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ORIGINAL ARTICLE

Simultaneous characterization and quantification of flavonoids in *Euonymus alatus* (*Thunb.*) *Siebold* from different origins by HPLC-PAD–MS

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HPLC-PAD–MS

Abstract A high-performance liquid chromatography coupled with photodiode array detection and electrospray ionization tandem mass spectrometry (HPLC-PAD–ESI-MS) method has been developed for the simultaneous identification and quantification of active compounds (rutin and quercetin) contained in Traditional Chinese Medicine (TCM) *Euonymus alatus* (*Thunb.*) *Siebold* (EAS). The herb samples from ten main origins and five medicinal portions (leaf, fruit, stem, pterygium and root) were investigated. The separation was performed on a Shim C_{18} column at 30 °C with an isocratic elution. Methanol (A) and water (0.5% methanoic acid, v/v) (B) were used as mobile phases. The recoveries of the two compounds were 100.184% and 100.417%, respectively, and all of them showed good linearity ($r^2 \geqslant 0.9993$) in relatively wide concentration ranges. The developed method was applied to identify and quantify the two major active compounds in the collected herb samples, and the results indicated that contents of the two compounds in EAS varied significantly from habitat to habitat. It was demonstrated that the proposed method was helpful for the quality evaluation of EAS.

1. Introduction

EAS, belonging to the genus of *Euonymus*, is a deciduous shrub which is native to China, far eastern Russia, Japan, and Korea and it was first introduced into the United States about 1860 (Ma, 2001). As a commonly used Chinese folk medicine, EAS was first recorded in *Shen Nong Ben Cao Jing* (Shen, 2005), a Chinese herbal book, and noted in *Ben Cao Gang Mu* (a dictionary of Chinese herbs) (Li, 2003). As one of the important TCMs, EAS exhibits a variety of biological activities, including anti-hyperlipidemia (Park et al., 2005a,b), ameliorating hyperglycemia (Park et al., 2005a,b), anti-cancer (Lee et al., 1993) and so on. Recently, such

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herb-derived drugs have gained more and more attention due to their good treatments and low toxicity. Therefore, analysis of the major components contained in these herbs is of importance for the research and quality evaluation of these botanical drugs. Especially, a comparative analysis on herbal drugs collected from different origins or different medicinal portions was very useful for choosing the best origin or medicinal portion.

With the development of analytical techniques, many approaches such as thin layer chromatography (TLC) (Maleš and Medić-šarić, 2001), capillary electrophoresis (CE) (Molnár-Perl and Füzfai, 2005), HPLC with PAD (Olszewska, 2008; Tan et al., 2008) or MS (Zhang et al., 2008) detection have been reported to analyze many TCMs. Previous phytochemical studies revealed that EAS contains a variety of phytochemical constituents, including flavonoids (Fang et al., 2008a; Ishikura and Sato, 1977), alkaloids (Huang and Williams, 1999; Shizuri et al., 1973), cardenolides (Kitanaka et al., 1996) and others (Jin et al., 2005; Park et al., 2005a,b). But few researches have been reported to identify and quantify the main active compounds in EAS collected from different origins and different medicinal portions.

The objective of the present study was to establish a highly selective and specific HPLC-PAD–MS method for the identification and quantification of rutin and quercetin in the extract of EAS. Method validation indicated that the proposed method was well fit for the quantitative analysis of these flavonoids in the investigated herb samples from different regions. Moreover, the content of flavonoids in different medicinal portions of EAS was also studied using the developed method.

2. Materials and methods

2.1. Materials and chemicals

The herb samples were collected from ten different provinces in China (Shandong, Heilongjiang, Shanxi, Beijing, Yunnan, Hunan, Henan, Jilin, Jiangsu and Shanghai), and identified by Prof. Chun-Min Li (Heilongjiang Institute for Drug Control). The dried samples were crushed into powder and stored in an exsiccator. Different medicinal portions (leaf, fruit, stem, pterygium, and root) collected from Heilongjiang were also for comparative analysis. The standards of rutin (batch number: 100080–200306; MWs: 610) and quercetin (batch number: 100081–200406; MWs: 302) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures are shown in Fig. 1. Methanol of chromatographic grade (TEDIA Company, USA) was used for the HPLC analysis. Deionized water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol and methanoic acid of A.R. grade were used for the plant extraction and mobile phase, respectively.

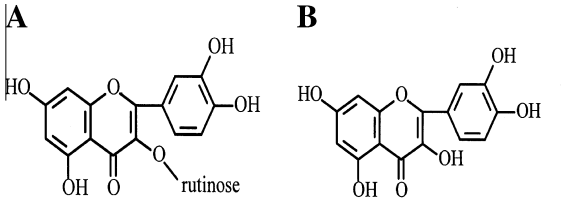

Figure 1 The chemical structures of rutin (A) and quercetin (B).

2.2. Apparatus and chromatographic conditions

A SHIMADZU HPLC system, consisting of a binary pump, an on-line degasser, and a PAD was used for acquiring chromatograms and UV spectra. For chromatographic analysis, a Shim C_{18} (4.6 mm × 150 mm, 5 μm) column with Kromasil C_{18} (4.6 mm × 10 mm, 5 μm) guard column was used at 30 °C. The mobile phase consisted of methanol–water–methanoic acid (42:57.5:0.5, v/v/v) and the flow rate was 1 mL min^{-1}. All solutions were filtered through a membrane filter (0.45 μm) prior to HPLC analysis and the injection volume was 10 μL. The compounds were monitored at 360 nm and UV spectra were recorded in the range 210–400 nm.

ESI–MS was performed by MICROMASS® Quattro Micro™ API. The flow rate was 1 mL min^{-1} with 14% of eluent being split into the inlet of the mass spectrometer. The mass spectra were recorded with ESI in the negative mode. The parameters were as follows: capillary voltage: 2.50 kV; cone voltage: 40 V; extractor voltage: 3 V; RF lens voltage: 0.3 V; source temperature: 100 °C; desolvation temperature: 200 °C; desolvation gas flow rate: 350 L h^{-1}; cone gas flow rate: 50 L h^{-1}; scanning range: from 100 to 1000 amu. These parameters were optimized in preliminary experiments to get the highest abundance of the targeted molecular-related ions. N_2 was used as both dry gas and nebulizer gas.

2.3. Stock and working standard solutions

Standard stock solutions of quercetin (0.08 mg mL^{-1}) and rutin (0.015 mg mL^{-1}) were prepared in 25 mL volumetric flasks and diluted with methanol to obtain appropriate concentrations for the establishment of calibration curves. The flasks were sealed by elastic plastic film (Parafilm, Chicago, IL, USA) and stored in a refrigerator (4 °C) until the analysis.

2.4. Sample preparation

Powdered samples (100 mesh, 2.5 g) were extracted three times with 25 mL of 60% (v/v) ethanol with reflux for 1.5 h. The extracts were filtered under vacuum and condensed into 20 mL. After resting for 12 h (4 °C), the concentrated solutions were filtrated under vacuum to remove chlorophyll. The extracts were dried to produce yellow powder, which were then mixed with 200 mL methanol.

2.5. Method validation

The proposed chromatographic method was validated to determine the linearity, limit of detection (LOD), precision, repeatability, stability and accuracy.

The calibration curve for each compound was established with six different concentrations by plotting the peak area versus concentration. Linear regression analysis was performed by the external standard method.

The LOD was defined as the lowest concentration of analytes in a sample that can be detected and was determined on the basis of the signal-to-noise ratios of 3:1. The standard solutions of the compounds for LOD were prepared by diluting them sequentially.

The precision, repeatability and stability of the method were assessed in this procedure. The precision test was carried out by injecting the same sample solution six times. The repeatability was assessed by analyzing six individual prepared samples. Stability was tested by analyzing one sample solution stored at room temperature for 0, 2, 4, 8, 16, and 24 h after preparation. The peak areas of rutin and quercetin were recorded and used to calculate the relative standard deviation (RSD).

The accuracy of the method was evaluated by analyzing the recovery percentage of compounds in the extract of EAS. The recovery assays of the two flavonoids were carried out by adding known quantities of the standard solutions to known amounts of samples of EAS.

2.6. Sample analysis

Ten microlitres of each sample solution was injected into the instrument. Identification of the different compounds was made by comparing the HPLC retention times, UV absorptions and MS spectra with those of the standards. Quantification was performed on the basis of linear calibration plots of the UV absorption peak area at 360 nm against concentration.

3. Results and discussion

3.1. Optimization of the HPLC–MS conditions

For LC–MS analysis, both positive and negative modes were attempted, only the negative mode afforded a high sensitivity of the target compounds.

3.2. Method validation

The results of the calibration curve and LOD were shown in Table 1. All the compounds displayed good linearity in the relatively wide concentration ranges.

The results of precision showed that RSDs of peak areas of rutin and quercetin were 1.629% and 0.493%, respectively. The RSDs of repeatability of the two compounds were 2.471% and 0.517%. In the stability test, the analytes were found to be rather stable within 24 h (RSD < 2.067%). The recoveries of rutin and quercetin were found in the range of 99.275–101.416% and 98.881–102.506%, and the RSDs of the recoveries were 0.905% and 1.362%, respectively.

3.3. Sample analysis

The spectroscopic and chromatographic characteristics of the compounds, including UV absorption, molecular ions, and LC retention times, were established under the experimental conditions for determining corresponding components in the samples.

The typical HPLC chromatograms of a mixed standard solution and sample solution collected from Heilongjiang Province were shown in Fig. 2. The UV and MS spectra of peaks 1 and 2 in the sample chromatogram were given in Figs. 3 and 4. The retention times, UVλ_{max} and MS signals of each compound were in good agreement to authentic compounds and reported literature values (Fang et al., 2008b; Lang et al., 2003; Wang et al., 2008).

Table 1 Linear relation between peak area and concentration (n = 6).

Compounds	Regression equation[a]	r^{2}[b]	Linear range (μg mL^{-1})	LOD[c] (μg mL^{-1})
Rutin	$y = 916.44x - 1346.6$	0.9996	1.60–25.60	0.06
Quercetin	$y = 1651.3x - 965.42$	0.9993	0.60–9.60	0.04

[a] In the regression equation $y = ax + b$, y refers to the peak area, x the concentration of the flavonoids.
[b] r^2: the square value of the correlation coefficient of the equation.
[c] LOD: the limit of detection (S/N = 3).

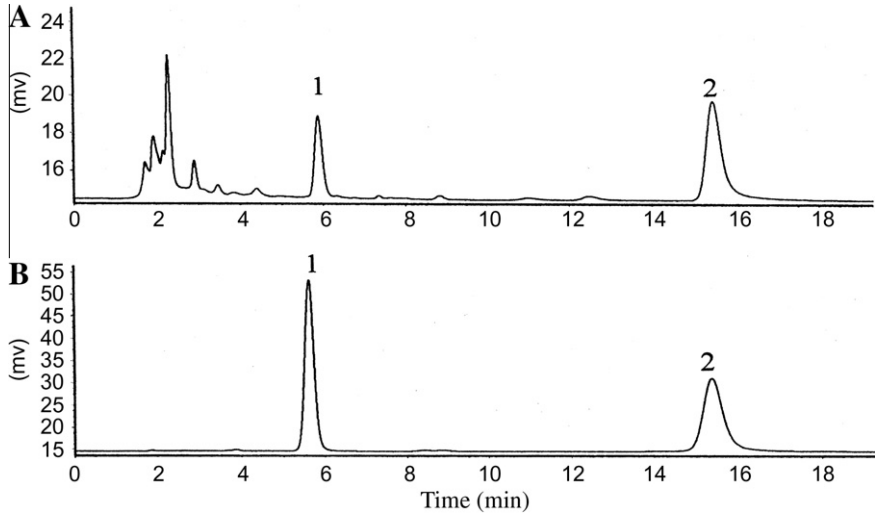

Figure 2 The HPLC chromatograms of the Heilongjiang sample (A) and the standards (B). Peaks: 1 = rutin; 2 = quercetin.

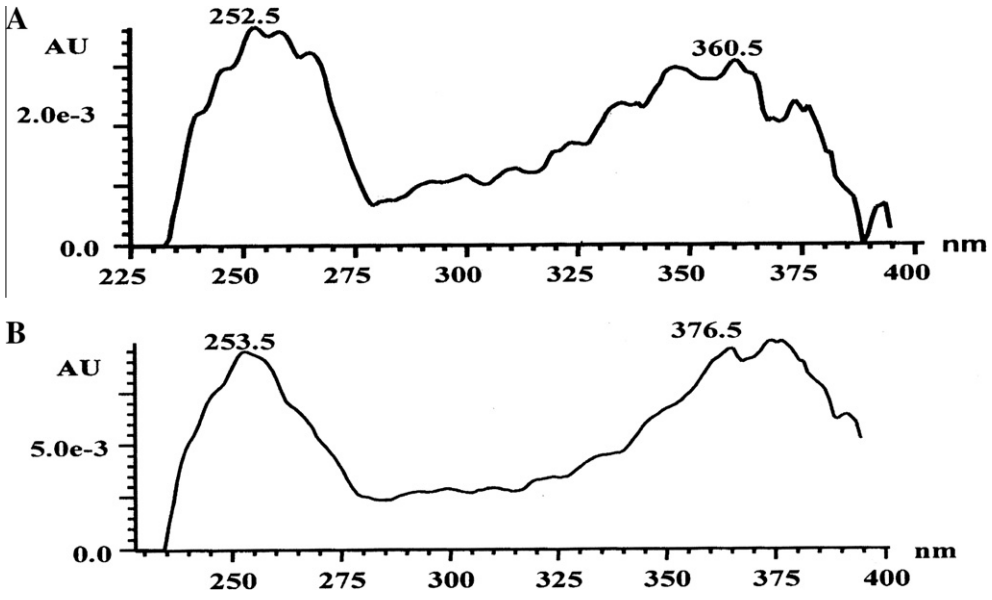

Figure 3 The UV spectra of peaks 1 (A) and 2 (B).

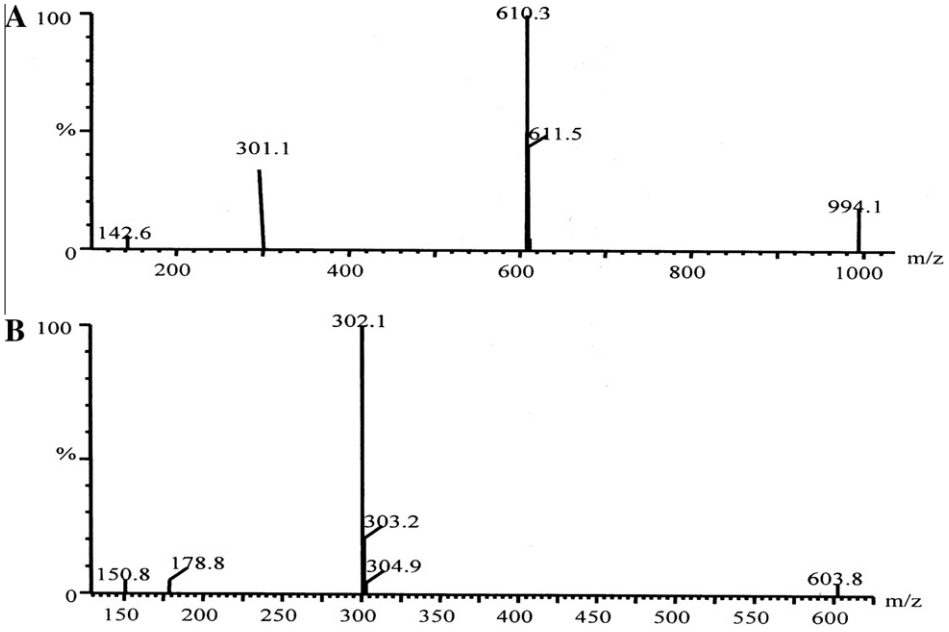

Figure 4 The MS spectra of peaks 1 (A) and 2 (B).

Quantification was performed on the basis of linear calibration. The contents of the two flavonoids in different samples from ten origins were calculated and the results were shown in Table 2 with the mean values of three replicate injections. The results indicated that the contents in this herb from different locations were varied. Especially, the compound of rutin in Yunnan sample was not detected. The contents in different medicinal portions were also given in Table 2 and there were the fewest contents of the compounds in the root among other parts which meant that this part need not be used, so this herb can be better protected and utilized continually.

4. Conclusions

A simple and validated method has been developed for the simultaneous characterization and quantification of the major flavonoids in EAS. According to the quantitative results, the variations of flavonoids in the samples of various locations and different parts were analyzed. This method could be used for both qualitative and quantitative analysis of EAS and their commercial products and could serve as a prerequisite for quality control and standardization of EAS products.

Table 2 The contents of rutin and quercetin in different samples ($n = 3$).

No.	Form and origin	Content (mg kg^{-1} crude drug)[a]		
		Rutin ($\times 10^3$)	Quercetin ($\times 10^3$)	Total ($\times 10^3$)
1	EP[b] (Shandong)	0.14 ± 0.01	0.22 ± 0.03	0.36 ± 0.03
2	EP (Heilongjiang)	0.24 ± 0.02	0.40 ± 0.03	0.64 ± 0.04
3	EP (Shanxi)	1.27 ± 0.12	0.42 ± 0.04	1.69 ± 0.11
4	EP (Beijing)	0.42 ± 0.06	0.40 ± 0.05	0.82 ± 0.09
5	EP (Yunnan)	ND[c]	0.03 ± 0.01	0.03 ± 0.01
6	EP (Hunan)	1.33 ± 0.11	0.18 ± 0.01	1.51 ± 0.08
7	EP (Henan)	0.54 ± 0.05	0.09 ± 0.02	0.63 ± 0.06
8	EP (Jilin)	0.23 ± 0.02	0.10 ± 0.01	0.33 ± 0.02
9	EP (Jiangsu)	0.66 ± 0.08	0.36 ± 0.02	1.02 ± 0.09
10	EP (Shanghai)	0.73 ± 0.10	0.04 ± 0.01	0.77 ± 0.07
11	Leaf (Heilongjiang)	1.03 ± 0.09	0.53 ± 0.03	1.56 ± 0.11
12	Fruit (Heilongjiang)	0.30 ± 0.01	1.51 ± 0.06	1.81 ± 0.05
13	Steam (Heilongjiang)	1.40 ± 0.10	1.25 ± 0.07	2.65 ± 0.13
14	Pterygium (Heilongjiang)	0.19 ± 0.02	0.13 ± 0.01	0.32 ± 0.02
15	Root (Heilongjiang)	0.07 ± 0.01	0.08 ± 0.01	0.15 ± 0.01

[a] Data were expressed as mean ± SD of three experiments.
[b] EP: entire plant.
[c] ND: not detected.

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