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Simultaneous quantification of flavonoids from *Achyrocline satureioides* by a polar-reversed phase LC method — application to skin permeation/retention studies

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A selective and sensitive polar-reversed phase LC method was validated for simultaneous quantification of the main *Achyrocline satureioides* flavonoids (quercetin, luteolin, and 3-*O*-methylquercetin) in skin samples after permeation/retention studies from topical nanoemulsions. The method was linear in a range of 0.25 to 10.0 μg/mL exhibiting a coefficient of determination higher than 0.999 for all flavonoids. No interference of the nanoemulsion excipients or skin components was observed in the retention times of all flavonoids. The R.S.D. values for intra- and inter-day precision experiments were lower than 6.73%. Flavonoids recovery from nanoemulsions and skin matrices was between 90.05 and 109.88 %. In a permeation/retention study with porcine ear high amount of 3-*O*-methylquercetin was found in the skin sample (0.92 ± 0.22 μg/g) after two hours. The proposed method was suitable to quantify the main flavonoids of *A. satureioides* in skin permeation/retention studies from topical nanoemulsions.

1. Introduction

Achyrocline satureioides Lam. DC. (Asteraceae), known as marcela or macela, is a medicinal plant native to the Southeastern region of South America. Recently, there has been an increasing interest in developing topical products containing marcela extracts. Retta et al. (2012) reported the use of phytotherapeutic and cosmetic formulations containing this medicinal plant due to the antioxidant, anti-inflammatory, and ultraviolet-blocking activities of its extracts. Such activities have most likely been related to the presence of flavonoid aglycones (i.e., quercetin, luteolin, and 3-*O*-methylquercetin) extracted from the inflorescences (Kadarian et al. 2002; Bettega et al. 2004; Polydoro et al. 2004; Morquio et al. 2005; De Souza et al. 2007). However, the poor water solubility of these flavonoids hampered the incorporation of the *A. satureioides* extract into topical formulations.

Our research group recently patented an original procedure intended to incorporate flavonoid aglycones from crude ethanol *A. satureioides* extracts into nanocarriers composed by various lipid and/or polymer combinations (Carvalho et al. 2008). Such procedures proved to be able to incorporate high amount of extracts of this medicinal plant in monodisperse nanoemulsions (200 nm range) composed especially by triglycerides as oil core and lecithins as surfactants. The proposed method, which is based on a spontaneous emulsification procedure, exhibits some advantages including the feasibility of producing in one step small batches of formulations at moderate temperatures.

Several studies have reported the determination of *A. satureioides* flavonoids by means of liquid chromatography (LC). For instance, De Souza et al. (2002) reported the validation of an isocratic reversed-phase LC method combined with ultraviolet detection for separating quercetin, luteolin and 3-*O*-methylquercetin from ethanol and aqueous extracts of *A. satureioides*. Such a method was inserted in the monograph of this plant in the fourth edition of the Brazilian Pharmacopeia. The analytical conditions of the proposed method were recently optimized by Retta et al. (2011) in relation to the extraction method and the quantitation of 3-*O*-methylquercetin using an external reference standard. However, the analysis of the *A. satureioides* extract using the method described above is an exhaustive process due to the run time of approximately one hour. Furthermore, low resolution of some peaks was observed. From these studies, Bica (2009) described an improvement in the method for assaying *A. satureioides* flavonoids based on the use of a polar-reversed phase column. Such a method exhibits a short analysis time (15 min) and high resolution for the flavonoid peaks, which was especially attributed to the use of a chromatographic column composed of ether linked phenyl bonded silica (Whelan et al. 2005; Kayillo et al. 2006).

In this study, our goal was to validate an LC method to quantify the skin retention of quercetin (QCT), luteolin (LUT), and 3-*O*-methylquercetin (3-*O*-MQ) in porcine ear skin after a retention assay from *A. satureioides*-topical nanoemulsions. Even though the literature describes the use of LC to assay *A. satureioides* flavonoids, as previously mentioned, a literature survey reveals the absence of methods to simultaneously quantify *A. satureioides* flavonoids in skin samples from nanostructured systems.

2. Investigations, results and discussion

This study was aiming to validate a polar-reverse phase LC method to determine the main *A. satureioides* flavonoids in porcine ear skin samples after permeation/retention studies from nanoemulsions. In a first step, the method specificity was estimated verifying interferences or overlaps between the *A. satureioides* flavonoids and nanoemulsion/skin components. This is a key issue in skin penetration studies, especially when UV detection is used (Liang et al. 2004; Taverniers et al. 2004), given that various skin and formulation components may absorb ultraviolet light. Figure A shows a typical chromatogram of a skin sample spiked with *A. satureioides* extract. The main retention time peaks (approximately 6.5, 7.5, and 8.5 min) and the diode array spectra led us to identify QCT, LUT, and 3-*O*-MQ. The absorption maxima are in accordance with previous literature (De Souza et al. 2002; Chen and Xiao 2005) and the purity index obtained for the three peaks was 1.0. The method seems to be specific for these flavonoids since no interference occured, which could be related to the absence of absorbance at set wavelength of the nanoemulsion (B) or skin components in the flavonoids' retention time and peak area (C).

2.1. Method validation

The linearity of this method was determined from linear regression of three calibration curves (Table 1). At the 0.25 to 10.0 μg/mL range, the coefficient of determination for all flavonoids was satisfactory (>0.999), which could be considered as highly significant. The confidence intervals for the intercept included zero in all cases, confirming the absence of a constant systematic error. Moreover, the ANOVA test showed no linearity deviation in the analyzed concentration range, and no significant variance between the curves obtained in different days ($p > 0.05$). The slope and *y*-intercepts of these curves were used to calculate the detection and quantitation limits. The quantitation limits varied from 0.34 to 0.49 μg/mL for all tested flavonoids.

The lower limit of quantitation (LLOQ) was 0.25 μg/mL for all tested flavonoids. This measurement is a great concern in the validation of bioanalytical methods, which are used to quantify low concentrations of drugs in biological matrices, such as in skin retention studies (Bansal and Destefano 2007). LLOQ can be defined as the lowest analyte concentration that can be accurately and precisely determined. According to FDA guidelines (FDA 2001), the relative standard deviation can be higher for accuracy and precision parameters at the LLOQ; however, it should not exceed 20%. Our results show intra- and inter-day precision at the LLOQ (at 0.25 μg/mL) for QCT, LUT, and *3-O*-MQ within 1.79 to 2.36 % and 2.35 to 6.73 % ranges, respectively. The accuracies of intra- and inter-day were 15.14 and 15.15 % for QCT, -11.69 and -8.48 % for LUT, and 16.68 and 9.52 % for 3-*O*-MQ (Table 2).

Precision and accuracy were also determined at 10.0 μg/mL to evaluate these parameters at the highest flavonoid concentration used in this study. As it can be observed in Table 2, the intra- and inter-day precision values were below 0.58 and 0.96 %, respectively. In turn, the accuracy results for all tested flavonoids varied between -6.06 and 7.14% for intra-day determination, and between -6.57 and -1.03 % for inter-day determination. The accuracy is an uncertainty measure of the method, representing the difference between the experimental and the true values. For the evaluation of the analytes (QCT, LUT, and 3-*O*-MQ) recovery from nanoemulsions and skin samples, five concentrations of each flavonoid were assayed, including the lowest and the highest concentrations of the linearity curves. Recoveries from 90.05 to 109.88% and from 90.81 to 107.41% for nanoemulsion and skin samples were observed, respectively (Table 3). The recovery at the lower limit of quantitation (0.25 μg/mL) was within FDA recommendations for bioanalytical method validation (FDA 2001). Taken together, the recovery yields are highly satisfactory and demonstrated no interference of the matrices' components on the quantification of the flavonoids under investigation.

2.2. Method application

The method was finally applied to the flavonoids assay into *A. satureioides* ethanol extract, nanoemulsions, as well as in the porcine ear skin sample after a permeation/retention study (Table 4). The QCT, LUT and 3-*O*-MQ contents into nanoemulsion were close to the amount of these flavonoids added into the formulation, considering the initial addition of 1% of dried residue of the extract. Such a result indicates that there is no significant loss or degradation of these compounds during the spontaneous emulsification process. Concerning the *in vitro* permeation/retention study, no flavonoids were detected in the receptor fluid after 2 h. Previous studies showed a very slow permeation profile of isolated quercetin from a similar nanoemulsion formulation that was related to different parameters, including both poor water solubility and low diffusion of quercetin (Fasolo et al. 2009). However, after 2 h, flavonoids were retained in the porcine ear skin from nanoemulsions containing *A. satureioides* (up to 0.63 μg of LUT and 0.92 μg of 3-*O*-MQ per g of skin).

In conclusion, the overall results showed that the method is specific, linear, precise, and accurate to simultaneously quantify quercetin, luteolin, and 3-*O*-methylquercetin in nanoemulsions containing *A. satureioides* extract. The method was also successfully employed to quantify released flavonoids from nanoemulsions containing extracts of this medicinal plant in the presence of the components of porcine ear skin samples.

3. Experimental

3.1. Materials

Egg-lecithin (Lipoid E-80®), and medium chain triglycerides (MCT) were purchased from Lipoid (Germany). Polysorbate 80 and vitamin E were supplied by Vetec (Brazil) and Alpha Química (Brazil), respectively. Methanol (J.T. Baker, USA), acetonitrile (Tedia, Brazil), and phosphoric acid (Merck) were HPLC grade. The standards QCT, LUT and 3-*O*-MQ were purchased from Sigma, Alfa Aesar (Germany) and Extrasynthese (France), respectively. All other chemicals and reagents were of analytical grade.

3.2. Preparation of the Achyrocline satureioides extract

The extract was prepared from inflorescences of *A. satureioides* by a maceration process in ethanol 80% (v/v) over eight days. The medicinal plant:solvent proportion used was 7.5:100 (w/v) and the extract obtained was filtered before to be used. The *A. satureioides* inflorescences were acquired from Chemical, Biological and Agricultural Pluridisciplinary Research Center of Campinas State University/ CPQBA-UNICAMP (São Paulo, Brazil). A voucher specimen was deposited in the herbarium of the CPQBA-UNICAMP (number 308).

3.3. Preparation of nanoemulsions

The nanoemulsions were prepared through spontaneous emulsification procedure as previously described by Carvalho et al. (2008). The organic phase (*A. satureioides* extract, MCT, egg-lecithin, vitamin E, and ethanol) was poured into an aqueous phase (Polysorbate 80, and water) under constant agitation. The excess of solvent was removed by distillation under reduced pressure at 40 °C until a desired final volume (10 mL). Final formulation contains 1% of dried residue of the extract.

3.4. Chromatographic conditions

The liquid chromatography equipment used was a Shimadzu LC-10A system, equipped with a LC-10 AD pump, a CBM- 10A system controller, a

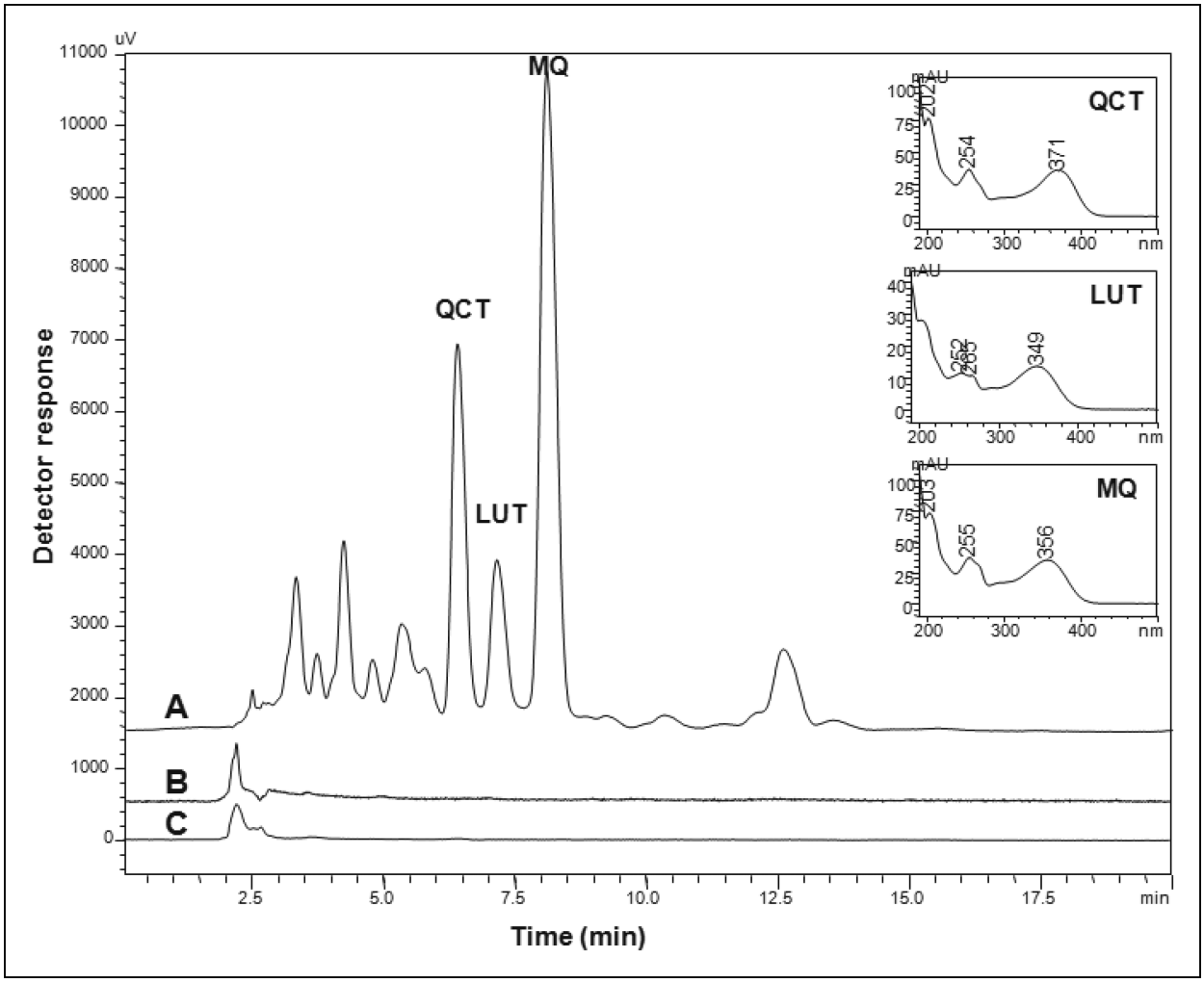

Fig. 1: Liquid chromatography profile at 362 nm and diode array spectra from 200 to 500 nm. (A) Chromatogram of *A. satureioides* extract spiked with skin sample at QCT, LUT and 3-O-MQ concentration of approximately 4.0, 3.0, and 10.0 μg/mL, respectively, (B) chromatogram of blank nanoemulsion, and (C) chromatogram of skin sample.

Table 1: Linearity curves data for QUE, LUT, and 3-O-MQ

	QCT	LUT	3-*O*-MQ
Equation	y = 99699x – 6162	y = 77017x + 535	y = 108364x – 9545
r^2	0.9997	0.9995	0.9999
LOD (μg/mL)	0.16	0.12	0.11
LLOQ (μg/mL)	0.25	0.25	0.25
LOQ (μg/mL)	0.49	0.38	0.34
Intercept (confidence interval)	[−21804; 9481]	[−17651; 19125]	[−15598; 16668]

Concentration range (μg/mL) 0.25–10.0

SIL-10A autosampler and a SPD-20AV UV/vis detector (set at 362 nm). The column used was a Synergi Polar-RP 150 x 4,6 mm i.d., 4 μm (Phenomenex, Torrance, CA) protected by a stainless steel pre-column previously filled with octadecylsilanized silica (150μm, 140A, Phenomenex). The mobile phase consisted of acetonitrile:methanol:phosphoric acid 0.16 M (46:44:10, v/v/v), and was eluted under isocratic flow of 0.8 mL/min. The temperature

Table 2: Precision and accuracy data of the method

Flavonoid (μg/mL)		Intra-day		Inter-day	
		Precision[a] (R.S.D.)	Accuracy[b] (%)	Precision (R.S.D.)	Accuracy (%)
QCT	0.25	1.79	15.14	2.35	15.15
	10	0.58	−6.06	0.96	−4.58
LUT	0.25	2.30	−11.69	2.46	−8.48
	10	0.23	6.22	0.29	−6.57
3-O-MQ	0.25	2.36	16.68	6.73	9.52
	10	0.24	7.14	0.37	−1.03

[a] Precision was expressed as relative standard deviation (R.S.D.) of replicate measurements. [b] Accuracy was expressed as relative error of measurement.

Table 3: Recovery (%) of the main flavonoids (QCT, LUT, and 3-O-MQ) of *A. satureioides* in nanoemulsions and skin samples

Flavonoids (μg/mL)		Nanoemulsions		Skin	
		Content[a] (μg/mL)	Recovery[a] (%)	Content[a] (μg/mL)	Recovery (%)
QCT	0.25	0.252 ± 0.011	100.83 ± 4.28	0.243 ± 0.012	97.18 ± 4.92
	0.5	0.501 ± 0.002	100.12 ± 0.43	0.487 ± 0.017	97.40 ± 3.44
	2.5	2.490 ± 0.068	99.58 ± 2.74	2.486 ± 0.055	99.43 ± 2.22
	5	5.076 ± 0.156	101.52 ± 3.12	4.873 ± 0.061	97.45 ± 1.21
	10	10.490 ± 0.122	104.90 ± 1.22	9.847 ± 0.123	98.47 ± 1.23
LUT	0.25	0.261 ± 0.008	104.28 ± 3.35	0.250 ± 0.019	99.85 ± 7.56
	0.5	0.476 ± 0.026	95.21 ± 5.16	0.467 ± 0.013	93.47 ± 2.66
	2.5	2.534 ± 0.076	101.38 ± 3.02	2.400 ± 0.120	95.98 ± 4.79
	5	5.253 ± 0.241	105.07 ± 4.81	0.474 ± 0.001	94.88 ± 0.19
	10	10.326 ± 0.436	103.26 ± 4.36	9.661 ± 0.084	96.61 ± 0.84
3-O-MQ	0.25	0.250 ± 0.010	99.82 ± 4.06	0.254 ± 0.009	101.6 ± 3.40
	0.5	0.499 ± 0.007	99.18 ± 1.39	0.505 ± 0.009	101.00 ± 1.90
	2.5	2.448 ± 0.043	97.93 ± 1.72	2.338 ± 0.038	93.52 ± 1.51
	5	5.153 ± 0.026	103.06 ± 0.52	5.108 ± 0.010	102.16 ± 0.21
	10	10.279 ± 0.061	102.79 ± 0.61	10.669 ± 0.078	106.69 ± 0.78

[a]expressed as mean ± s.d.

Table 4: Flavonoid determination in *Achyrocline satureioides* extract, nanoemulsion and skin

	Flavonoid content[a]		
	Extract (μg/ml)	Nanoemulsion (μg/ml)	Skin[b] (μg/g)
QCT	304.82 ± 15.30	300.07 ± 2.01	ND[c]
LUT	198.38 ± 5.67	191.07 ± 2.31	0.63 ± 0.05
3-O-MQ	805.09 ± 23.78	718.62 ± 6.05	0.92 ± 0.22

[a] expressed as mean ± s.d.; [b]after 2 h of permeation study; [c]ND: not detected

of the system was controlled at 30 ± 1 °C and the programmed injection volume was 20 μL. The samples were diluted in methanol:phosphoric acid 16 mM (50:50, v/v). The specificity of the method was determined with a Shimadzu LC-20A system, equipped with a LC-20 AT pump, a CBM- 20A system controller, a SIL-20A autosampler and a SPD-M20A diode array detector.

3.5. Validation

The validation parameters used for the determination of *A. satureioides* flavonoids into both nanoemulsions and porcine ear skin samples were assessed according to FDA (2001) and ICH (2005) guidelines, as follows:

Specificity: The specificity was evaluated by analyzing skin sample or blank nanoemulsions interference or overlaps in the retention time of the *A. satureioides* flavonoids. The purity index of the flavonoids peaks was also estimated from the analysis of the *A. satureioides* extract in the presence of blank nanoemulsions or skin samples.

Linearity: Three stock solutions (100 μg/ml) containing QUE, LUT, or 3-*O*-MQ in methanol were prepared and diluted to obtain concentrations of 0.25, 0.5, 1.0, 5.0 and 10 μg/mL of the three flavonoids. The last dilution for each point was performed using a methanol:0.16 mM phosphoric acid (1:1, v/v) solution. Calibration curves were prepared on three consecutive days (triplicate) and independently for each of the flavonoids. The areas of the flavonoid peaks were plotted against the analyte concentrations and the curves were analyzed by linear regression.

Detection and quantitation limits: The detection (LOD) and quantitation (LOQ) limits were determined from equations as described in ICH guidelines. Furthermore, the lower limit of quantitation (LLOQ) was experimentally obtained by the determination of the lowest concentration of flavonoids that could be precisely and accurately assayed (FDA 2001). Precision and accuracy of this point (LLOQ) was assessed by analyzing five replicates in this sample concentration (FDA 2001) and the relative standard deviation accepted for these parameters was less than 20%.

Precision: The intra-day precision of the method was evaluated from six determinations of 0.25 μg/mL (LLOQ) and 10.0 μg/mL of QCT, LUT, and 3-*O*-MQ, all of them performed at the same day. The inter-day precision was obtained from the analysis of the same concentrations on three different days. The results were expressed as relative standard deviation (R.S.D.).

Accuracy: Accuracy was evaluated as the standardized correlation between the measured value and the theoretical value, as follows: RE% = [(mean calculated concentration – theoretical value)/theoretical value] × 100 (Causon 1997).

Recovery: QCT, LUT, and 3-*O*-MQ solutions at 0.25, 0.5, 2.5, 5.0 and 10.0 μg/mL were added in blank nanoemulsions and in skin samples, and were immediately evaluated. The recovery was determined comparing these results with the values obtained from the analyses of the same concentrations in absence of the matrices, expressed as percentages.

3.6. Skin permeation/retention study

Prior to permeation/retention studies, the amount of flavonoids was determined in *A. satureioides* ethanol extract and nanoemulsion. Aliquots were adequately diluted and analyzed by LC as described above. The QCT, LUT, and 3-*O*-MQ contents were expressed as μg/ml of each flavonoid directly assessed into the extract or the nanoemulsion.

The permeation/retention of *A. satureioides* flavonoids from the nanoemulsion was evaluated using Franz type diffusion cells. Briefly, a circular cut of porcine ear skin was set between the donor and receptor compartments on a surface area of 2.54 cm^2. The receptor compartment (10 mL) was supplied with ethanol:PBS (30:70). The system was kept under controlled temperature (32 ± 1 °C) and constant stirring. The nanoemulsion (500 μL) was applied in the upper facing of the skin and after two hours the fluid receptor was withdrawn for analysis. Then, the skin was removed from the Franz cell, cleaned, cut and the retained amounts of QCT, LUT and 3-*O*-MQ were extracted with methanol using ultrassom bathing for 30 min. Results were expressed as the amount of each flavonoid (μg) retained per weight of skin (g).

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