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ORIGINAL ARTICLE

Simultaneous identification and quantification of five flavonoids in the seeds of *Rheum palmatum* L. by using accelerated solvent extraction and HPLC–PDA–ESI/MS^n

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Abstract In this study, flavonoids were extracted using accelerated solvent extraction (ASE) with 80% aqueous methanol. A high-performance liquid chromatography equipped with photodiode array detection and coupled with an electrospray ionization tandem mass spectrometry (HPLC–PDA–ESI/MS^n) method has been developed for rapid identification and quantification of flavonoids in the plant extract of *Rheum palmatum* L. (RPL) seeds from Qinghai. Ten flavonoids from methanolic extract of RPL seeds were screened, of which epicatechin, myricetin, hyperoside, quercitrin and quercetin were identified and quantitatively analyzed for the first time. The absorbance was monitored at 280 nm for epicatechin and 360 nm for other four flavonoids. It was found that the calibration curves for all five analytes showed a good linearity ($r^2 > 0.999$) within the test range and the data of the limit of detection (LOD) and limit of quantification (LOQ) for all investigated compounds were less than 2.98 μg mL^{-1} based on PDA. The relative standard deviation (R.S.D) values of the intra- and inter-day precisions were 0.98% and 1.65%, respectively. The range of mean recoveries of the five components was 91.3–93.9%, and the R.S.D was 1.3–2.9%. It was also found that the content of quercitrin is the highest in RPL seeds while the contents of epicatechin and myricetin are relatively lower. In addition, the validation procedure confirmed that this method was suitable for providing quality control evaluation of RPL seeds to ensure the therapeutic benefits.

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1. Introduction

Rheum palmatum L. (RPL) grows in forest margins in mountainous regions. It is mainly distributed in Sichuan, Gansu, Qinghai and Tibet. *Rheum tanguticum* Maxim. ex Balf. (RTM) and *Rheum officinale* Baill. (ROB) grow in well-drained

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mountainous areas, mainly in Hubei, Sichuan, Yunnan and Guizhou. The roots and rhizomes of the genus of Rheum (RPL, RTM or ROB) of the family polygonaceae were collected in late autumn or winter, when the stems and leaves were withered (ChPC, 2010). RPL is a well established medicinal plant used to treat a variety of ailments, including diarrhea (Ma and Qi, 2003; Zhou et al., 2003), choleretic, hepatoprotective (Cheng and Kang, 1998; Fu et al., 2011) and immunomodulatory (Zhou, 2011; Wang, 2013). It also exhibits a variety of biological qualities, such as, anti-diabetic, anti-inflammatory, anti-viral, anti-ischemic (Sato et al., 2000; Song, 2012), improvement on hyperlipidemia (He et al., 2013; Huang et al., 2013; Fan and Zhu, 2014; Zhao et al., 2014) and it scavenges oxygen free radicals (Wang et al., 2013; Yan, 2014). As an infusion, or in the form of extracts, the herbal drug is used for the treatment of hepatitis, hepatic coma, acute cholecystitis, peptic ulcer, pregnancy-induced hypertension syndrome and acute bacillary dysentery (Li, 2009). Therefore, the analysis of the major chemical components contained in this herb is important for the research and quality evaluation of botanical drugs.

With the development of analytical techniques, many approaches such as thin layer chromatography scanning (TLCS; Zhao and Ou, 2001), polarography (Pournaghi-Azar et al., 1995), capillary electrophoresis (CE; Liu et al., 2011) and high-performance liquid chromatography (HPLC; Yin et al., 2010) have determined the contents of many chemical components such as anthraquinones (Jia et al., 2008; Liu et al., 2008), stilbene glycosides (Li et al., 2010), polysaccharides (Ni et al., 2007; Li et al., 2013; Liu et al., 2013) and flavonoids (Zhang et al., 2005; Gao, 2012) in recent years. Photodiode array (PDA) detector can collect multiple wavelength of chromatograms and corresponding spectra at the same time. The purity of chromatographic peaks can also be determined and it can be used for spectra retrieval. In addition, the qualitative information of chemical constituents can be provided (Justesen et al., 1998). The advantage of electrospray ionization (ESI) combined with tandem mass spectrometry has permitted ready study of the flavonoids, their ion chemistry and the determination of flavonoids in low concentrations. Furthermore, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS) technique is able to separate each other single component in complex mixtures and to perform their identification and quantification. Due to these several advantages, HPLC–MS has gained widespread interest as a favorable technique for the determination of pharmacologically interesting compounds in biological matrices (Marín et al., 2002; Caristi et al., 2003; Gil-izquierdo et al., 2004; Caristi et al., 2006; Abad-García et al., 2008). It is a strong, powerful tool for the rapid identification and quantification of natural compounds in complex extracts. Therefore, it is expected to obtain good natural compounds with the potential of preventing diseases from this plant resource.

So far there have been several studies on some chemical components such as amino acids, polysaccharide, protein and starch in rhubarb seeds (Liu et al., 2013, 2014). To the best of our knowledge, the application of methods for the simultaneous identification and quantification of individual flavonoids in RPL seeds have not been investigated hitherto. As an essential traditional Chinese herbal medicinal, with the growing shortage of wild herbs and the expansion of the market demand, the planting areas of rhubarb were increased gradually and rhubarb seeds were circulated frequently in different regions. Although there are cultivated rhubarbs in some areas of Gansu, Sichuan and Qinghai, but because it is lack of the uniform quality standard and the rhubarb introduction is a mess, can result in biology confusion of rhubarb seeds and declining in the quality of medicinal materials. At present, the rhubarb can be propagated mainly from seeds and the seed quality is closely related to the quality of rhubarb. In order to ensure and improve the quality of seeds and realize the quality standardization of medicinal plants, it must has solved and guaranteed a source of quality problems from fountainhead.

The objective of the present study was to gain new insights into the ASE for on-line purification and HPLC–PDA–ESI/MS^n method for simultaneous identification and quantification of flavonoids in RPL seeds from Qinghai. Furthermore, the resulting flavonoids components in RPL seeds were determined for the first time by a HPLC–PDA method. Based upon our validation results, the developed method was used for rapid separation, convenient identification and quantification of the multiple flavonoids in RPL seeds, featuring good quantification parameters, accuracy and precision. In addition, the presented study elucidates the main active ingredients for RPL seeds, which also provide evidence for developing excellent components from this plant resource.

2. Materials and methods

2.1. Pretreatment of sample and preparation of chemicals

The RPL seeds were collected from Qinghai in late autumn/winter and they were dried in a dryer, cut into segments, crushed into powder and then loaded into an air-tight container and stored in an exsiccator.

Petroleum ether (Analytical grade, 60–90 °C), methanol and glacial acetic acid were supplied by Tianjin Baishi Chemical Co., Ltd. The former two of analytical reagent grade were used for the plant extraction, the latter one and methanol (Shandong Yuwang Pharmaceutical Co., Ltd. Chemical Branch, China) of chromatographic grade were used for the mobile phase of HPLC analysis. Ultrapure water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

The standards of epicatechin (batch number: 110878–200102; MWs: 290), hyperoside (batch number: 111521–201406; MWs: 464), quercitrin (batch number: 111538–20040; MWs: 448), quercetin (batch number: 100081–200406; MWs: 302) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with purity $\geqslant$98%. Myricetin (batch number: E-0298; MWs: 318) was purchased from Shanghai Tanto Biotech CO., Ltd. (Shanghai, China) with purity $\geqslant$98%. Their structures are shown in Fig. 1.

2.2. Sample preparation using ASE 350 system

Sample preparation was performed using a Dionex ASE 350 system (Dionex Corp., Sunnyvale, CA, USA). 2.0 g of the air-dried RPL seed powder (20–30 mesh) was mixed with diatomaceous earth and placed into a 34 mL stainless steel extraction cell. The extraction was carried out under the optimized conditions. Lipids were removed at room temperature by petroleum ether and then the five flavonoids were extracted by 80% aqueous methanol. The extract was concentrated to obtain the residue in a water-bath at 60 °C by using a rotary

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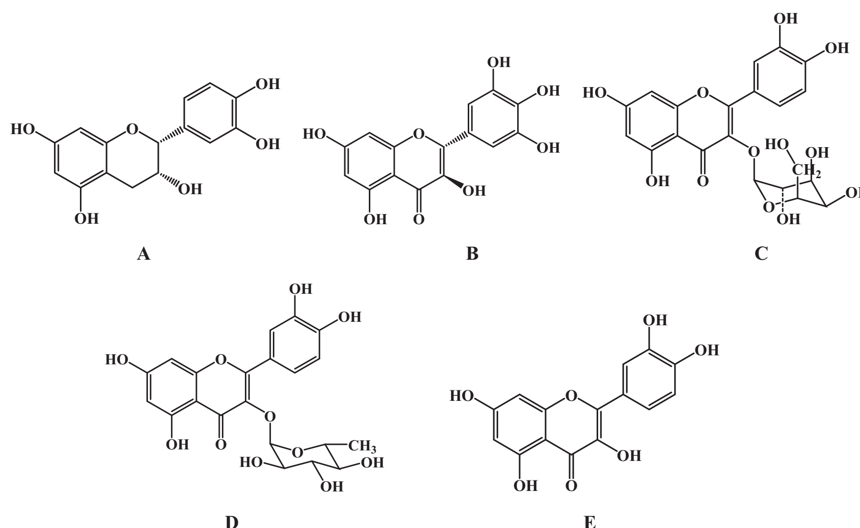

Fig. 1 Chemical structures of epicatechin (A), myricetin (B), hyperoside (C), quercitrin (D) and quercetin (E).

evaporator, followed by being transferred into a 25 mL volumetric flask and made up to its volume with extraction solvent and filtered through a 0.45 μm Econofilter (Agilent Technologies, USA) prior to analysis. 20 μL of the sample solution was injected into the HPLC system.

2.3. Apparatus, chromatographic and mass spectroscopic conditions of HPLC–PDA–ESI/MS^n

Waters 515 HPLC system was equipped with Waters 515 HPLC pump, a Waters 2996 PDA detector, a Waters column temperature controller, a pump control module coupled with an empower chromatography workstation. For chromatographic analysis, an Agilent Eclipse XDB-C_{18} column (4.6×250 mm, 5 μm), with gradient elution as the mobile phase, was adopted. The standards and samples were separated using a gradient mobile phase which consisted of 0.5% aqueous acetic acid (A) and methanol (B). The gradient condition was as followed: 0–28 min, 35–43% B; 28–45 min, 43–60% B. The flow rate was 1.0 mL min^{-1} and the column temperature was 30 °C. The chromatography was recorded use a PDA detector at 280, 320 and 360 nm to provide real time chromatograms and on-line Ultraviolet (UV) spectra from 190 to –400 nm were recorded for plant components identification. The solution was filtered through a membrane filter (0.45 μm) prior to HPLC analysis and the injection volume was 20 μL.

The quantitation was simultaneously achieved by MS^n detection in the positive (PI) and negative ionization (NI) modes for all the analytes. The HPLC flow was introduced into the ESI interface following detection by UV absorption measured from 200 to 400 nm. The flow rate was 1.0 mL min^{-1} with 25% of eluent being split into the inlet of the mass spectrometer. The parameters were as follows: the collision gas was argon (Ar), the flow rate was 0.18 mL min^{-1}; high purity nitrogen (N_2) was used as the sheath gas; the capillary temperature was 350 °C; the desolvation gas flow rate was 650 L h^{-1}; the cone gas flow rate was 50 L h^{-1}; the radiofrequency (RF) lens voltage was 0.50 V; and the scanning range was from 50 to 1000 *m*/*z*. For a full-scan mass spectrum the capillary voltage was 3.0 kV and the cone voltage was 50 V. The MS^n spectra were obtained with the collision energy (CE) adjusted to 30–45% of maximum. These parameters were optimized in preliminary experiments.

2.4. Stock and preparation of working standard solutions

Standard stock solutions of epicatechin (0.226 mg mL^{-1}), myricetin (0.121 mg mL^{-1}), hyperoside (0.414 mg mL^{-1}), quercitrin (0.885 mg mL^{-1}) and quercetin (0.336 mg mL^{-1}) were prepared in 5 mL volumetric flasks and diluted with methanol to obtain appropriate concentrations for content determination. The solutions were transferred to 10 mL glass brown vials, sealed using elastic plastic film (Parafilm, Chicago, IL, USA) and stored in a refrigerator (4 °C) for analysis.

2.5. Method validation

Methanol stock solution containing epicatechin, myricetin, hyperoside, quercitrin and quercetin were diluted with methanol to obtain a series of concentrations of working solutions, and subjected to HPLC analysis. The calibration curves of compounds 1, 2, 4, 7 and 9 were constructed with five different concentrations by determining the best fit of peak area ratios versus concentration and fitted to linear regression using a weighing factor ($1/\chi^2$).

The estimate for the limit of quantification (LOQ) was calculated using signal-to-noise (S/N) ratio of 10. The limit of detection (LOD) represents the lowest concentration that can be reliably determined at a signal-to-noise (S/N) ratio of about 3. The LOD and LOQ were established for the sensitivity. The stock solutions mentioned above were diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions were injected into HPLC for analysis.

The precision, repeatability and stability of the method were assessed as follows. The precision of the method was evaluated by calculation of the percent difference of assayed values from known concentrations of standard solutions. Methanol stock solutions above were diluted for five times with methanol as high concentration of quality control samples solutions, ten

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times as medium concentration solutions and two hundred times as low concentration solutions. The intra- and inter-day precisions were determined by assay of five replicates in each different concentration levels on one day and three different consecutive days, respectively. The results were expressed with the relative standard deviation (R.S.D). To assess the repeatability of the method, five different sample working solutions prepared from the same sample was subjected to an HPLC analysis. The stability was tested by analyzing the sample solution of RPL seeds. The peak areas of the five analytes, stored at room temperature for 0, 2, 4, 8, 12, 16 and 24 h, were recorded and compared in terms of R.S.D.

Spike recovery was used to evaluate the accuracy of the assay. Known amounts of individual standards were added into accurately weighed samples. The mixtures were extracted and analyzed in triplicate using the method described in Section 2.2.

2.6. Identification and quantification of five flavonoids in sample

The different compounds were identified by comparing the retention time (t_R), UV λ_{max} and MS^n data with those of known standard references. Quantification was performed on the basis of external standardization of the UV absorption peak area at 280 nm for epicatechin and 360 nm for other flavonoids.

3. Results and discussion

3.1. Online purification using ASE 350 system

The purification process can be simplified through on-line purification. ASE is capable of efficient extraction of multiple components. Some lipids were extracted out when the RPL seeds were extracted with methanol directly and the chromatographic column could be polluted with these lipids. Petroleum ether and chloroform were investigated as degreasing solvents. Petroleum ether had a suitable polarity and did not cause the loss of the components like as flavonoids, so it was selected to remove fat-soluble components.

3.2. Optimization of ASE procedure

The optimization of the ASE procedure was performed using the RPL seeds. The parameters, included the species and concentrations of solvent (70%, 80%, 90%, 100% ethanol; 70%, 80%, 90% and pure methanol), temperature (70, 80, 90 °C), static extraction time (5, 10, 15, 20 min) and number of cycles (1, 2, 3), were studied using a univariate approach. The peak areas of the five flavonoids were used as markers for evaluation of extraction efficiency. The results showed that 80% aqueous methanol had the highest extraction efficiency (87.2%, 90.3%, 90.9%, 87.6% for 70%, 80%, 90%, 100% ethanol, respectively; 91.4%, 95.5%, 94.1%, 92.9% for 70%, 80%, 90%, 100% methanol, respectively) and the peak areas were stable at 80 °C. The exhausted extraction for the ASE procedure was determined by performing consecutive accelerated solvent extractions on the same sample under the optimized ASE conditions, until no investigated compounds were detected by the analysis. Taking into account above, the conditions of the ASE method proposed were as follows: solvent, 80% aqueous methanol; temperature, 80 °C; static extraction time, 10 min and the number of cycles, 1. The extraction time was reduced and the efficiency was greatly improved.

3.3. Optimization of the HPLC–PDA–ESI/MS^n conditions

The chromatographic conditions were optimization as follows. (1) Analysis time was longer using isocratic elution and there were more components of similar polarity which interfered with the determination of target components. Therefore, gradient elution was the best choice that not only achieved peak separation requirements, but also greatly reduced the analysis time. (2) Acetonitrile–water, methanol–water and methanol–0.5% aqueous acetic acid as elution systems were investigated. The elution system of methanol–0.5% aqueous acetic acid could achieve separation requirements and a better peak shape. (3) The wavelengths at 280, 320 and 360 nm were selected within the range of 190–400 nm for UV scanning. Although there were bigger chromatographic peaks at the wavelength of 320 and 360 nm, there was lack of epicatechin peak in both of them. Considering each compound was monitored at its maximum absorption for better selectivity and sensitivity, 280 nm was chosen as the detection wavelength for epicatechin and 360 nm for others. (4) For ESI/MS^n analysis, both positive and negative modes were attempted. Only the negative mode afforded a high sensitivity of the target compounds.

3.4. Analysis the MS^n spectra of flavonoids based on the fragmentation mechanism

The five components (peaks 1, 2, 4, 7 and 9) were identified unambiguously as epicatechin, myricetin, hyperoside, quercitrin and quercetin respectively by comparing the retention time (t_R), UV λ_{max} and MS^n data with those of known standard references. Purity angle of each component was less than purity threshold and it demonstrated that the five peaks were pure. The data of t_R, masses of the $[M-H]^-$ ions, elemental composition, the MS^n experiments, the UV λ_{max} and the peak purity values of the five identified components in the extracts of the RPL seeds were established in Table 1. However, the other peaks 3, 5, 6, 8 and 10 cannot be identified exactly due to be lack of its available standard references. The fragmentation mechanism of precursor → product ion pairs were assumed as followed.

For compound **1**, (−) ESI-MS provided the $[M-H]^-$ ion at m/z 288.9, indicating the molecular formula $C_{15}H_{14}O_6$. The MS^2 spectrum: m/z 270.8 ($[M-H-H_2O]^-$, $C_{15}H_{11}O_5^-$), m/z 244.9 ($[M-H-CO_2]^-$, $C_{14}H_{13}O_4^-$), m/z 178.8 ($[M-H-C_6H_6O_2]^-$, $C_9H_7O_4^-$), m/z 204.9 ($[M-H-2(C_2H_2O)]^-$, $C_{11}H_9O_4^-$), m/z 136.9 ($[^{1,3}A^-]^-$, formed by the fragment containing A ring and the rest C ring after breakdown of carbon 1—carbon 3 bond) and m/z 124.9 ($[^{1,4}A^-]^-$, formed by the fragment containing A ring and the rest C ring after breakdown of carbon 1—carbon 4 bond). The MS^3 spectrum: m/z 160.9 ($[M-H-H_2O-C_6H_6O_2]^-$, $C_9H_5O_3^-$), m/z 186.8 ($[M-H-CO_2-C_3H_6O]^-$, $C_{11}H_7O_3^-$) and m/z 150.9 ($[M-H-C_6H_6O_2-CO]^-$, $C_8H_7O_3^-$). On the basis of these data, compound **1** was identified as epicatechin.

For compound **2**, (−) ESI-MS provided the $[M-H]^-$ ion at m/z 316.8, indicating the molecular formula $C_{15}H_{10}O_8$. The MS^2 spectrum: m/z 178.8 ($[M-H-C_7H_5O_3$(B ring and C-2 in

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C ring)]$^-$ at $C_8H_3O_5^-$, formed by the fragment containing A ring and the rest C ring after breakdown of carbon 2—carbon 1 bond and carbon 2—carbon 3 bond at the same time), *m/z* 270.9 ([M−H−H$_2$O−CO]$^-$, $C_{15}H_{11}O_5^-$), *m/z* 136.9 ($C_7H_5O_3^-$), *m/z* 191.8 ([M−H−C$_6$H$_5$O$_3$(B ring)]$^-$, $C_9H_4O_5^-$) and *m/z* 125.0 ($C_6H_5O_3^-$). The MS^3 spectrum: *m/z* 150.8 ([M−H−C$_7$H$_5$O$_3$−CO]$^-$, $C_7H_3O_4^-$). The MS^n of the protonated ion showed the same fragmentation pattern as that of myricetin, indicating that compound **2** was myricetin.

Compound **3** showed the [M−H]$^-$ ion at *m/z* 462.9, indicating the molecular formula $C_{21}H_{20}O_{12}$. The MS^2 spectrum: *m/z* 300.7 ([M−H−162]$^-$ at $C_{15}H_9O_7^-$, formed by the loss of a hexose moiety), *m/z* 178.9 ([M−H−162(hexose)−C$_7$H$_6$O$_2$(B ring and C-2 in C ring)]$^-$, $C_8H_3O_5^-$). The MS^3 spectrum: *m/z* 150.9 ([M−H−162(hexose)−C$_7$H$_6$O$_2$−CO]$^-$, $C_7H_3O_4^-$). As a result, the compound **3** maybe was quercetin-3-*O*-hexoside or an isomer of hyperoside.

Compound **4** showed the [M−H]$^-$ ion at *m/z* 462.9, indicating the molecular formula $C_{21}H_{20}O_{12}$. The fragmentation patterns were analyzed as above. The MS^2 spectrum: *m/z* 300.8 ([M−H−162]$^-$ at $C_{15}H_9O_7^-$, formed by the loss of a galactose moiety), *m/z* 178.8 ([M−H−162(gal)−C$_7$H$_6$O$_2$(B ring and C-2 in C ring)]$^-$, $C_8H_3O_5^-$). The MS^3 spectrum: *m/z* 150.9 ([M−H−162(gal)−C$_7$H$_6$O$_2$−CO]$^-$, $C_7H_3O_4^-$). Therefore, it was indicated that compound **4** was hyperoside.

Compound **5** showed the [M−H]$^-$ ion at *m/z* 625.3, indicating the molecular formula $C_{27}H_{30}O_{17}$. The MS^2 spectrum: *m/z* 463.3 ([M−H−162]$^-$ at $C_{21}H_{19}O_{12}^-$, formed by the loss of a hexose moiety), *m/z* 341.3 ([M−H−162(hexose)−C$_7$H$_6$O$_2$(B ring and C-2 in C ring)]$^-$, $C_{14}H_{13}O_{10}^-$), a fragment ion [M−H−162–60]$^-$, [M−H−162–90]$^-$ and [M−H−162–120]$^-$ at *m/z* 403.3 ($^{0,4}X^-$), 373.2 ($^{0,3}X^-$) and 343.2 ($^{0,2}X^-$), respectively (Fig. 2). The MS^3 spectrum: *m/z* 315.3 ([M−H−162–120−28]$^-$ at $^{0,2}X^-$-CO, $C_{16}H_{11}O_7^-$). The data above demonstrated that the compound **5** maybe was isoorientin-3-*O*-hexoside.

Compound **6** showed the [M−H]$^-$ ion at *m/z* 432.9, indicating the molecular formula $C_{20}H_{18}O_{11}$. The MS^2 spectrum: *m/z* 300.9 ([M−H−132(pentose)]$^-$ at $C_{15}H_9O_7^-$, formed by the loss of a pentose moiety), *m/z* 178.9 ([M−H−132(pentose)−C$_7$H$_6$O$_2$(B ring and C-2 in C ring)]$^-$, $C_8H_3O_5^-$). The MS^3 spectrum: *m/z* 150.9 ([M−H−132(pentose)−C$_7$H$_6$O$_2$−CO]$^-$, $C_7H_3O_4^-$). According to the data above, the compound **6** maybe was quercetin-3-*O*-pentoside.

The compound **7** had a similar fragmentation pattern as compound **4** (*m/z* 446.9 [M−H]$^-$ → *m/z* 300.8 [M−H−146(rha)]$^-$ → *m/z* 178.8 [M−H−146(rha)−C$_7$H$_6$O$_2$(B ring and C-2 in C ring)]$^-$ → *m/z* 150.9 [M−H−146(rha)−C$_7$H$_6$O$_2$ −CO]$^-$) with hyperoside due to their identical flavone

Table 1 Peak identification assignments of the five flavonoids in the RPL seed extract.

Peak no.	t_R (min)	[M−H]$^-$ (*m/z*)	Error (×10^{-6})	Elemental composition	(−) ESI/MS^n (*m/z*)	UV λ_{max} (nm)	Identification	Purity angle	Purity threshold
1	5.01	288.9	2.05	$C_{15}H_{14}O_6$	288.9 → 270.8 → 160.9, 288.9 → 244.9 → 186.8, 288.9 → 178.8 → 150.9, 288.9 → 204.9, 288.9 → 136.9, 288.9 → 124.9	279.8	Epicatechin[a]	0.177	0.459
2	12.85	316.8	−1.64	$C_{15}H_{10}O_8$	316.8 → 270.9, 316.8 → 178.8 (136.9) → 150.8, 316.8 → 191.8 (125.0)	254.2, 376.5	Myricetin[a]	0.678	1.172
4	16.21	462.9	1.39	$C_{21}H_{20}O_{12}$	462.9 → 300.8 → 178.8 → 150.9	256.2, 357.1	Hyperoside[a]	1.545	2.115
7	22.05	446.9	3.42	$C_{21}H_{20}O_{11}$	446.9 → 300.8 → 178.8 → 150.9	256.2, 352.3	Quercitrin[a]	0.069	0.233
9	35.15	300.8	−2.27	$C_{15}H_{10}O_7$	300.8 → 178.8 → 150.9, 300.8 → 256.8	256.2, 371.2	Quercetin[a]	0.565	1.138

[a] Compound identified in the RPL seeds exact.

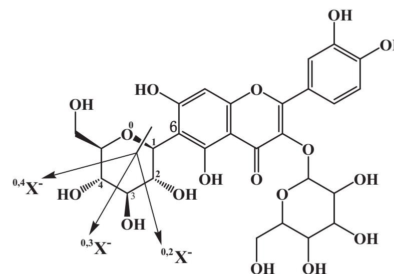

Fig. 2 Fragmentation pathways of the 6-*C*-glucosides.

aglycone structure. Therefore, we determined that compound **7** was quercitrin.

Compound **8** and **10** showed the same $[M-H]^-$ ion at *m/z* 489.1, indicating the molecular formula $C_{23}H_{22}O_{12}$. The MS^2 spectrum: *m/z* 447.1 ($[M-H-42]^-$ at $C_{21}H_{19}O_{11}^-$, formed by the loss of a acetyl moiety), *m/z* 285.2 ($[M-H-42–162 (hexose)]^-$, $C_{15}H_9O_6^-$), *m/z* 257.2 ($[M-H-CO]^-$, $C_{14}H_9O_5^-$), *m/z* 185.1 ($[M-H-C_4H_4O_3]^-$, $C_{11}H_5O_3^-$). The MS^3 spectrum: *m/z* 229.2 ($[M-H-CO-CO]^-$, $C_{13}H_9O_4^-$) and *m/z* 211.1 ($[M-H-CO-HCOOH]^-$, $C_{13}H_7O_3^-$). As a result, one or the other maybe was kaempferol-3-*O*-(6-*O*-acetyl)-hexoside or compound **8** was the isomer of compound **10**.

Compound **9** had a similar fragmentation pattern (*m/z* 300.8 $[M-H]^- \rightarrow$ *m/z* 178.8 $[M-H-C_7H_6O_2$(B ring and C-2 in C ring)$]^- \rightarrow$ *m/z* 150.9 $[M-H-C_7H_6O_2-CO]^-$) with hyperoside. In addition, the MS^2 spectrum showed another fragment ion $[M-H-44]^-$ at *m/z* 256.8 (base peak, $C_{14}H_9O_5^-$), formed by the loss of a CO_2 moiety. Based on the basis data above, we determined that compound **9** was quercetin.

3.5. The validation results of linearity, sensitivity, precision, stability and accuracy

The method was validated in terms of linearity, sensitivity, precision, stability and accuracy. The regression equation, linearity range, LOD and LOQ of the five analytes are summarized in Table 2. It was found that the calibration curves for all five analytes showed a good linearity ($r^2 \geqslant 0.999$) within the test range and the data of LOD and LOQ for all investigated compounds were less than 2.98 μg mL^{-1} for PDA, indicating that this method is sufficiently sensitive.

The repeatability of the developed method which was analyzed as mentioned above was evaluated that the R.S.D was less than 2.52% for the analytes. The R.S.D values of the intra- and inter-day precisions were 0.98% and 1.65%, respectively. The range of mean recoveries of the five components was 91.3–93.9%, and the R.S.D was 1.3–2.9%. It is demonstrated that this method was also very accurate. Furthermore, the sample was stable for at least 24 h (R.S.D < 2.0%).

3.6. Quantification of five flavonoids in sample

The extract of RPL seeds was separated efficiently by using the optimized chromatographic conditions with a PDA set at 280 nm for epicatechin (Fig. 3A) and 360 nm for other flavonoids (Fig. 3B). The total ion current chromatogram of RPL seed methanolic extract in negative ion mode is shown in Fig. 3C.

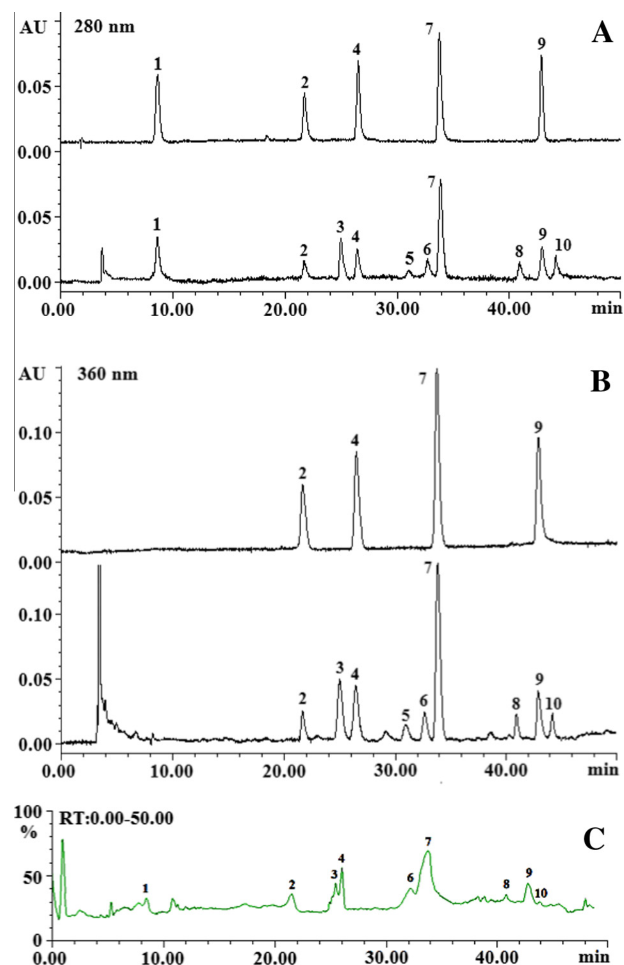

Fig. 3 HPLC–PDA chromatograms of the standards and RPL seeds extract (A) monitored at 280 nm, (B) monitored at 360 nm. (C) The total ion current chromatograms of RPL seeds methanolic extract in negative ion mode. Peak 1 = epicatechin; peak 2 = myricetin; peak 4 = hyperoside; peak 7 = quercitrin and peak 9 = quercetin.

The UV absorption, molecular ions, and retention times were established under the optimal experimental conditions for determining corresponding components in RPL seeds. The HPLC chromatograms of a mixed standard solution and sample solution are shown in Fig. 3.

Table 2 Calibration curves, linearity range, LOD and LOQ by HPLC–PDA ($n = 6$).

Compounds	Regression equation[a]	r^2[b]	Linear range (μg mL^{-1})	LOD[c] (μg mL^{-1})	LOQ[d] (μg mL^{-1})
Hyperoside	$y = 31525\ x - 19960$	0.9994	5.65–45.20	0.11	0.36
Rutin	$y = 49732\ x - 16884$	0.9990	3.03–24.20	0.14	0.45
Myricetin	$y = 38640\ x + 60655$	0.9990	10.35–82.80	0.47	1.15
Quercitrin	$y = 50979\ x - 92773$	0.9993	22.13–177.0	0.98	2.98
Quercetin	$y = 68916\ x - 33543$	0.9992	8.40–67.20	0.17	0.57

[a] In the regression equation $y = ax + b$, x denotes the concentration of compound injected, y is the peak area.
[b] r^2 is the correlation coefficient of the equation.
[c] Limit of detection.
[d] Limit of quantification.

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The linear calibration was performed to enable quantification of the flavonoids. The concentrations determined of the five flavonoids (epicatechin, myricetin, hyperoside, quercitrin and quercetin), in RPL seeds from Qinghai in China were, respectively, 0.296, 0.260, 0.553, 2.175 and 0.598 mg g^{-1} (mean values of three replicate injections). The concentrations of these analytes were great variant in the same sample. The results demonstrated that multiple flavonoids were detected and quantified in the RPL seeds and that, of the five flavonoids, the concentration of quercitrin was the highest while the contents of epicatechin and myricetin are relative lower. The results suggested that the quercitrin should be used as an important index for quality evaluation of RPL seeds. Generally, the five flavonoids were the major components in RPL seeds. The content of total identified flavonoids was 0.388% and it plus the content of unknown flavonoids, the data maybe was more than 0.50%. It was bigger than the content of medicinal parts (the rhizome and root) in rhubarb (Wang, 2005). The result demonstrated that the RPL seeds also had a good medicinal function.

The other peaks 3, 5, 6, 8 and 10 were not phenolic acids components by comparing the retention time (t_R), UV λ_{max} and MS^n data with those of known standard references like as protocatechuic aldehyde, caffeic acid, ferulic acid, gallic acid, chlorogenic acid and so on in our laboratory. There might be some phenolic acids compounds in RPL seeds, but they were not found in the experiment. The reason could be because the contents of phenolic compounds were low and they could not be extracted efficiently by using 80% aqueous methanol.

4. Conclusions

In this study, a reliable ASE and HPLC–PDA–ESI/MS^n method was reported for simultaneous identification and quantification of five flavonoids in RPL seeds. Lipids were removed using petroleum ether through on-line purification and effective components were extracted using ASE with the best optimization procedure: solvent, 80% aqueous methanol; temperature, 80 °C; static extraction time, 10 min and the number of cycles, 1. PDA detector can collect multiple wavelengths of chromatograms and corresponding spectra at the same time. The purity of chromatographic peaks can also be determined and it can be used for spectra retrieval. The advantage of ESI combined with tandem mass spectrometry has permitted ready study of the flavonoids, their ion chemistry, and the determination of flavonoids in low concentrations. Furthermore, HPLC–MS technique is able to separate each other single components in complex mixtures and to perform their identification and quantification. The chromatographic method was carried out on an Agilent Eclipse XDB-C_{18} column (4.6 × 250 mm, 5 μm) and methanol–0.5% aqueous acetic acid with gradient elution as the mobile phase was adopted. Ten flavonoids in the 80% aqueous methanolic extract of RPL seeds were extracted and separated, five of them were according identified as epicatechin, myricetin, hyperoside, quercitrin and quercetin for the first time with the negative mode in ESI/MS^n, under the experimental conditions illustrated, represents an important method. Considering each compound was monitored at its maximum absorption for better selectivity and sensitivity, 280 nm was chosen as the detection wavelength for epicatechin and 360 nm for myricetin, hyperoside, quercitrin and quercetin. Another five flavonoids were carried on the preliminary analysis of structures due to be lack of its available standard references. Based upon our validation results, the developed method was used for rapid separation, convenient identification and quantification of these flavonoids in RPL seeds, featuring good quantification parameters, accuracy and precision. It was found that the calibration curves for all five analytes showed a good linearity (r^2 > 0.999) within the test range and the data of LOD and LOQ for all investigated compounds were less than 2.98 μg mL^{-1} based on photodiode array. The repeatability of the developed method was evaluated that the R.S.D was less than 2.52% for the analytes. The R.S.D values of the intra- and inter-day precisions were 0.98% and 1.65%, respectively. The range of mean recoveries of the five components was 91.3–93.9%, and the R.S.D was 1.3–2.9%. Furthermore, the sample was stable for at least 24 h (R.S.D < 2.0%). It was found that the content of quercitrin is the highest in RPL seeds while the contents of epicatechin and myricetin are relative lower. The validation procedure confirmed that this method was suitable for not only the identification and quantification of five flavonoids, but also for providing quality control evaluation of RPL seeds to ensure the therapeutic benefits. In order to ensure and improve the quality of seeds and realize the quality standardization of medicinal plants, it must have solved and guaranteed a source of quality problems from fountainhead. It should be universally carried out the quality testing work of medicinal plant seeds for the sake of laying the foundations for establishment and development of rhubarb seed quality standards.

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