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LC determination of flavonoids: separation of quercetin, luteolin and 3-*O*-methylquercetin in *Achyrocline satureioides* preparations

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Abstract

The pharmacological activities of the flavonoids show the interest in quantifying these constituents in phytopharmaceutical preparations, as well as in the validation of the analytical methodologies. LC methods have been reported to quantify isolated flavonoids or these compounds in complex biological matrices, such as herbal raw materials and extractive preparations. This work was designed, therefore, to develop an LC system to separate quercetin, luteolin and 3-*O*-methylquercetin and to quantify them in extractive solutions from *Achyrocline satureioides*. The main validation parameters of the method are also determined. The method showed linearity for quercetin and luteolin in the range 1–10 μg/ml. The aqueous and ethanol 80% extractive solutions showed linear response in the range 2.5–20 μl/ml and ethanol 40% extractive solution in the range 2.5–10 μl/ml. Precision and accuracy were determined for ethanol 80% extractive solution, in the concentration of 10 μl/ml. The LC method showed an excellent performance in separating the flavonoids quercetin, luteolin and 3-*O*-methylquercetin in *A. satureioides* extracts, since the presence of interference has been previously evaluated.

Keywords: LC; Flavonoids; *Achyrocline satureioides*; Quercetin; 3-*O*-Methylquercetin; Luteolin

1. Introduction

The reports on *Achyrocline satureioides* (Lam.) D.C., Compositae, have shown that its extracts present anti-inflammatory, antioxidant and immunomodulatory activities [1–3]. The investigations on *A. satureioides* have demonstrated that the flavonoids, quercetin, luteolin and 3-*O*-methylquercetin are the main constituents of its ethanol extracts [4] and the relationship among these flavonoids with some pharmacological activities. Moreover, the literature points out that some activities can be especially related to these flavonoids: antioxidant, anti-inflammatory, antiulcerative, antihepatotoxic and antispasmodic for quercetin [5–8], antiplatelet and vasodilatatory activities for luteolin [3,9] and antiviral activity reported for 3-*O*-methylquercetin [10]. The rela-

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tionship between the flavonoids and the biological effects reveal the interest of quantifying these constituents in phytopharmaceutical preparations, as well as of the validation of the analytical methodologies.

The reported LC system employed to quantify quercetin, luteolin and 3-*O*-methylquercetin in *A. satureioides* preparations [11] did not achieve the separation of the luteolin from 3-*O*-methylquercetin. The retention time of the last two compounds was the same, consequently, luteolin and 3-*O*-methylquercetin were assayed together and the concentration of both expressed in luteolin.

The high potential of utilization of *A. satureioides* extracts in viral infection [12], as well as anti-inflammatory medicines [1] which has been related to 3-*O*-methylquercetin, quercetin and luteolin, demonstrate the interest in the separation of these flavonoids for the standardization of phytopharmaceutical preparations. This work was, therefore, designed to develop an LC system in order to separate and quantitate the three main *A. satureioides* flavonoids, quercetin, luteolin and 3-*O*-methylquercetin, in ethanol and aqueous extracts. The main validation parameters of the method are also determined for these complex matrices.

2. Experimental

2.1. Chemicals and reagents

Methanol (LC grade, Merck, Darmstadt, Germany), phosphoric acid (Merck) and LC-grade water (Milli-Q system, Millipore, Bedford, MA) were used for the mobile phase preparation. Quercetin (Merck) and luteolin (Sigma, St. Louis, MO) were used as external standard. 3-*O*-Methylquercetin (isolated from the plant) was only used as reference to the corresponding peak in the sample extracts.

2.2. Apparatus and chromatographic conditions

LC analysis was performed using equipment from Waters (Milford, MA): a pump Waters 510, an automatic controller of flow Waters 600, a Rheodyne 7125 injection valve with a 20 μl loop, a 486 UV variable-wavelength detector (set at 362 nm) and a Waters 746 integrator. Flavonoids were analyzed using a Shim-pack column CLC-ODS (M) RP-18, 5 μm, 250×4 mm i.d. The mobile phase consisted of a mixture, methanol–phosphoric acid 0.16 M (53:47, v/v) and the solution was degassed by suction-filtration through a nylon membrane (MFS, CA). The flow was 0.6 ml/min and the sensitivity was 0.05 AUFS. The LC system was operated at ambient temperature (23 ± 1 °C).

The analysis of the flavonoids in the extractive solutions were, additionally, monitored with a Waters Millenium (Milford), which measured absorbance (200–800 nm) every 1 s with 4.8 nm resolution. In this analysis, the same mobile phase, column and the other chromatographic conditions were employed.

2.3. Flavonoid calibration curves

Quercetin and luteolin standard were dissolved in methanol–water (53:47, v/v) yielding concentrations of 1, 1.5, 2, 2.5, 5 and 10 μg/ml. The solutions were filtered through a 0.45 μm membrane filter (Millipore-HVHP). Evaluation of each point was repeated three times and each calibration curve was fitted by linear regression.

2.4. Preparation and analysis of extractive solution

2.4.1. Preparation of the extractive solutions (ES)

The aqueous extractive solution (ESAQ) was prepared by decoction. Two other extractive solutions, ES40 and ES80 were, respectively, prepared by maceration in ethanol 40 and 80% (v/v). The plant:solvent ratio of 0.75:10 was employed for all extractive solutions. All the extractive solutions were filtered through filter paper (grade 1: 11 μm, Whatman, UK) and the volume was made up to 500 ml with the solvent.

2.4.2. Preparation of extractive solutions curves

Samples of 0.5, 1.0, 2.0, 3.0 and 4.0 ml of the ESAQ were diluted in methanol–water (53:47,

v/v) to 20 ml, yielding concentrations of 2.5, 5, 10, 15 and 20 μl/ml. The solutions were filtered through a 0.45 μm membrane filter (Millipore-HVHP). The same procedure was employed for ES40 and ES80. Evaluation of each point was repeated three times.

2.5. Validation

The linearity was determined for the calibration curves obtained by LC analysis of quercetin and luteolin and for the extractive solution curves (ESAQ, ES40 and ES80). 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.

The detection limit (DL) and quantitation limit (QL) were calculated based on the S.D. and the slope (S) of the calibration curves [13].

Precision of the method was determined following ICH guideline [13]. 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 and luteolin to extractive solutions. The recovery experiment was performed at three concentration levels (80, 100 and 120%). The recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100% [13].

3. Results and discussion

In this report, a method based on reversed-phase LC separation combined with UV spectrometric detection was developed for flavonoid assay in *A. satureioides* extracts. An isocratic system was chosen to minimize the variation of the baseline and ghost peaks. The mobile phase, a mixture of methanol–phosphoric acid 0.16 M (53:47, v/v), as well as the other chromatographic conditions, showed high performance in the separation of the three flavonoids, quercetin, luteolin and 3-*O*-methylquercetin.

For validation of analytic methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) [13] and USP 24 [14] 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 LC method was investigated for quercetin and luteolin in the range 1–10 μg/ml at six concentration levels. The linearity of the method was also investigated employing different amounts of extractive solutions ESAQ, ES40 and ES80, obtaining three calibration curves in the range 2.5–20 μl/ml.

Quercetin and luteolin presented, respectively, retention times of 24.2 and 29.2 min. The calibration curves for quercetin and luteolin were linear in the range 1–10 μg/ml, with excellent correlation coefficients (r). The representative linear equation for quercetin and luteolin were, respectively, $y = 29913.6x + 322238.4$ ($n = 6$; $r = 0.9991$; P-value $= 1.044$) and $y = 7110.2x + 314226.3$ ($n = 6$; $r = 0.9992$; P-value $= 0.963$). The R.S.D. of the slope of the three lines was, respectively, 6.0 and 8.0% for quercetin and luteolin. The retention time of 3-*O*-methylquercetin (authentic sample), as well as the diode array spectra, were used to identify the corresponding peak in the *A. satureioides* extractive solutions. Unfortunately, 3-*O*-methylquercetin is not available on the market and the amount isolated from *A. satureioides* by Simões [1], was only enough to use as reference to the corresponding peak. The concentration of this flavonoid was expressed in luteolin.

The detection limits, taken as the lowest absolute concentration of analyte in a sample which can be detected but not necessary quantified under the stated experimental condition, were, respectively, 0.32 and 0.31 μg/ml, for quercetin and luteolin. 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, 0.92 and 0.96 μg/ml, for quercetin and luteolin.

To date, no efficient method for the separation of 3-*O*-methylquercetin from luteolin and quercetin in *A. satureioides* extracts has been reported. 3-*O*-Methylquercetin and luteolin has been quantified together and the concentration of the 3-*O*-methylquercetin has been expressed in luteolin [12].

The selectivity of the proposed method was evaluated by the analysis of the chromatograms of extractive solutions (ESAQ, ES40 and ES80). The chromatograms of the three extractive solutions presented high resolution of the peaks of quercetin, luteolin and 3-*O*-methylquercetin, indicating that the proposed method could be applied for the selective determination of three flavonoids in the *A. satureioides* liquid preparations (Fig. 1).

Fig. 2 shows the LC profile at 362 nm of the flavonoids quercetin, luteolin and 3-*O*-methylquercetin (Fig. 2a) with the respective diode array spectra and Fig. 2(b) shows the peaks of these flavonoids in the SE80. The identical spectra of the three flavonoids isolated with the flavonoids present in the SE80 permits the inference on the absence of interference. The UV spectrum of the peak with a retention time of 60 min (Fig. 2b) indicates that it corresponds, probably, to a flavonoid with a chalcone structure. The precise identification of this compound is being investigated.

Considering that *A. satureioides* preparations are biological samples and therefore, complex matrices, in these cases the presence of interference is possible, the extractive solution curves were used to determine the sample amount of ESAQ, ES40 and ES80, where linearity is observed. The calibration curve of the quercetin, luteolin and 3-*O*-methylquercetin in these extractive solutions are shown in Tables 1 and 2.

Table 1 shows the concentration of the three flavonoids in five levels of *A. satureioides* extracts. The ES80 presented the highest concentration of quercetin and luteolin, at all the points of the calibration curve. The quercetin and luteolin concentrations in ESAQ were the smallest, demonstrating that the flavonoid aglycones were better extracted from the inflorescences with the solvent of lower polarity.

The regression equations, the correlation coefficients and the *P*-value of the aqueous extractive solution (ESAQ) and extractive solution obtained

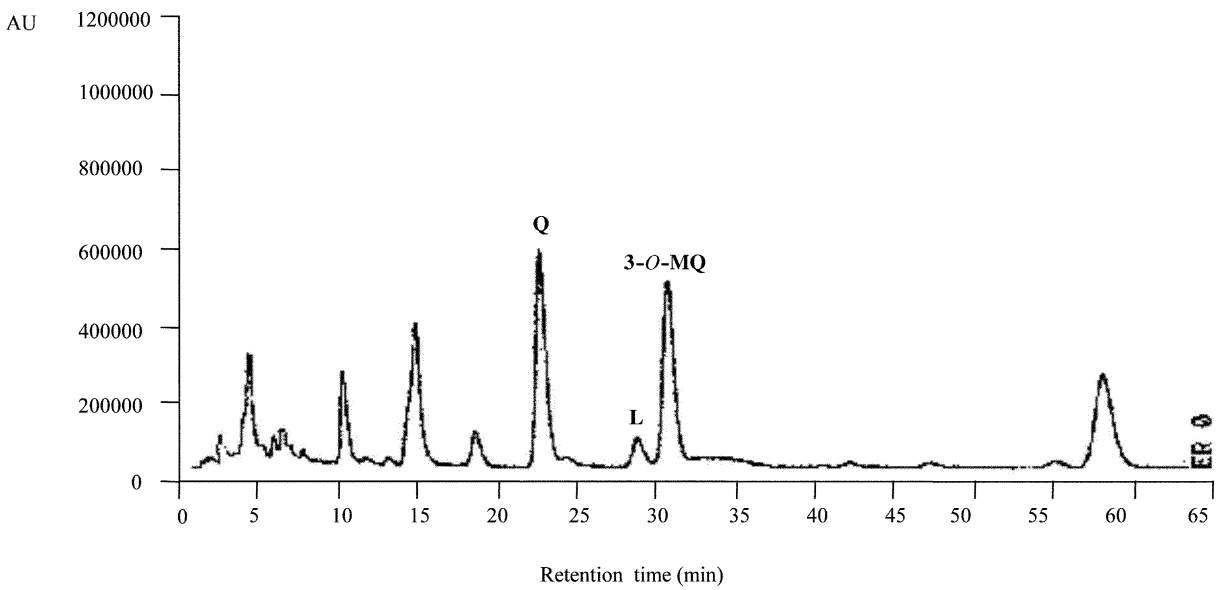

Fig. 1. Chromatograms of the flavonoids quercetin (Q), luteolin (L) and 3-*O*-methylquercetin (3-*O*-MQ) in the extractive solution ES80. Chromatographic conditions: column RP18 CLS-ODS (250 × 4.6 mm i.d., 5 μm); mobile phase: methanol–phosphoric acid 0.16 M (53:47, v/v); flow-rate, 0.6 ml/min; detection at 362 nm.

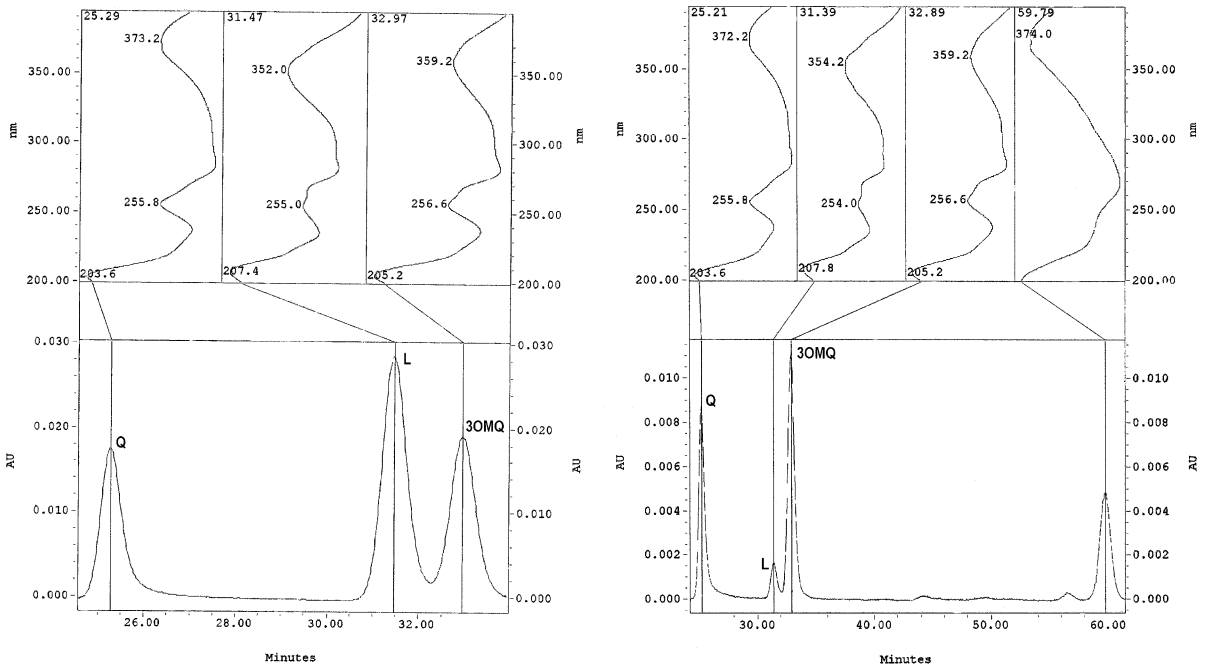

Fig. 2. LC profile at 362 nm and diode array spectra 200–400 nm. (a) Flavonoids quercetin (Q), luteolin (L) and 3-*O*-methylquercetin (3-*O*-MQ); (b) extractive solution ES80.

Table 1
Calibration curve of the extractive solutions ESAQ, ES40 and ES80 by LC with the corresponding concentrations (μg/ml) of quercetin, luteolin and 3-*O*-methylquercetin

ESC (μl/ml)	ESAQ			ES40			ES80		
	Q	L	3-*O*-MQ	Q	L	3-*O*-MQ	Q	L	3-*O*-MQ
2.5	0.127	0.008	0.153	0.290	0.074	0.612	0.650	0.140	0.858
5.0	0.362	0.019	0.338	0.650	0.170	1.220	1.310	0.200	1.469
10	0.672	0.061	0.691	1.410	0.330	2.452	2.860	0.400	2.916
15	1.044	0.092	1.067	3.120	0.700	5.226	4.540	0.640	4.447
20	1.370	0.145	1.429	2.520	0.550	4.106	5.590	0.850	5.575

ESC, extractive solution concentration; ESAQ, aqueous extractive solution; ES40, extractive solution obtained from ethanol 40% (v/v); ES80, extractive solution obtained from ethanol 80% (v/v); Q, quercetin concentration (μg); L, luteolin concentration (μg); 3-*O*-MQ, 3-*O*-methylquercetin expressed in luteolin.

with ethanol 80% (ES80) are presented in Table 2. Excellent linearity was obtained for all three flavonoids in these extractive solutions, demonstrated by the correlation coefficients of the calibration curves from 0.9940 to 0.9999 for ESAQ and 0.9972 to 0.9988 for ES80.

However, the extractive solution obtained with ethanol 40% (v/v) (ES40) showed linearity deviation in concentrations higher than 15 μl/ml, yielding correlation coefficients of 0.9083, 0.8953 and 0.8971, for quercetin, luteolin and 3-*O*-methylquercetin, respectively (Table 2). These *r* values

Table 2
Linearity data for the extractive solutions (ES) evaluated by regression analysis

ES	Slope			Intercept			Correlation coefficient		
	Q	L	3-*O*-MQ	Q	L	3-*O*-MQ	Q	L	3-*O*-MQ
ESAQ	22597.8	2444.7	22902.3	22113.8	1923.0	−2244.8	0.9989	0.9940	0.9999
ES40	49324.2	10253.8	76166.0	27067.6	14223.3	63009.6	0.9083	0.8953	0.8971
ES40*	48315.5	10765.3	77152.3	524.3	4531.2	5734.8	0.9998	0.9994	0.9999
ES80	93765.1	13156.8	86704.8	8609.6	8298.9	56045.9	0.9974	0.9972	0.9988

ESAQ, aqueous extractive solution; ES40, extractive solution obtained with ethanol 40% (v/v); ES40*, extractive solution obtained with ethanol 40% (v/v) in the range 2.5–10 μl/ml; ES80, extractive solution obtained with ethanol 80% (v/v); Q, quercetin; L, luteolin; 3-*O*-MQ, 3-*O*-methylquercetin.

could be attributed to the presence of interference in the extractive solution ES40, in concentrations higher than 15 μl/ml. However, the correlation coefficients were, respectively, 0.9998, 0.9993 and 0.9999 for quercetin, luteolin and 3-*O*-methylquercetin, in the range 2.5–10 μl/ml of ES40.

Table 1 also shows that ES80 present the higher flavonoid concentration, which is why the precision (repeatability and intermediary precision) and accuracy (recovery) were determined only for this solution. The repeatability of the LC analysis of ES80 was demonstrated with R.S.D. of 4.0% for ES80 in the concentration of 10 μl/ml. This result could be considered satisfactory since the majority of phytochemicals present a range of 3–6% [15]. The intermediary precision of this sample showed a R.S.D. of 6.2%.

The R.S.D. of the areas obtained by LC was 0.9% for the quercetin, 10.2% for luteolin and 2.3% for 3-*O*-methylquercetin, in the ES80. The results demontrated high reproducibility between areas for the flavonoids, quercetin and 3-*O*-methylquercetin. The high R.S.D. for the luteolin peak can be explained by its low concentration in ES80.

The accuracy of the LC method for the assay analysis of recovery was determined by preparing samples adding 80, 100 and 120% of quercetin and luteolin in the ES80. The recoveries of added flavonoids standards were, respectively, 100.7, 100.5 and 112.4% for quercetin, and 83, 103.6 and 81.9% for luteolin.

4. Conclusions

The LC method developed in this work allowing the separation of the three main flavonoids present in *A. satureioides*, is the first report of the separation of luteolin from 3-*O*-methylquercetin, by LC method.

The extractive solution curves showed linear response for ESAQ and ES80 in the range 2.5–20 μl/ml and for ES40 in the range 2.5–10 μl/ml. Precision and accuracy were demonstrated for the solution containing the highest flavonoid concentration, ES80.

In conclusion, the proposed LC method shows an excellent performance to separate and quantitative the flavonoids quercetin, luteolin and 3-*O*-methylquercetin in *A. satureioides* extracts.

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References

[1] C.M.O. Simões, E.P. Schenkel, L. Bauer, A. Langeloh, J. Ethnopharmacol. 22 (1988) 281–293.
[2] C. Desmarchelier, J. Coussio, G. Ciccia, Braz. J. Med. Biol. Res. 31 (1998) 1163–1170.

[3] A.L.G. Santos, D. Ripoll, N. Vardi, V.L. Bassani, Phytother. Res. 13 (1999) 65–66.
[4] G.E. Ferraro, C. Norbedo, J.D. Coussio, Phytochemistry 20 (8) (1981) 2053–2054.
[5] L.E. Pelzer, T. Guardia, A.O. Juarez, E. Guerreiro, Farmaco 53 (1998) 421–424.
[6] J.B. Harbone, C.A. Willians, Phytochemistry 55 (2000) 481–504.
[7] S.R. Husain, J. Cillard, P. Cillard, Phytochemistry 26 (9) (1987) 2489–2491.
[8] C. Morand, V. Crespy, C. Manach, C. Besson, C. Demigné, C. Rémésy, Am. J. Phys. 275 (1998) 212–215.
[9] G. di Carlo, N. Mascolo, A.A. Izzo, F. Capasso, Life Sci. 65 (4) (1999) 337–353.
[10] J.V. Formica, W. Regelson, Food Chem. Toxicol. 33 (12) (1995) 106–108.
[11] E. Lemos-Senna, P.R. Petrovick, G. Gonzalez Ortega, V.L. Bassani, Phytother. Res. 2 (1997) 123–127.
[12] C.M.O. Simões, M.B. Falkenberg, A. Mentz, E.P. Schenkel, M. Amoros, L. Girre, Phytomedicine 6 (3) (1999) 205–214.
[13] International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) Q2B. Validation of analytical procedures. Methodology, 1996.
[14] US Pharmacopeia 24 (USP 24). Validation of Compendial Methods. United States Pharmacopeia Convention, Rockville, 2000.
[15] W. Andlauer, M.J. Martena, P. Fürst, J. Chromatogr. A 849 (1999) 341–348.