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Original Article

RP-HPLC-DAD Detrmination of Flavonoids: Separation of Quercetin, luteolin and Apigenin in *Marchantia Convoluta*

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Abstract

High performance liquid chromatography coupled with photodiode array detector (HPLC-DAD) has been reported to quantify isolated flavonoids or these compounds in complex biological matrices, such as Chinese herbal drugs and products from factories. This work was designed, therefore, to develop an HPLC-DAD system to separate quercetin, luteolin and apigenin and to quantify them in extractive solutions from *Marchantia convoluta*. Flavonoids were analyzed on a Kromasil RP-C_{18} column; using a mobile phase, consisted of methanol-acetonitrile-acetic acid-phosphoric acid-H_2O (200:100:10:10:200, *V/V*); under the following conditions: detecting wavelength, 352 nm; flow rate, 0.60 ml/min; the sensitivity, 0.05 AUFS and the volume of injecting sample, 6.0 µl. The HPLC system was operated at ambient temperature (28±1°C). The method showed linearity for quercetin, luteolin and apigenin in the range 2.0-20.8, 2.2-24.0 and 1.6-20.0 µg/ml respectively, and the R.S.D. of the slope of the three lines was, respectively, 0.33%, 1.21% and 2.49% for quercetin, luteolin and apigenin. The aqueous and ethanol 80% extractive solutions showed linear response 1.5-15 µl/ml and ethanol 50% extractive solution in range 1.0-10 µl/ml. Precision and accuracy were determined for ethanol 80% extractive solution, in concentration of 10 µl/ml. The recoveries were 95.92-98.10%, 92.18-95.13% and 98.72-103.19% for quercetin, luteolin and apigenin respectively. RSD of results was 2.83-3.62%. The HPLC method showed an excellent performance in separating the flavonoids quercetin, luteolin and apigenin in *Marchantia convoluta* extracts, since the presence of interference has been previously evaluated and the mobile phase was chose carefully.

Keywords: HPLC-DAD; *Marchantia convoluta*; flavonoids; quercetin; luteolin; apigenin.

Introduction

Marchantia plants (Chinese name Di Qian) is a well-known traditional Chinese medicine and has been extensively used for the treatment of tumefaction of skins (1). It can protect liver and treat hepatitis (2). Flavonoids are almost universal pigments of plants. They are important parts of the human diet and considered as active principles of many medical plants. There are few reports about constituents of *Marchantia convoluta* living in China have been performed. Through our investigations on *Marchantia convoluta*, we found that the flavonoids, quercetin, luteolin and apigenin are the main constituents of its ethanol extracts. Moreover, the literature points out that some activities

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can be especially related to these flavonoids: antioxidant, anti-inflammatory, antiulcerative, antihepatotoxic and antiangiogenesis for quercetin (3-6), antiplatelet and vasodilatatory activities for luteolin (7-8) and anticarainogenic, anti-inflammatory, and antimutagenic for apigenin (9-12).

The methods of determination of flavonoids include UV-Vis spectrophotometry (13), HPLC (14) and LC-ESI-MS (15). Determination of contents of quercetin, luteolin and apigenin in *Marchantia convoluta* was not reported.

The high potential of utilization of *Marchantia convoluta* extracts in anti-HBV, antibiosis, diuresis in mice, as well as anti-inflammatory effect, which have been related to quercetin, luteolin and apigenin, demonstrate the interest in the separation of phytopharmaceutical preparations. This work was, therefore, designed to develop an HPLC system in order to separate and quantify the three flavonoids, namely, quercetin, luteolin and apigenin, in ethanol and aqueous extracts. The main validation parameters of the method are also determined for these complex matrices.

Experimental

Chemicals, reagents and materials

Methanol (**C**hromatographic grade, Jiangsu Hanbon Sci. & Tech. Co., Ltd), phosphoric acid (Analytical grade, Hanbon), acetonitrile (**C**hromatographic grade, Hanbon) and acetic acid (Analytical grade, Hanbon) were used for the mobile phase preparation. Quercetin (Sigma, St.Louis), luteolin (Sigma, St.Louis) and apigenin (Chinese Medicine Checking Institute) were used as external standards.

The whole plants of *Marchantia convoluta* were collected in Shangling City of Guangxi Zhuang Autonomous District in August 2003. The specimen (No 20041364) was identified by Zhou Zi-jing at Biology Department of Guangxi Chinese Medical University. The leaves, after being washed with water and dried in the shade for several days, were powdered.

Apparatus and chromatographic conditions

HPLC analysis was performed using equipment from Shimadazu (Japan): a Shimadazu LC-2010A liquid chromatograph, a Shimadazu SPD-M10A Diode Array Detector and a Shimadazu Class-vp V6.12 SP4 offline processing system. Flavonoids were analyzed using a Kromasil RP-C_{18} column (250×4.6mm i.d, 5μm, Hanbon Science &Technology Co., Ltd). The mobile phase consisted of a mixture, methanol-acetonitrile-acetic acid-phosphoric acid-H_2O (200:100:10:10:200, *V/V*) and the solution was degassed by suction-filtration through a nylon membrane. The detecting wavelength was 352 nm. The flow rate was 0.60 ml/min and the sensitivity was set at 0.05 AUFS. The quantity volume of injecting sample was 6.0 μl. The HPLC system was operated at ambient temperature (28±1ºC).

The analysis of the flavonoids was, additionally, monitored with a Shimadaz SPD-M10A Diode Array Detector, which measured absorbance (200-800nm) every 1 s with 4.8 nm. In this analysis, the same mobile phase, column and the other chromatographic conditions were employed.

Flavonoids calibration curves

Quercetin, luteolin and apigenin standards were dissolved in the mobile phase yielding concentrations of 26, 30 and 25 μg/ml. Accurately measuring fixed standard solutions of 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 ml to six 10 ml volumetric flasks were transferred and made up to the volume by mobile phase. The solutions were filtered through a 0.45 μm membrane filter. Evaluation of each point was repeated three times and each calibration curve was fitted by linear regression.

Preparation and analysis of testing solution

The aqueous extractive solution (AS) was prepared by decoction. Two other extractive solutions, S50 and S80, respectively, prepared by maceration in ethanol 50 and 80% (*V/V*). The plant: solvent ratio of 1:15 was employed for all extracts. All extracts were filtered through filter paper and the volume was made up to 500 ml with the mobile phase.

Preparation of extractive solutions curves

Samples of 1.0, 2.0, 3.0, 4.0 and 5.0 ml of the AS were diluted in the mobile phase up to 20 ml, yielding concentrations of 5, 10, 15, 20,

25 μl/ml. The solutions were filtered through a 0.45 μm membrane filter. The same procedure was employed for S50 and S80. Evaluation of each point was repeated three times.

Validation

The linearity was determined for the calibration curves obtained by HPLC analysis of quercetin, luteolin and apigenin and for the extractive solutions (AS, S50 and S80). The range of the appropriate amount of samples was then determined. The slope and the other statistics of the calibration curves were calculated by linear regression method.

The detection and quantitation limits were calculated based on S.D. and slope of the calibration curves.

Precision of the method was determined following Analytical Chemistry Guideline. For evaluation of the repeatability, the S.D. and R.S.D of six injections were considered. The intermediate precision was evaluated in triplicate for 3 days.

Accuracy was determined by recovery, adding measured amounts of quercetin, luteolin and apigenin to the extractive solutions. The recovery experiment was performed at three concentration levels (adding different flavonoids standards). The recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that were pre-preparation with the added standards, divided by amount added and then multiplied by 100%.

Determination of total flavone content in Marchantia convoluta

After carried out an orthogonal test, the best extraction condition for total flavones in *Marchantia convoluta* was discovered as following: temperature, 70°C; time, 30 min; times, 3; solvent, 80% alcohol; material to solvent ratio, 1:15. The whole plant (63.2144 g) was extracted with 80% alcohol (1000 ml) for 30 min at 70°C. The solvent was distilled off under reduced pressure. The residue was constant volume to 250 ml by methanol. Accurately measuring 2.5 ml above solution to 100 ml measuring flask was constant volume to 100 ml by the mobile phase. Then the test solution used for quantitative analysis was prepared.

Results and discussion

Calibration curves and detection limits

In this report, a method based on reversed-phase HPLC separation combined with UV spectrophotemetric detection was developed for flavonoids assay in *Marchantia convoluta* extracts. An isocratic system was chosen to

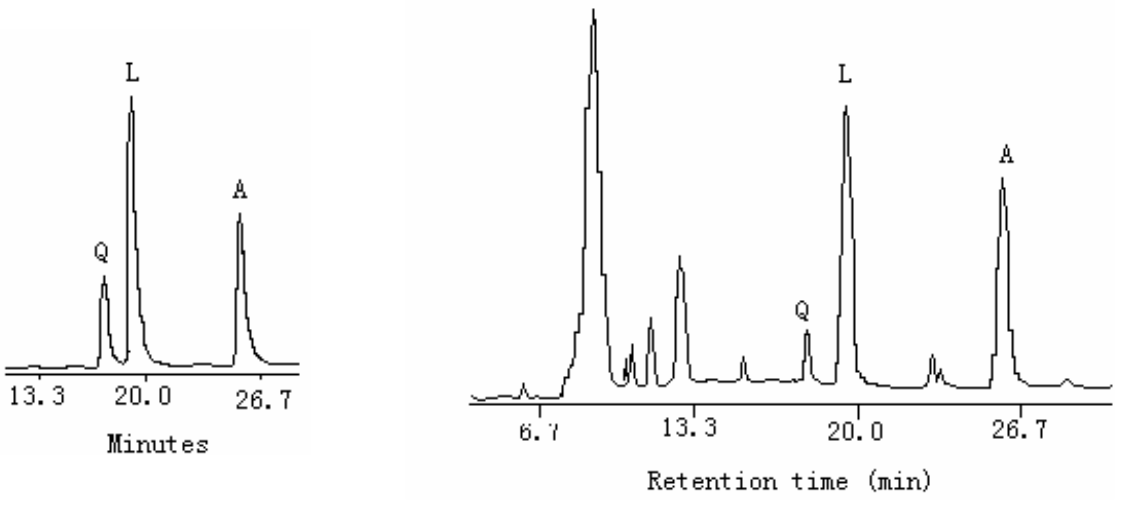

Figure 1. HPLC profile at 352 nm. (a) Flavonoids quercetin (Q), luteolin (L) and apigenin (A); (b) extraction solution S80. HPLC system: column, Kromasil RP-C18 column (250×4.6mm i.d, 5μm);mobile phase, methanol-acetonitrile-acetic acid-phosphoric acid-H2O (200:100:10:10:200, *V/V*); detecting wavelength, 352 nm; flow rate, 0.60 ml/min; sensitivity, 0.05 AUFS; quantity volume of injecting sample, 6.0 μl.

Table 1. Calibration curve of the extractive solutions AS, S50 and S80 by HPLC with the corresponding concentration (μg/ml) of quercetin, luteolin and apigenin

ES(μl/ml)	AS			S50			S80		
	Q	L	A	Q	L	A	Q	L	A
5	0.14	0.03	0.06	0.33	0.18	0.13	0.49	0.21	0.35
10	0.33	0.09	0.11	0.59	0.29	0.31	1.09	0.47	0.64
15	0.49	0.13	0.16	0.89	0.35	0.51	1.62	0.68	0.93
20	0.65	0.18	0.22	1.95	0.44	1.36	2.15	0.94	1.24
25	0.82	0.22	0.27	1.63	0.41	1.39	2.71	1.14	1.57

ES, extractive solution concentration; AS, aqueous extractive solution; S50, extractive solution obtained from ethanol 50% (*v/v*); S80 extractive solution obtained from ethanol 80% (*v/v*); Q, quercetin concentration (μg/ml in 20 ml solution); L, luteolin concentration (μg/ml in 20 ml solution); A, apigenin concentration (μg/ml in 20 ml solution).

minimize the variation of the baseline and ghost peaks. The mobile phase, a mixture of methanol-acetonitrile-acetic acid-phosphoric acid-H_2O (200:100:10:10:200, *V/V*), as well as the other chromatographic conditions, showed high performance in the separation of the three flavonoids, namely quercetin, luteolin and apigenin.

For validation of analytic methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Human Use (ICH) (16) and USP 24 (17) recommend the accomplishment of tests of accuracy, precision, specificity, linearity, work strip and robustness of the method. The type of method and its respective use determines what parameters should be evaluated, especially when the samples are complex biologic matrices, as in the case of extractive solutions from plants.

In this work, the linearity of the HPLC method was investigated for quercetin, luteolin and apigenin in the range 1.0-20.8 μg/ml, 0.6-24.0 μg/ml and 0.4-20.0 μg/ml respectively at six concentration levels. The linearity of the method was also investigated employing different amounts of extractive solutions AS, S50 and S80, obtained three calibration curves in the range 5-25 μl/ml.

Quercetin, luteolin and apigenin, respectively, retention times were 18.3, 20.1 and 26.9 min. The calibration curves for quercetin, luteolin and apigenin were in range 1.0-20.8, 2.2-24.0 and 1.6-20.0 μg/ml respectively, with excellent correlation coefficients (*r*). The representative linear equation for quercetin, luteolin and apigenin were, respectively, y=4232.7x-1672.2 (n=6; r=0.9995), y=12970.1x+941.5 (n=6; r=0.9995) and y=7572.7x-1075.9 (n=6; r=0.999 1). The R.S.D. of the slope of the three lines were, 0.33%, 1.21% and 2.49% for quercetin, luteolin and apigenin, respectively.

The detection limits, taken as the lowest absolute concentration of analysis in a sample, which can be detected but not necessary quantified under the stated experimental condition, were, respectively, 0.52, 0.48 and 0.60 μg/ml for quercetin, luteolin and apigenin. The limits of quantitation, taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, were, respectively, 1.91, 2.02 and1.46 μg/ml for quercetin, luteolin and apigenin.

Separation by HPLC

Selectivity of the proposed method was evaluated by analysis of the chromatograms of extractive solutions (AS, S50 and S80). The chromatograms of the three extractive solutions presented high resolution of the peaks of luteolin and apigenin, but resoulation of the peak of quercetin is low. These three flavonoids were separated well from each other and other constituents. It indicated that the proposed method could be applied for selective

Table 2. The recovery of standard addition obtained by HPLC (n=9)

Sample	Quantity (μg)	Added (μg)	Determined (μg)	Recovery (%)
apigenin	4.91	1.04 2.08 4.16	5.92 7.21 9.21	98.72 103.19 101.48
luteolin	3.58	1.20 2.40 4.80	4.41 5.55 7.97	92.18 92.83 95.13
quercetin	8.57	1.00 2.00 4.00	9.18 10.37 12.11	95.92 98.10 96.34

Table 3. Results of HPLC analysis

Sample	Percentage Determined (%)	Average found (%)	RSD(%,n=3)
apigenin	0.0045 0.0048 0.0049	0.0047	2.83
luteolin	0.0034 0.0036 0.0036	0.0035	2.80
quercetin	0.0077 0.0085 0.0082	0.0081	3.62

determination of the three flavonoids in the *Marchantia convoluta* liquid preparations (Fig. 1b).

Fig. 1 shows the HPLC profile at 352 nm of flavonoids quercetin, luteolin and apigenin in both standard and S80 solutions with the respective diode array spectra which are shown in (Figure 2a) and Figure 2b ; respectively. The identical spectra of the three standard flavonoids with the isolated flavonoids present in the S80 demonstrates minimum inferences from the matrix. The UV spectrum of the peaks with retention times of 12.7 and 8.9 min (Figure 2. x1 and x2) indicates that they correspond, probably, to flavonoids with *O*- or *C*-glycosides.

Determination of all extractive solutions

Considering that *Marchantia convoluta* preparations are biological samples and therefore, complex matrices, and the presence of interferences is possible, the extractive solution curves were used to determine the flavonoid contents of AS, S50 and S80, where linearity is observed. The concentration of the quercetin, luteolin and apigenin in these extractive solutions are showed in Table 1.

Table 1 shows the concentration of the three flavonoids in five levels of *Marchantia convoluta* extracts. The S80 presented the highest concentration of quercetin, luteolin and apigenin, at all the points of the calibration curve. It demonstrated that the flavonoid aglycones were better extracted from the inflorescences with the solvent of lower polarity.

The correlation coefficients of the calibration curves of AS and S80 were from 0.9975 to 0.9995 for AS, from 0.9991 to 0.9997 for S80. However, the extractive solution obtained with

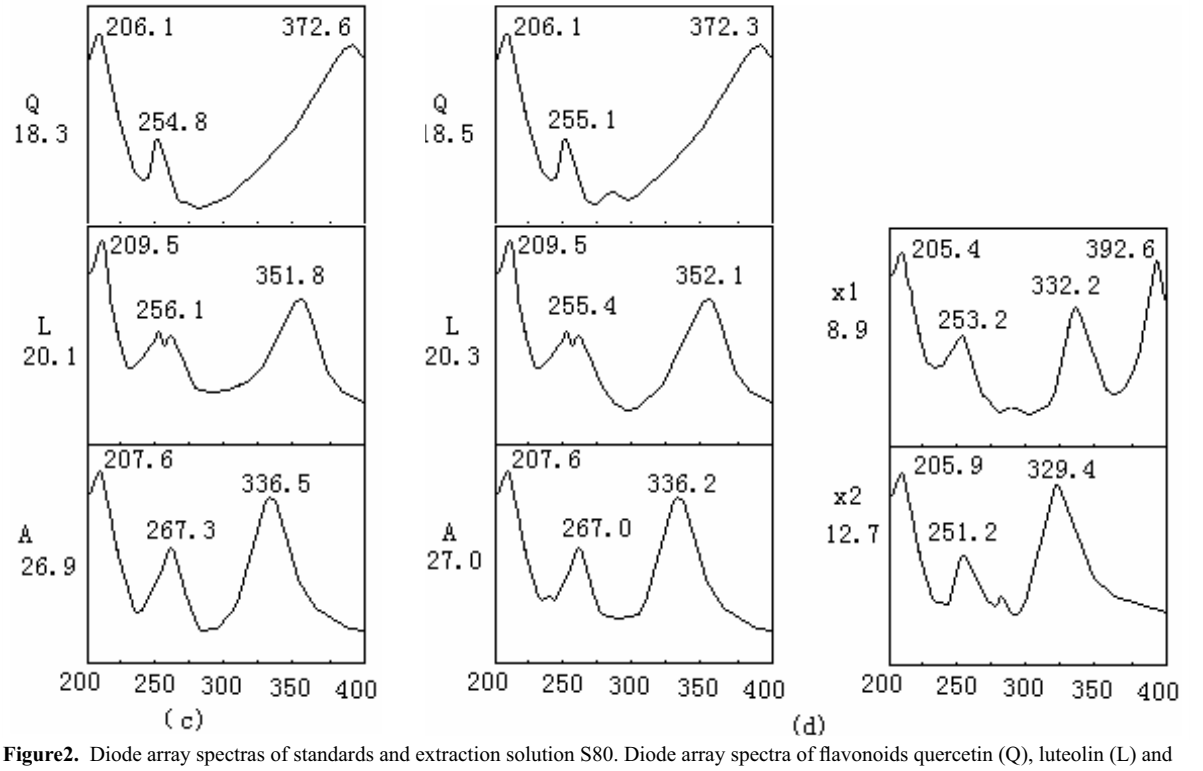

Figure2. Diode array spectras of standards and extraction solution S80. Diode array spectra of flavonoids quercetin (Q), luteolin (L) and apigenin (A) were Fig. 2 (a). The UV spectrums of the peaks with retention times of extractive solution S80 were Fig. 2 (b). X1 and x2 were unknown flavonoids.

ethanol 50% (S50) showed linearity deviation in concentrations higher than 20 µl/ml, yielding correlation coefficients of 0.9089, 0.9473 and 0.9037, for quercetin, luteolin and apigenin, respectively. These *r* values could be attributed to the presence of interferences in the extractive solution S50, in concentrations higher than 20 µl/ml. However, the correlation coefficients were, respectively, 0.9992, 0.9959 and 0.9995 for quercetin, luteolin and apigenin, in the range 5-15 µl/ml for S50.

Table 1 also shows that S80 presents the higher flavonoids concentration. Thus, the precision (repeatability and intermediary precision) and accuracy (recovery) were determined only for this solution. The repeatability of the HPLC analysis of S80 was demonstrated with R.S.D. of 3.52% for S80 in concentration of 10 µl/ml. This result could be considered satisfactory since the majority of phytochemical studies present a range of 3-6% for RSD. The intermediary precision of this sample showed a R.S.D. of 5.6%.

The R.S.D of the peax areas obtained by HPLC was 0.3% for the quercetin, 1.21% for luteolin and 2.49% for the apigenin, in the S80. The results showed high reproducibility between areas for the flavonoids, quercetin, luteolin and apigenin.

The accuracy of the HPLC method for the assay analysis, based on percent recovery, was determined by adding different flavonoids standard solutions for the samples. Table 2 showed the results of the recovery experiment. The recoveries were 95.92-98.10%, 92.18-95.13% and 98.72-103.19% for quercetin, luteolin and apigenin, respectively.

Determination results of quercetin, luteolin and apigenin in Marchantia convoluta

The results of the content of the flavonoids, quercetin, luteolin and apigenin in *Marchantia convoluta* were given in Table 3. The average contents of the three flavonoids, quercetin, luteolin and apigenin in *Marchantia convoluta* were 8.1×10^{-5}, 3.5×10^{-5} and 4.7×10^{-5} (%).

The HPLC method developed in this work allowing the separation of three HPLC method present in quercetin, luteolin and apigenin in *Marchantia convoluta*, is the first reported of determination of flavonoids in *Marchantia convoluta*.

The extractive solution curves showed linear response for AS and S80 in the range 5-25 µl/ml and for S50 in the range 5-15 µl/ml. Precision and accuracy were demonstrated for the solution containing the highest flavonoid concentration, S80.

In conclusion, the proposed HPLC method shows an excellent performance to separate and quantitative the flavonoids, quercetin, luteolin and apigenin in *Marchantia convoluta* extracts.

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