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Simultaneous determination of catechin, rutin, quercetin kaempferol and isorhamnetin in the extract of sea buckthorn (*Hippophae rhamnoides* L.) leaves by RP-HPLC with DAD

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Abstract

A rapid and specific reversed-phase high performance liquid chromatography (RP-HPLC) method with diode array detection (DAD) at room temperature was used and validated for the simultaneous determination of five flavonoids (catechin, CA; rutin, RU; quercetin, QU; kaempferol, KA; isorhamnetin, IS) in the extract of sea buckthorn (*Hippophae rhamnoides* L.) leaves. The sample pretreatment process involved ultrasonic extraction with 85% ethanol under the frequency of 80 kHz, at a temperature of 45 °C for 30 min and with the ratio of liquor to material of 15 mL g^{-1}, followed by separation on HIQ SIL C18V column with methanol–acetonitrile–water (40:15:45, v/v/v) containing 1.0% acetic acid as a mobile phase. The extract was detected by DAD at the wavelength of 279 nm for CA, 257 nm for RU, 368 nm for QU, KA and IS. Calibration curves were found to be linear with the ranges of 0.011–0.520 mg ml^{-1} (CA), 0.007–0.500 mg ml^{-1} (RU), 0.019–0.280 mg ml^{-1} (QU), 0.010–0.440 mg ml^{-1} (KA) and 0.008–0.400 mg ml^{-1} (IS). The correlation coefficients of linear regression analysis and detection limits were between 0.9963–0.9999 and 0.00079–0.00290 mg ml^{-1}. The contents of CA, RU, QU, KA and IS in sea buckthorn leaves were successfully determined with 3.8, 5.2, 7.3, 10.9 and 11.9 min with satisfactory reproducibility and recovery. Recoveries of the five flavonoids were between 97.27 and 99.98%. The method was applied to the determination of flavonoids in sea buckthorn leaves and was found to be simple, rapid and efficient.

Keywords: Sea buckthorn (*Hippophae Rhamnoides* L.) leaves; RP-HPLC; Catechin; Rutin; Quercetin; Kaempferol; Isorhamnetin

1. Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is a thorny nitrogen fixing deciduous shrub, naturally distributed throughout Asia and Europe [1]. In China sea buckthorn, grown widely in the northern and southwestern regions, is a phytoprotective agent to human health. All parts of the plant are considered to be a good source of a large number of bioactive substances. The bioactive substances are reputed to have considerable medicinal value and are frequently used for cure cough, skin wounds, cardiovascular diseases, improving blood circulation and have antioxidant activity [2–4]. The chemical compositions of sea buckthorn have attracted considerable attentions in the world. The flavonoids in sea buckthorn are bioactive constituents, which are reported to suppress platelet aggregation, relieve cardiac disease and have antioxidants, hepato-protective and immunomodulatory properties [5–9]. CA, RU, QU, KA and IS are five major flavonoids in the extract of sea buckthorn leaves [10], which have similar molecular structures as shown in Fig. 1.

Several chromatographic methods have been documented for the determination of chemical compositions present in sea buckthorn [11–15]. All of the chromatographic methods, HPLC is applied widely [16–19]. CA, RU, QU, KA and IS are five major flavonoids in the extract of sea buckthorn leaves. Therefore, it is important for the simultaneous determination of the five major flavonoids. So far, no report on simultaneous determination of the five major flavonoids in sea buckthorn leaves by HPLC method.

In the study, HPLC with DAD was used for the simultaneous determination of CA, RU, QU, KA and IS in the extract of sea buckthorn leaves. The results indicate that this method is fast, sensitive and suitable to quantitative assessment in the flavonoids of sea buckthorn leaves.

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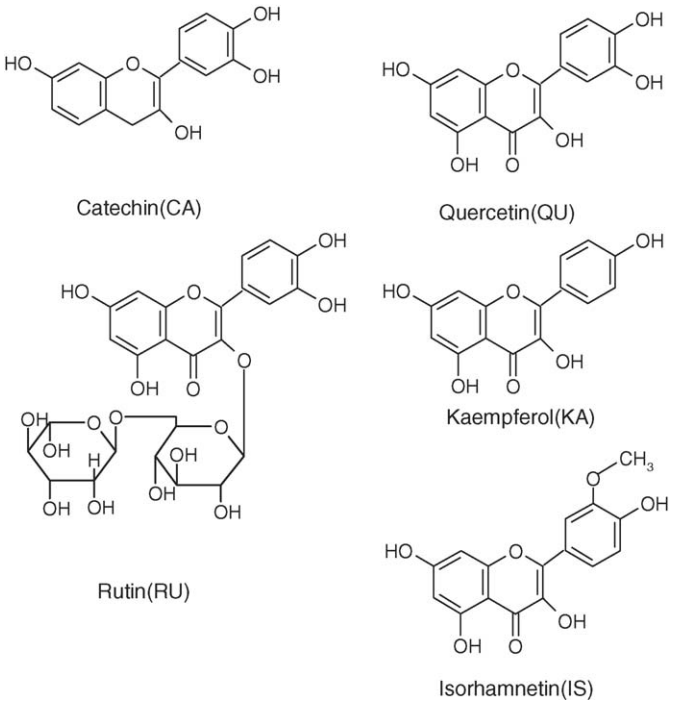

Fig. 1. Structures of five flavonoids in the extract of sea buckthorn leaves.

2. Experimental

2.1. Equipments

The chromatographic system consisted of Millennium32 system software(Waters company, USA), Waters 717 plus Autosampler (Waters company, USA), Model Waters Delta 600 pump (Waters company, USA), and Model Waters 2996 Diode Array Detector (Waters company, USA), HIQ SIL C18 V column(ø 4.6 mm × 250 mm, KYA TECH Corporation, Japan).

2.2. Reagents and materials

2.2.1. Reagents and chemicals

Methanol, acetonitrile and acetic acid were of HPLC grade (Tedia Company, USA). Ethanol was of analytical grade (Beijing Chemical Reagents Company, China). Deionized water was prepared by a Milli-Q Water Purification system (Millipore, MA, USA). CA, RU, QU, KA and IS standards were purchased from Sigma company (USA).

2.2.2. Plant material

Leaves of sea buckthorn were collected from the hilly Da He Kou forestry centre of Sunwu county, Heilongjiang province.

2.3. Preparation of standard solutions

Standard stock solutions of five flavonoids were prepared in ethanol, at concentration of 0.520 mg ml^{-1} for CA, 0.500 mg ml^{-1} for RU, 0.280 mg ml^{-1} for QU, 0.440 mg ml^{-1} for KA and 0.400 mg ml^{-1} for IS. All sample solutions were filtered through 0.45 μm membrane filter (Millipore), and injected directly.

2.4. Preparation of sample solution

The fresh leaves were cleaned and dried at 60 °C. The extraction was carried out using 2 g of powdered leaves with 30 ml of 85% ethanol under 80 kHz, 45 °C in an ultrasonic extraction device for 30 min, repeated for twice. The extract was collected and filtered, the filtrate was dried at 50 °C under reduced pressure in a rotary evaporator. The dried extract was dissolved in the mobile phase. After filtering through a filter paper and a 0.45 μm membrane filter (Millipore), the extract was injected directly.

2.5. Chromatographic conditions

Chromatographic analysis was carried out by HIQ SIL C18V reversed-phase column (ø 4.6 mm × 250 mm) packed with 5 μm diameter particles, the mobile phase was methanol–acetonitrile–water (40:15:45, v/v/v) containing 1.0% acetic acid. This mobile phase was filtered through a 0.45 μm membrane filter (Millipore), then deaerated ultrasonically prior to use. CA, RU, QU, KA and IS were quantified by DAD following RP-HPLC separation at 279 nm for CA, 257 nm for RU, 368 nm for QU, KA and IS. Flow rate and injection volume were 1.0 ml/min and l0 μl, respectively. The chromatographic peaks of the analytes were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using external standard method. All chromatographic operations were carried out at ambient temperature.

3. Results and discussion

3.1. Optimization of sample preparation

Reflux, soxhlet and ultrasonication (all with 85% ethanol as extractants, leaves powder dried under 60 °C, 60 meshes sifter) were, respectively, used to extract total flavonoids in the sea buckthorn leaves, repeated twice. The extraction yield of total flavonoids in sea buckthorn leaves was determined according to the method of reference [20]. The results are shown in Fig. 2.

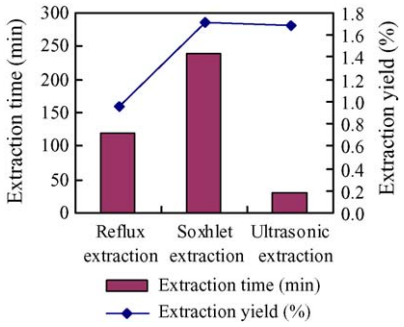

Fig. 2. Effect of different sample preparation methods on extraction yield of total flavonoids in the sea buckthorn leaves.

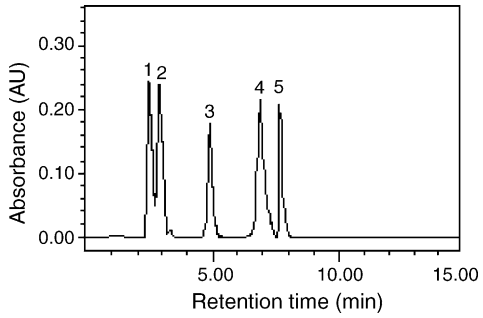

Fig. 3. Chromatogram of CA, RU, QU, KA extract, methanol, acetonitrile or water is and IS standard mixture (adding no acetic acid to mobile phase), Peak 1 for CA, usually used as a component of the mobile Peak 2 for RU, Peak 3 for QU, Peak 4 for KA, Peak 5 for IS.

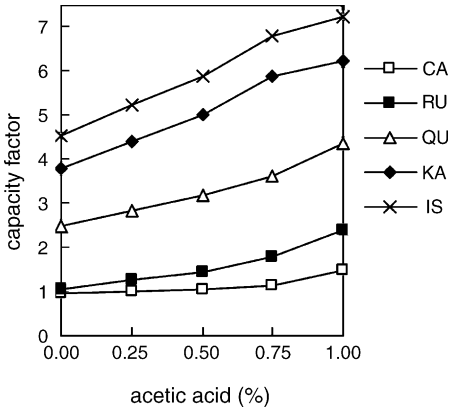

Fig. 4. Effect of acetic acid concentration on the values of the capacity factor.

Fig. 2 shows the extraction yield by reflux is obvious low, extraction yields by soxhlet and ultrasonication are higher and the results with different the extraction completeness by soxhlet depended to some extent on the extraction time, whereas that by ultrasonication was almost independent on the extraction time. Moreover, ultrasonication for 30 min gave the almost same result as soxhlet for 240 min did. Therefore, in this work, ultrasonic extraction is considered a simpler and more effective method for extraction of flavonoids in the sea buckthorn leaves and consequentially used in the following tests.

3.2. Optimization of chromatographic conditions

3.2.1. Effect of mobile phase

The five compounds, CA, RU, QU, KA, and IS are polar molecules. For polar phase. In the beginning, various proportions methanol–water or acetonitrile–water system was chosen as a mobile phase but separation was not satisfactory. In succession, a mixture of methanol, acetonitrile and water in different ratios was tested. Eventually, it was found that methanol–acetonitrile–water (40:15:45, v/v/v) system gave a much better separation for CA, RU, QU, KA, and IS except it couldn't baseline separate CA and RU. Under this system, slight trailing peaks were observed in the chromatograph (see Fig. 3.)

The presence of acid in a mobile phase system can improve peak trailing of compounds and change pH value of the mobile phase, having a significant effect on the resolution of compounds. Therefore, on the basis of methanol–acetonitrile–water (40:15:45, v/v/v) system, adding acetic acid to the mobile phase was considered at this point. The results obtained are illustrated in Fig. 4.

The concentration of acetic acid in the mobile phase was varied from 0.00 to 1.00%. The calculated capacity factor increases as the concentration of acetic acid increases (see Fig. 4). The significant difference is in the capacity factor between CA, RU and QU, KA, IS. The change in the capacity factor is very small for CA and RU. It is necessary to choose a proper volume percent acetic acid that gives enough differences in the values of the capacity factor for a good resolution in the RP-HPLC. In addition, volume percent of acetic acid in a mobile phase is crucial for column lifetime. The higher concentration acid added in mobile phase may produce more better sample separation, which in turn shorts the HPLC column lifetime. For above these reasons, 1.0% of acetic acid was chosen.

As a result, a mixture system of methanol–acetonitrile–water (40:15:45, v/v/v) containing 1.0% acetic acid was confirmed as the optimum mobile phase. Under this system, the chromatogram of CA, RU, QU, KA and IS standard mixture is shown in Fig. 5.

It can be seen from Fig. 5 that a good separation can be achieved within 15 min using the conditions described. Symmetrical, sharp and well-resolved peaks were observed for CA, RU, QU, KA and IS. The retention time for CA, RU, QU, KA and IS was 3.8, 5.2, 7.3, 10.9 and 1.9 min, respectively.

3.2.2. Effect of detection wavelength

The choice of proper detection mode is crucial to ensure that all the components are detected. With DAD, this problem can be overcome by using a multiple wavelength scanning program which is capable of monitoring several wavelengths simultaneously. It provides assurance that all the UV–vis absorbing components are detected, if present in sufficient quantity. DAD are used to record spectro-chromatograms of compounds simultaneously. So DAD was used to optimize determination wavelength in the work.

The UV–vis spectrums of CA, RU, QU, KA and IS standards dissolved in the mobile phase were obtained by DAD.

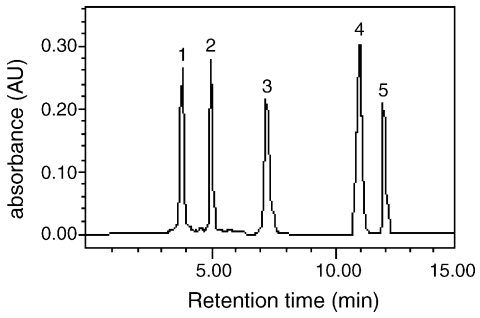

Fig. 5. Chromatogram of CA, RU, QU, KA and IS standard mixture (adding acetic acid to mobile phase), Peak 1 for CA, Peak 2 for RU, Peak 3 for QU, Peak 4 for KA, Peak 5 for IS.

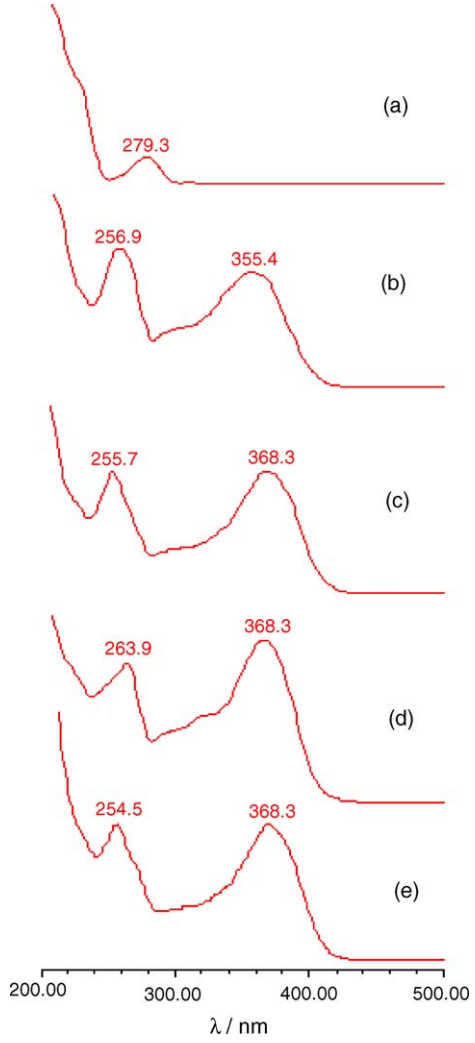

Fig. 6. UV–vis absorbing spectrograms of a mixture of CA, RU, QU, KA and IS standardard: (a) for CA; (b) for RU; (c) for QU; (d) for KA; (e) for IS.

UV–vis absorbing spectrograms of the five flavonoids are shown in Fig. 6.

From the spectrogram of CA (see Fig. 6(a)), we can see only a peak appeared at 279 nm. Two hundred and seventy-nine nanometers was confirmed as determination wavelength for CA. While from the spectrogram of RU (see Fig. 6(b)), we can see two clear peaks appeared at 257 and 355 nm separately. The peak at 257 nm is higher than the peak at 355 nm, that is RU's UV-absorbing value at 257 nm is higher than the value at 355 nm. So 257 nm was confirmed as determination wavelength for RU. Alike, on the spectrogram of QU (see Fig. 6(c), KA (see Fig. 6 (d) and IS (see Fig. 6(e)), also two peaks separately appeared at two different wavelength. 368 nm was confirmed as determination wavelength for QU, KA and IS according to their corresponding UV–vis absorbing value.

3.3. System suitability test

3.3.1. Reproducibility, linearity and detection limit

A standard mixture solution of 0.26 mg ml^{-1} CA, 0.25 mg ml^{-1} RU, 0.14 mg ml^{-1} QU, 0.22 mg ml^{-1} KA and 0.20 mg ml^{-1} IS was analyzed five times to determine the reproducibility of the peak areas and retention time under the optimum conditions in this experiment. The relative standard deviations (R.S.D.) of the peak areas and retention time were 1.02 and 1.05% for CA, 1.46 and 1.58% for RU, 2.06 and 2.23% for QU, 1.73 and 2.04% for KA, 1.69 and 1.85% for IS.

A series of the standard mixture solutions of these five flavonoids were tested to determine the linearity between the standard mixture concentration and peak areas. The results of regression analysis on calibration curves and detection limits are presented in Table 1. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3 (S/N = 3), the detection limits was between 0.00079 and 0.00290 mg ml^{-1} for five compounds.

3.3.2. Precision and stability

The intra-day and inter-day precisions (expressed as the relative standard deviation (R.S.D.) for retention time and peak area were determined for all five flavonoids standards by repeated analysis ($n = 5$) (see Table 2).

The results obtained from Table 2 show that intra-day and inter-day relative standard deviations for retention time and for peak area are both quite low and the precision is good.

For stability test, a sample solution (ethanol extract) was analyzed every 12 h in 2 days, and the sample solution was found to be rather stable within 48 h (R.S.D. < 2.0%).

3.3.3. Sample analysis and recovery

Ethanol solutions in the extract of sea buckthorn leaves were injected directly and separated under the optimum condition mentioned earlier. The typical chromatograms in the extract of sea buckthorn leaves were shown in Fig. 7.

The calculated contents of the five flavonoids were given in Table 3.

The recovery experiments of the five flavonoids were performed by adding CA, RU, QU, KA and IS standards to the extract of sea buckthorn leaves, which were treated according to

Table 1
Validation data from calibration curves of five flavonoids in the extract of sea buckthorn leaves

Compound	Regression equation	Correlation coefficient (R)	Linear range (mg ml^{-1})	Detection limit (mg ml^{-1})
CA	$y = 3.09 \times 10^{-8}x - 0.0261$	0.9963	0.011–0.520	0.00092
RU	$y = 2.08 \times 10^{-8}x + 0.0629$	0.9981	0.007–0.500	0.00079
QU	$y = 9.60 \times 10^{-9}x + 0.0145$	0.9995	0.019–0.280	0.00290
KA	$y = 1.44 \times 10^{-8}x + 0.0088$	0.9999	0.010–0.440	0.00115
IS	$y = 1.15 \times 10^{-8}x + 0.0103$	0.9993	0.008–0.400	0.00233

x, peak area; y, concentration (mg ml^{-1}).

Table 2
Precision ($n = 5$)

Compound	Intra-day R.S.D. for retention time (%)	Intra-day R.S.D. for peak area (%)	Inter-day R.S.D. for retention time (%)	Inter-day R.S.D. for peak area (%)
CA	1.12	1.20	1.93	1.53
RU	1.04	1.32	1.73	1.68
QU	1.36	1.36	1.82	1.42
KA	1.26	1.19	1.60	1.22
IS	1.51	1.46	1.72	1.56

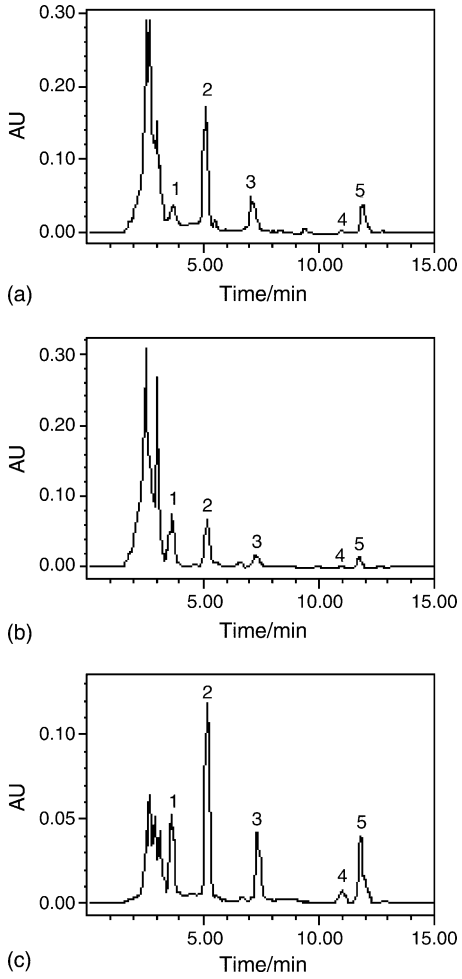

Fig. 7. Chromatograms of a sample under different detection wavelength. 256 nm for (a), 279 nm for (b), 368 nm for (c), Peak 1 for CA, Peak 2 for RU, Peak 3 for QU, Peak 4 for KA, Peak 5 for IS.

Table 3
Contents of five flavonoids in the extract of sea buckthorn leaves ($n = 5$)

Component	Content (%)	R.S.D. (%)
CA	0.04	2.14
RU	0.30	1.72
QU	0.49	1.55
KA	0.37	1.28
IS	0.42	1.41

Table 4
Recoveries of the five flavonoids in the extract of sea buckthorn leaves ($n = 5$)

Compound	Amount added (mg/ml)	Recovery (%)	R.S.D. (%)
CA	0.10	98.59	0.68
	0.30	97.27	0.54
RU	0.10	99.57	0.58
	0.30	98.04	0.24
QU	0.10	98.37	0.91
	0.30	97.52	0.56
KA	0.10	99.98	0.32
	0.30	97.93	0.25
IS	0.10	99.19	0.82
	0.30	99.02	0.23

the procedure described in Section 2.4 for five times. The recoveries for the five flavonoids was between 97.27 and 99.98% (see Table 4).

4. Conclusion

The RP-HPLC method mentioned here represented an excellent technique for simultaneous determination of CA, RU, QU, KA and IS in the extract of sea buckthorn leaves, with good sensitivity, precision and reproducibility. The method gives a good resolution among CA, RU, QU, KA and IS with a short analysis time (15 min). The sample preparation involving ultrasonic extraction is very simple. Furthermore, the method can be used as quality control of flavonoids in sea buckthorn and will play a reference role on the determination of flavonoids in other medicinal plants or pharmaceutical preparations.

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