selectively applied to the extraction of different types of components presented in the medicine herbs. Considering the extraction yield, cost, and time, comparative evaluations of these methods will be conducted for optimizing an efficient one which suitable for extraction of flavonoids.

The aims of the present study were to: 1) identify and quantify flavonoid aglycones in the hydrolyzed extracts of RBP from the Qinghai-Tibetan Plateau by HPLC-DAD-APCI/MS; and 2) select the most efficient extraction method of flavonoids in RBP among four different extraction methods, i.e. microwave-assisted extraction (MAE), Soxhlet extraction (SE), cold-soaked extraction (CSE), and heat reflux extraction (HRE).

2. Materials and methods

2.1. RBP source

RBPs were collected from the Menyuan region on the Qinghai-Tibetan Plateau, northwest China (3500–4400 m above sea level) in July 2012. The RBPs were freeze-dried under N_2 gas, and then ground into 40 mesh. The powder was stored at 4 °C in a refrigerator.

2.2. Standards, reagents, and other materials

Standards of rutin, quercetin, kaempferol, isorhamnetin were purchased from the National Institutes for Food and Drug Control (Beijing, China). HPLC-grade acetonitrile was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol, hydrochloric acid and formic acid were of analytical grade.

2.3. Preparation of flavonoid standard solution

Individual stock solutions of four standard flavonoids were prepared by dissolving 0.0611 g rutin, 0.0302 g quercetin, 0.0286 g kaempferol, and 0.0316 g isorhamnetin with 1 mL of 70% methanol, respectively. The standard solution for HPLC analysis was prepared by diluting the corresponding stock solution to 1.0×10^{-2} mol/L. The four standard flavonoids were prepared in serial concentrations (1×10^{-3} mol/L, 5×10^{-4} mol/L, 2.5×10^{-4} mol/L, 1.25×10^{-4} mol/L, 6.25×10^{-5} mol/L, 3.125×10^{-5} mol/L, 1.5625×10^{-5} mol/L, 7.8125×10^{-6} mol/L, 3.9063×10^{-6} mol/L, and 1.9531×10^{-6} mol/L) by diluting the stock standard flavonoid solution. All reagents and solutions were stored at 4 °C prior to use.

2.4. Conditions of the HPLC-DAD-APCI/MS

Determination of flavonoid aglycones was done with an Agilent 1100 HPLC series equipped with a quaternary pump (G1311A), a vacuum degasser (G1322A), an auto-sampler (G1329A), a thermostated column compartment (TCC, G1316A), a diode array detector (DAD, G1315A), and an Agilent HPLC workstation. HPLC separation of flavonoids was conducted on a reversed-phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies, Ltd., USA) with gradient elution using a binary mobile phase (phase A: 0.1% formic acid in 5% acetonitrile; and phase B: 100% acetonitrile). The mobile phase was filtered through a 0.2-μm membrane filter (Alltech Associates, Inc., USA). The gradient elution program was as follows: 0 min, 100:0 (A:B); 10 min, 75:25; 21 min, 75:25; 22 min, 0:100; and 30 min, 0:100. The injection volume was 10 μL. The flow rate was maintained at 1.0 mL/min and the column temperature was set at 30 °C. The appropriate wavelength was selected after scanning. Before injection of the next samples, the column was equilibrated with mobile phase A for 10 min.

MS analyses were performed on a Mass spectrometer 1100 Series LC/MSD Trap-SL (ion trap) which was purchased from Bruker Daltonnik (Bermen, Germany) and equipped with an atmospheric pressure chemical ionization (APCI) source in the negative ion mode. HPLC effluent was directed into the APCI of the MS operated under optimum conditions: nebulizer pressure 60 pis, dry gas temperature 350 °C, dry gas flow rate 5.0 mL/min, APCI Vap temperature 450 °C, capillary voltage 3500 V, and corona current 4000 nA (pos).

2.5. Validation of analytical method

The method was validated for linearity, limits of detection (LOD), and reproducibility. Based on the optimum HPLC conditions with an injection volume of 10 μL, the peak areas of four standard flavonoids at each concentration level were obtained. Linear regression equations were constructed by establishing calibration graph with the peak area (y-axis) versus the flavonoid concentration (x-axis). LOD was defined as the flavonoid concentration that produced a signal-to-noise ratio of 3. To examine the repeatability of the method, a standard solution was prepared with the four standard flavonoids and repeatedly injected into the HPLC system for 6 times.

2.6. Extraction of flavonoid from RBP

Four methods as below were employed for extracting flavonoids components from RBP.

(1) Microwave-assisted extraction (MAE): 5.0 g of RBP powder was extracted with 60 mL of 65% ethanol at 25 °C under refluxing for 30 min in an XH-100A microwave-assisted extractor (100–1000 W, output power 500 W) purchased from Beijing Xianghu Science and Technology Development Co. (Beijing, China).
(2) Soxhlet extraction (SE): 5.0 g of RBP powder was wrapped with a filter paper and then extracted with 60 mL of 65% ethanol at 65 °C under refluxing in a Soxhlet assembly fitted with a 250-mL flask until the reflux fluid was colorless.
(3) Cold-soaked extraction (CSE): 5.0 g of RBP powder was extracted with 60 mL of 65% ethanol at room temperature in the cold-soaked way for 24 h. The extraction procedure was repeated three times.
(4) Heat reflux extraction (HRE): 5.0 g of RBP powder was extracted with 60 mL of 65% ethanol at 75 °C under refluxing for 2 h. The extraction procedure was repeated three times.

The flavonoid extracts were centrifuged at 3000 rpm for 5 min. Then the supernatant was respectively collected and concentrated under reduced pressure on a RE-5298 rotary evaporation apparatus (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) to obtain the residue. The residue was re-dissolved in 100 mL of 65% ethanol for the analysis of acid hydrolysis.

2.7. Acid hydrolysis of flavonoid extracts

Acid hydrolysis was performed on flavonoid extracts for investigating the aglycone composition. Briefly, 2 mL of each re-dissolved residue solution was mixed with 0.5 mL of 20% hydrochloric acid in a 5-mL glass vial. Then, the vials were sealed and incubated in a water-bath at 85 °C with mild shaking for 90 min. After the reactions of acid hydrolysis were completed, each mixture was allowed cooling down at room temperature, filtered

through a 0.45-μm membrane filter, and then injected into the chromatograph system.

2.8. Analysis of flavonoid aglycones in RBP

The flavonoid aglycones in the extracts with acid hydrolysis were analyzed by the selected HPLC-DAD-APCI/MS condition. The identification of flavonoid aglycones in RBP were carried out by comparing the retention time and MS data with standard flavonoids. The contents of flavonoid aglycones in RBP were quantified based on the regression equation.

3. Results and discussion

3.1. Optimization of HPLC chromatographic conditions

The choice of proper detection mode is crucial for the determination of components in extracts from plants by HPLC analyses. As to DAD, this problem can be solved by simultaneously monitoring several wavelengths with a multiple wavelength-scanning program. This guarantees that all of the UV–vis absorbing components can be detected if present in sufficient quantity. To select an appropriate wavelength for HPLC, we obtained the maximum absorbance of four standard flavonoids in UV spectrum by HPLC-DAD on line spectrum scanning. Results were as follows: Rutin (204 nm, 258 nm, 355 nm); Quercetin (202 nm, 257 nm, 373 nm); Kaempferol (196 nm, 258 nm, 368 nm); and Isorhamnetin (202 nm, 256 nm, 373 nm). Despite the three main absorbance peaks of the standard flavonoids at 200 nm, 258 nm, and 370 nm, the solvent had a strong absorbance around 200 nm, which caused serious background interference. In addition, the absorbance peak at 370 nm was obviously weaker than that at 258 nm. Thus, 258 nm was chosen as the determination wavelength for flavonoids according to their corresponding maximum UV–vis absorption.

It has been reported that formic acid used in the HPLC mobile phase can minimize peak tailing. Despite its slight negative effects on the ionization efficiency and detection limits, formic acid remains the first choice of MS-compatible additive mainly for the volatility (Prokudina et al., 2012). In the present study, 0.1% formic acid was added to mobile phase A to achieve better chromatographic separation and peak shapes.

3.2. HPLC-DAD-APCI/MS analysis of standard flavonoids

Taking all factors into consideration, a baseline separation of four standard flavonoids was achieved within 25 min under the optimized conditions. The sharp, symmetrical and well-resolved peaks are shown in Fig. 1a.

The ionization and fragmentation of flavonoids were studied by MS with an APCI source in the negative ion mode. In crude plant extracts, flavonoids often exist in the form of O- or C-glycosides with sugar units directly bonded to hydroxyl group or a carbon (generally 6-C and 8-C) of the aglycone. The mass spectra of O-glycosides usually generate abundant aglycone ions by loss of the neutral mass of sugar while C-glycosides generate characteristic ions of the fragmentation of its own C-glycoside unit (Cuyckens and Claeys,

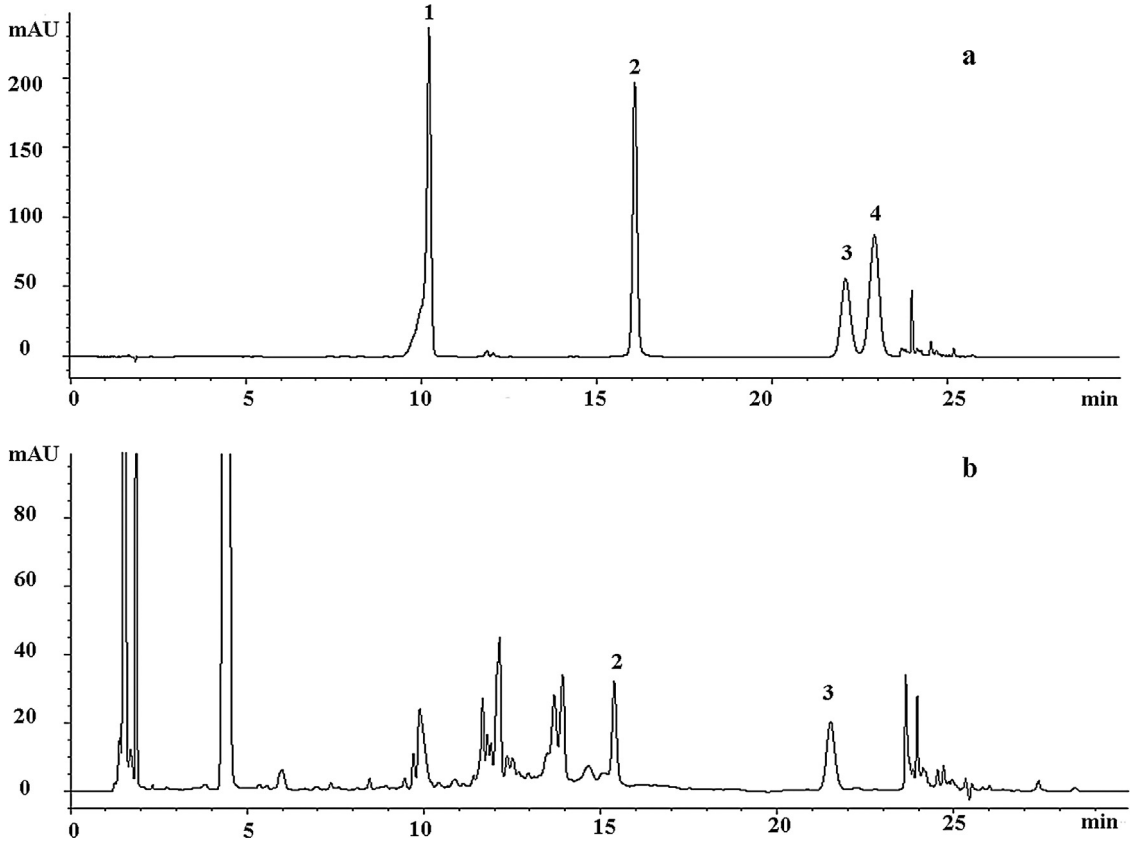

Fig. 1. HPLC-DAD chromatograms of four standard flavonoids and the hydrolyzed flavonoid extracts of rape bee pollen obtained by microwave-assisted extraction. (a) HPLC-DAD chromatograms of standard flavonoids (1, Rutin, 10.3 min; 2, Quercetin, 16.1 min; 3, Kaempferol, 22.1 min; and 4, Isohamnetin, 22.9 min); and (b) HPLC-DAD chromatograms of the hydrolyzed flavonoid extracts of rape bee pollen obtained by microwave-assisted extraction. HPLC conditions: Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies, Ltd., USA); mobile phases A (5% acetonitrile containing 0.1% formic acid) and B (100% acetonitrile) in gradient mode (0–10 min, 75% A and 25% B; 10–21 min, 75% A and 25% B; 21–22 min, 100% B; and 22–30 min, 100% B); injection volume, 10 μL; flow rate, 1.0 mL/min; column temperature, 30 °C; detection wavelength, 258 nm.

2004; Rak et al., 2010). Thus, it is easy to distinguish O- and C-glycosides by MS spectra. MS can provide important information on the structure of flavonoids and is of particular value for determining the nature and site of attachment of the sugar in O-glycosides (Waridel et al., 2001).

The peak order of four standard flavonoids in the MS ion current profile (Fig. 2a) was the same as that in the HPLC chromatogram (Fig. 1a) based on the retention time. For compound 1, APCI/MS gave 3 fragments at *m/z* 609, *m/z* 463 and *m/z* 301, respectively (Fig. 2b). The presence of $[M-H]^-$ fragment ion at *m/z* 609 suggested that the molecular mass of compound 1 was 610. The existence of $[M-H-146]^-$ and $[M-H-146-162]^-$ indicated that Rha and Glc were linked to the flavonoid aglycone. The most intensive fragment at *m/z* 301 suggested that compound 1 was an O-glycoside by losing two sugar units. Accordingly, compound 1 was identified as quercetin 3-O-rhamnopyranosyl-(1→6)-glucopyranoside (rutin).

Table 1
Peak assignments of four standard flavonoids.

Peak number	t_R (min)	$[M-H]^-$ (*m/z*)	Identification
1	10.3	609.5	Rutin
2	16.1	301.7	Quercetin
3	22.1	285.5	Kaempferol
4	22.9	315.5	Isohamnetin

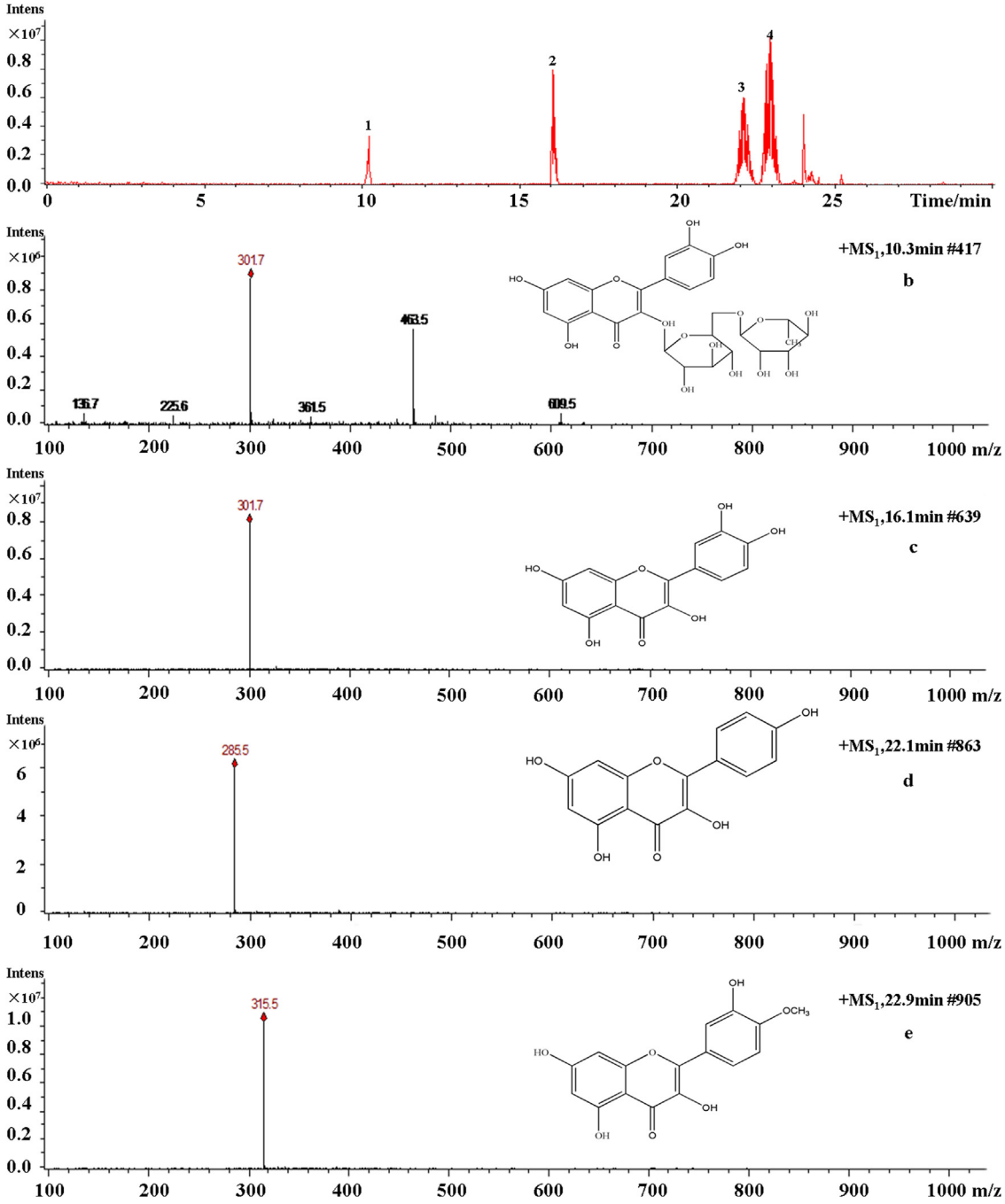

Fig. 2. Mass spectra of four standard flavonoids (APCI/MS). (a) Total ion chromatograms; (b) MS spectrum of rutin; (c) MS spectrum of quercetin; (d) MS spectrum of kaempferol; and (e) MS spectrum of isohamnetin. MS Conditions: Ion source type, atmospheric pressure chemical ionization (negative mode); nebulizer pressure, 60 pis; dry gas temperature, 350 °C; dry gas flow rate, 5.0 mL/min; APCI Vap temperature, 450 °C; capillary voltage, 3500 V; corona current, 4000 nA (pos).

Table 2
Linear regression equations, correlation coefficients, linear range, and detection limits of four standard flavonoids.

Flavonoids	Regression equation[a]	r^2 [b]	Linear range	LOD[c] (pmol)
Rutin	$y = 0.26x + 11.31$	0.9999	1.95325 μmol/L–1.0 mmol/L	0.77
Quercetin	$y = 0.17x - 7.76$	0.9998	1.95325 μmol/L–1.0 mmol/L	0.92
Kaempferol	$y = 0.10x - 0.02$	0.9999	3.90625 μmol/L–1.0 mmol/L	15.50
Isohamnetin	$y = 0.17x - 3.47$	0.9998	3.90625 μmol/L–1.0 mmol/L	9.70

[a] In the regression equation $y = kx + m$, y refers to the peak area, and x refers to the injection amount (pmol).
[b] r^2 is the correlation coefficient of the equation.
[c] LOD (the minimum limit of detection, S/N = 3) is expressed in concentration unit with an injection volume of 10 μL.

Compound 2 had an intensive $[M-H]^-$ fragment ion at m/z 301 (Fig. 2c) with a molecular mass was 302, thus was confirmed as quercetin. Compound 3 had an intensive $[M-H]^-$ fragment ion at m/z 285 (Fig. 2d) with a molecular mass of 286, thus was identified as kaempferol. Based on the MS data and retention time (Fig. 2e), compound 4 was identified as isorhamnetin. Table 1 lists the retention time and MS data for the standard flavonoids, i.e. rutin, quercetin, kaempferol, and isohamnetin.

The results showed that HPLC-DAD-APCI/MS in negative mode was applicable for separation and identification of the mixture of rutin, quercetin, kaempferol, and isorhamnetin.

3.3. Method validation

The results for the method validation are shown in Table 2. The linearity of the procedures was evaluated in the range of 1.9531 μmol/L to 1 mmol/L. As presented in Table 2, within the range of linearity between 1.9531 μmol/L and 1 mmol/L, the four standard flavonoids gave excellent linear responses and their correlation coefficients were greater than 0.9998. With a 10-μL injection volume for each standard flavonoid, the calculated detection limits (at signal-to-noise of 3:1) ranged from 0.77 pmol to 15.50 pmol. The results of method repeatability showed that the relative standard deviations (RSDs) of observed peak areas and retention times were less than 3.94% and 1.35%, respectively.

3.4. Analysis of hydrolyzed flavonoid extracts from RBP

The established analytical method was subsequently applied to simultaneous determination of flavonoid aglycones in hydrolyzed flavonoid extracts from RBP. The hydrolyzed flavonoid extracts were analyzed using optimized HPLC-DAD-APCI/MS condition. The extracts of RBP that originally contained several flavonoid derivatives of given aglycones produced only the chromatographic peaks of free aglycones after acid hydrolysis. The identification of flavonoid aglycones was based on retention time and mass spectra by comparison with standard flavonoids. As shown in Fig. 1b, the signals of compounds 2 and 3 appeared at almost the same position as those of peaks 2 and 3 in the spectrum of the standard flavonoids. Accordingly, compounds 2 and 3 were identified as quercetin and kaempferol. Isohaemnetin was not detected in the RBP samples. As to rutin (quercetin 3-O-rhamnopyranosyl-(1→6)-glucopyranoside), there are Rha and Glc linked to the flavonoid aglycone. When the flavonoid extracts were completely hydrolyzed, the glycosides were breakdown and quercetin aglycone was free. Thus, rutin can not be detected in completely hydrolyzed flavonoid extracts, and its aglycone, quercetin can be detected.

3.5. Selection of optimal extraction method

Flavonoids are considered to be an effective, natural and functional dietary food supplement because of the remarkable abundance and significant radical scavenging capacity. Therefore, it is desirable to obtain as much flavonoids as possible, and selection of an appropriate extraction method is a key point. Here, four methods were comparatively evaluated for extraction of flavonoids components from RBP with respect to the yield, cost, and time. The flavonoid aglycone contents in different hydrolyzed extracts from RBP were qualitatively analyzed. The contents of quercetin and kaempferol aglycones extracted by MAE reached 1.37 ± 0.059 mg/g and 23.44 ± 0.544 mg/g, respectively (Table 3), indicating that the highest extraction efficiency of flavonoid from RBP was achieved by MAE.

As a flavonoid extraction method, MAE combined microwave with the use of traditional solvent. Its high extraction efficiency can be related to the two-layer structure of the bee pollen wall, with the outer layer made of sporopollenin and the inner layer constructed of cellulose (Blackmore et al., 2007, 2009; Xu et al., 2009). In the MAE method, heating was performed in a targeted and selective manner with practically no heat loss to the environment. RBP powder was impregnated in 65% ethanol with water as modifiers can achieve a high dielectric constant of the extraction solvent under microwave. High temperature attained by microwave radiation can hydrolyze the linkages of cellulose, enhance the dehydration of cellulose, reduce its mechanical strength, and weaken or broken down the cell wall, thereby rendering the intracellular compounds more accessible for extraction. The mechanism of MAE was based on analytes exposure to the solvent through cell rupture (Mandal et al., 2007). Compared to conventional extraction techniques, MAE was more advantageous in terms of the short extraction time, low solvent consumption, and homogeneous extraction conditions by stirring. Thus, the content of flavonoid components extracted from RBP by MAE was relatively higher than those extracted by the other three methods (Table 3).

In order to examine the accuracy of the method, the recovery of the method was investigated by adding known amounts of standard flavonoids to the RBP samples and followed by the same extraction procedure, acid hydrolysis and HPLC analysis. The

Table 3
Contents of flavonoid aglycones in hydrolyzed extracts obtained from RBPs by four extraction methods.

Flavonoid aglycone	Regression equation	Extraction methods	Peak areas	Content (mg/g)
Quercetin	$y = 0.17x - 7.76$	MAE[a]	300.2 ± 13.48	1.37 ± 0.059
		SE[b]	188.1 ± 5.72	1.09 ± 0.031
		CSE[c]	167.6 ± 7.82	0.97 ± 0.043
		HRE[d]	238 ± 10.92	1.36 ± 0.061
Kaempferol	$y = 0.10x - 0.02$	MAE[a]	327.5 ± 7.61	23.44 ± 0.544
		SE[b]	215.4 ± 10.18	19.27 ± 0.911
		CSE[c]	232.2 ± 4.54	20.77 ± 0.406
		HRE[d]	193.7 ± 9.47	13.33 ± 0.847

Data are expressed as mean ± standard deviation ($n = 4$).
[a] MAE, microwave-assisted extraction.
[b] SE, Soxhlet extraction.
[c] CSE, cold-soaked extraction.
[d] HRE, heat reflux extraction.

Table 4
Determination of recovery for this method ($n = 3$).

Flavonoid	Mean level in hydrolyzed RBP sample (mg/g)	Added amount (mg/g)	Detected amount (mg/g)	Recovery %	R.S.D. %
Rutin	0	5	3.98[a]	104.4[b]	1.25
Quercetin	1.37	5	6.72	103.5	1.35
Kaempferol	23.44	5	28.32	98.8	1.01
Isohamnetin	0	5	4.76	97.6	1.32

[a] The amount of quercetin contains the amount of quercetin in the hydrolyzed RBP and the amount transformed from rutin.
[b] The % recovery of quercetin.

analysis was carried out in triplicate. The method accuracy was found to be in the range of 97.6%–104.4% (Table 4).

4. Conclusions

We have successfully developed an accurate and precise HPLC-DAD-APCI/MS analysis method to determine the flavonoid aglycones in RBP collected from the Qinghai-Tibetan Plateau. The identified flavonoid aglycones in RBP were quercetin and kaempferol. Then we selected the most efficient extraction method (microwave-assisted extraction) of flavonoids in RBP among four extraction methods by HPLC-DAD-ACPI/MS. The proposed method is capable of providing higher sensitivity and repeatability. It is believed that the determination of flavonoids in RBP from Qinghai-Tibetan Plateau has important value for the application of food science.

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