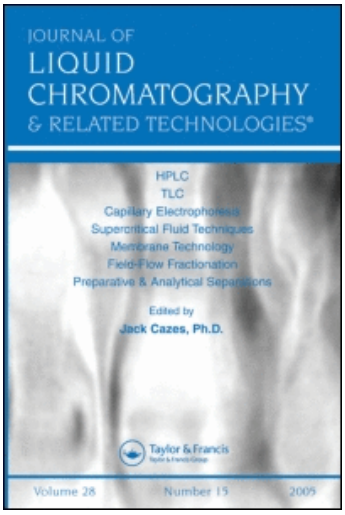

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Development and Validation of an HPLC Method for the Characterization and Assay of the Saponins from *Ilex paraguariensis* A. St.-Hil (Mate) Fruits

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Abstract: Two improved HPLC methods for the characterization and assay of the saponin content in green fruits of *Ilex paraguariensis* (erva-mate) are described. Both HPLC methods consisted of isocratic separation at room temperature, using a RP-18 column with UV-Vis detection. The first system was intended for the characterization of the main *I. paraguariensis* saponin fraction, while the second one was specifically developed and validated for its quantitation. Matesaponin-3 was used as external standard. From the freeze dried extract, ten peaks were characterized as saponins by TLC and HPLC analysis. The matesaponin-3 content was 5.13 g%, and the total saponin content, calculated by the sum of the areas related to the peaks previously characterized as saponins, was 30.48 g%.

Keywords: *Ilex paraguariensis*, Fruits, Saponins, HPLC assay, Validation

INTRODUCTION

Ilex paraguariensis A. St.-Hil. (Aquifoliaceae) is a tree known as "erva-mate" or "mate", which is widely cultivated in South America because of the popular use of their leaves by the food, beverage and pharmaceutical industries.[1] Together with the methylxanthines caffeine, theobromine, and theophylline, it has a high

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content of triterpenoid saponins in it aerial parts, to which diuretic, anti-inflammatory, and hypocholesterolemic properties were ascribed.[2] Besides that, the mate saponins are typically surface active compounds with a potential use as absorption enhancers and cosmetic excipients.[3]

The chemical composition of erva-mate leaves and fruits was an earlier matter of interest of many studies, especially with regard to the isolation and structure elucidation of the saponin fraction.[4,5] Recent studies have shown, however, that the saponin content in the green fruits of erva-mate is higher than in their leaves and ripe fruits.[6] Since the erva-mate fruits are regarded as an unwanted byproduct of the mate process, it opens new perspectives from a technological point of view, namely, the use of the erva-mate fruits as an alternative saponin raw material source.

There are only a few methods specifically described for the assay of the saponin content in erva-mate. They are performed usually by HPLC and, occasionally, by spectrophotometric methods.[6,7] In general, the LC separation methods of saponins become rather intricate and time consuming because of the lack of noticeable chromophers attached to the sapogenin moiety. These methods include chemical oxidation and derivation of the triterpenoidal moiety, as well as the previous acid hydrolysis of the heterosidic saponins. Differently than other saponins from medicinal plants, no erva-mate saponins are available on the market. For that reason, this work was designed to develop an easy and robust HPLC method for the characterizations of the most hydrophilic saponin fractions, as well as the saponin content in erva-mate green fruits. Among several saponins previously isolated by researchers of our Pharmacy College from leaves of erva-mate, the bidesmosidic matessaponin-3 was chosen as the external standard because it also occurs in their fruits.

EXPERIMENTAL

Aerial parts containing green fruits of *Ilex paraguariensis* were harvested in January 2003, in Barão de Cotegipe County, RS, Brazil. The raw material was oven dried (Memmert, Germany) at 40°C, for three days. The fruits were picked and, afterwards, comminuted in a hammer mill (Retsch SK1, Germany).

Acetonitrile, phosphoric acid, and methanol were HPLC grade (Merck, Germany). Ultrapure water was obtained from a Milli-Q® system (Millipore, USA). Matessaponin-3 and matesaponin-5 were previously isolated from *Ilex paraguariensis* leaves[4] and used for characterization purposes with the HPLC method coded as MP-I (see below). The matesaponin-3 (MS-3) was also used as external standard (97% pure, HPLC). An enriched saponin fraction (EMFS-050), obtained from *Ilex paraguariensis* fruits was used in order to confirm the identity of the saponin peaks. The product consisted of a purified fraction obtained by solid phase separation, which is a matter of patent request (Brazilian Pat. PI 0501510-3; April 22, 2005).

HPLC Analysis

HPLC analysis was performed using a Shimadzu LC-10 Class equipment (Kyoto, Japan), provided with a FCV-10 AL system controller, a LC-10 AD pump system, a SIL-10 A automatic injector (20 μL-loop), and a SPD-10-A ultraviolet-visible detector. The data were processed by LC-10 CLASS software. A reverse phase column Nova-pak® RP-18, 300 × 3.9 mm i.d., 4 μm (Waters, USA) was used. Two methods based on different mobile phases were coded as MP-I and MP-II. The MP-I (acetonitrile:phosphoric acid 0.15M; 25.4:74.6, v/v) was used for the qualification and characterization of the chromatographic behavior of the saponin fraction of green fruits. The MP-II (acetonitrile:phosphoric acid 0.15M; 30.4:69.6, v/v) was intended for the quantitative separation of the main saponin fraction from green fruits and validated afterwards. In both cases, isocratic conditions were applied; the flow rate was 0.7 mL/min. The detection was at 205 nm and, when necessary, at 275 and 340 nm. The separations were performed at 23 ± 1°C.

Freeze-Dried Extract Preparation

The extractive solution was prepared by turbo-extraction (IKA, Germany) for 15 min, at 10,000 rpm using an ethanolic solution (40%; v/v) and a drug:solvent ratio of 1.0:10 (w/w). After the extraction, the mixture was pressed, filtered, and concentrated under vacuum, at 50°C, up to a half of its original volume. The concentrate was immediately freeze-dried (Modulyo 4L, Edwards, USA).

Reference Substance

The reference substance matessaponin-3 (MS-3) was dissolved in a mixture of acetonitrile:water (25:75, v/v). The MS-3 was diluted so as to obtain standard solutions within a concentration range from 6.06 to 60.60 μg/mL. The saponin content was calculated from the peak area and expressed as the mean value of at least three injections.

Freeze-Dried Extract Calibration Curve

Samples of the freeze-dried extract were dissolved in a mixture of acetonitrile: water (25:75, v/v) and subsequently diluted in such a way to yield five solutions of 240.0; 320.0; 400.0; 800.0, and 1200.0 μg/mL, of the dried drug. Each curve point represents the mean value of at least three sample injections. The total saponin concentration was calculated by MP-II method, taking into consideration the sum of the area under the peaks 1 to 10, and expressed as matessaponin-3.

Method Validation

The linearity tests consisted of regression analysis, ANOVA test, Durbin-Watson test for residues, and the confidence interval for the linear coefficient (constant systematic error). The precision parameters reproducibility and intermediate precision were evaluated by intra-day and inter-day tests. In the last case, it was a three day interval with the same analysis. The results were analyzed by ANOVA. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as directed by the ICH guidelines.[8]

The method validation was carried out considering the more conspicuous peak 1 and the sum of the areas of the peaks 1 to 10. The individual and total saponin contents were expressed as matesaponin-3.

RESULTS AND DISCUSSION

Analytically, saponins are a troublesome group of plant substances because of their chemical complexity and physicochemical properties. Owing to the lack of specific chromophores in the saponin structures, their HPLC analysis requires the detection at a low wavelength or the use of a refractive index detector. Because many vegetal substances and solvents show a significant absorption at 205 nm, the saponin analysis becomes a hard task for the analyst. Moreover, at low wavelengths, the baseline is rather sensible, particularly, by using a gradient technique. Because of that, we opted for a relative low detection wavelength but under isocratic conditions, which overcomes the baseline variation. By means of the MP-I method, we were able to achieve a satisfactory separation of matessaponin-3 (MS-3) and matessaponin-5 (Figure 1).

The separations by MP-I comprised, simultaneously, saponins with different polarities in an easy way, but the variation of the baseline was indeed so troublesome that the method validation could not be fully achieved. Therefore, the MP-I method appears to be suitable only for the HPLC characterization of the main saponin fraction (Figure 2).

The MP-II method represents a simple variation of the composition of the MP-I one. It offers a better separation of the hydrophilic saponins fraction, as well as a more stable baseline, better signal/noise ratio, and more suitable conditions for the method validation (Figure 3). Notwithstanding, its use implies the exclusion of some of the less polar saponins from the analysis; the comparison of the sum of the MP-I and MP-II peak areas showed that the main fraction of the erva-mate more hydrophilic saponin was still comprised by the method MP-II.

The presence of matessaponins 2, 3, G3, J1, and J3-J4 could not be established in neither the freeze-dried extract MP-II chromatogram, nor in the EMFS-050 enriched saponin fraction after a spiking technique (Figure 4). Ilexoside-2, a bidesmosidic saponin previously isolated from erva-mate fruits,[5] was a possible exception in both cases, with a 21 min retention time.

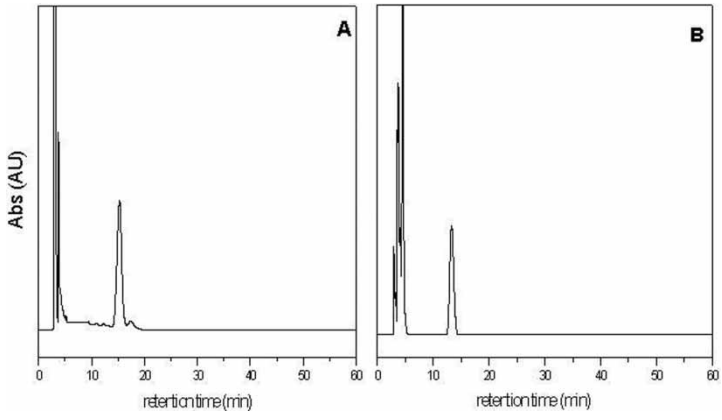

Figure 1. HPLC chromatogram of reference substance matessaponin-3 (A) and matessaponin-5 (B), at 205 nm by MP-I method.

The HPLC analysis showed that some peaks noticed in the freeze-dried extract chromatogram at 205 nm were absent in the saponin fractions, EMFS-050 (Figure 5). Moreover, when the detection was performed at 275 and 340 nm (two typical detection wavelengths where saponins show no absorption as a rule) the same peaks could be detected in the freeze-dried

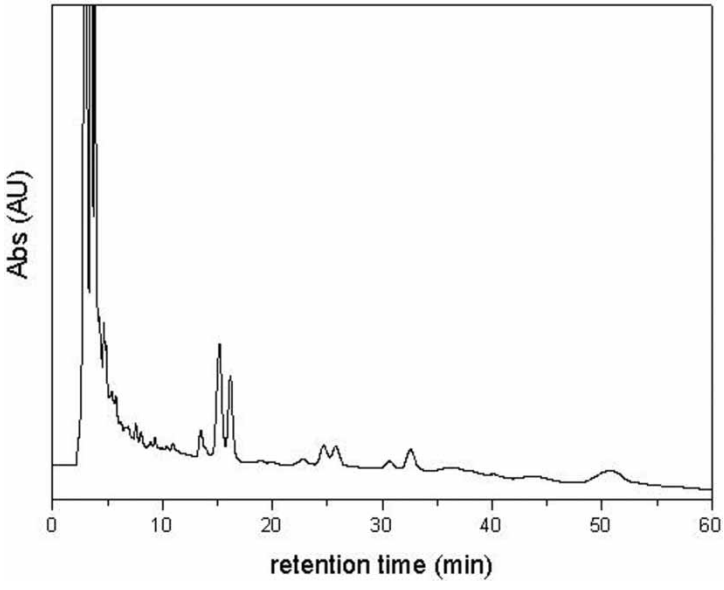

Figure 2. HPLC chromatogram of *Ilex paraguariensis* fruits freeze-dried extract, at 205 nm by MP-I method.

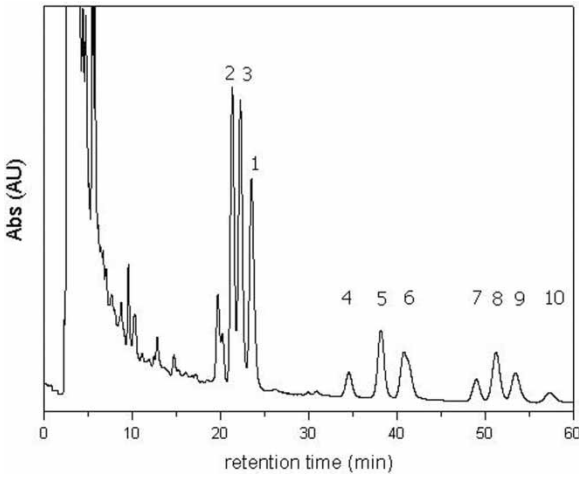

Figure 3. HPLC chromatogram of *Ilex paraguariensis* fruits freeze-dried extract, at 205 nm by MP-II method.

extract chromatogram, but not in the saponin fractions, EMFS-050. By additional TLC analyses performed by detection with anisaldehyde-sulfuric acid, it was possible to conclude that the peaks coded as 1 to 10 are almost certainly saponin or similar triterpenic compounds (results omitted).

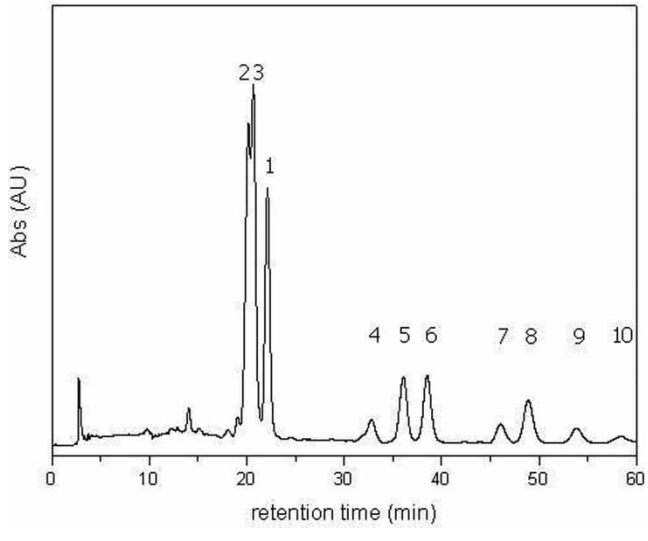

Figure 4. HPLC chromatogram of the saponin fraction EMFS-050 from *Ilex paraguariensis* green fruits, at 205 nm by MP-II method.

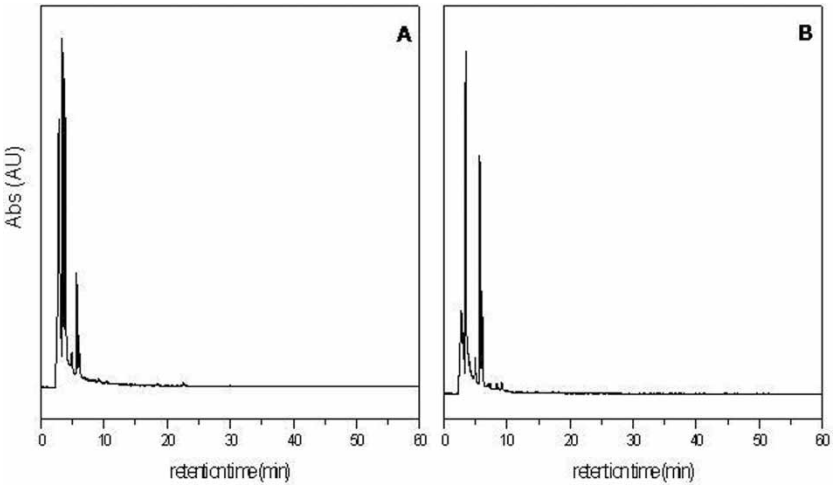

Figure 5. HPLC chromatograms of *Ilex paraguariensis* green fruits extract, at 275 nm (A) and 340 nm (B) by MP-II method.

The separation parameters for peak 1, and the results from their linearity tests and the sum of the peaks 1 to 10, are shown in Tables 1 and 2, respectively.

For peak 1, the linearity test was thoroughly satisfactory (R = 0.9994) and no evidence of proportional systematic error could be detected after analysis of the linear coefficient confidence limits, which included the zero within it. No colinearity was evidenced by the Durbin-Watson test for regression residues ($dv = 2.04$; α 0.05). The LOD and LOQ values were clearly below the lowest curve concentration (0.362 μg/mL and 1.09 μg/mL, respectively) and, therefore, suitable for analytical purposes.

Similarly, good results were achieved for the sum of the peaks 1–10 (R = 0.9982) and the Durbin-Watson test ($dv = 2.09$; α 0.052). In this case, LOD and LOQ were not calculated.

Table 1. Method MP-II separation chromatographic parameters related to the peak 1

	Parameter			
	R_S	N	k′	α
Result	1.65[a]; 3.12[b]	9560	4.27	1.20

Note: R_S: resolution; N: Column plate number; k′: capacity factor; selectivity. The parameter values were calculated after SNYDER et al.[9]

[a]Resolution related to peaks 1 and 3;

[b]Resolution related to peaks 1 and 4.

Table 2. Linearity parameters of the validation of the HPLC method MP-II

Parameter	Regression equation	R	ANOVA $F_{(f.d.;0.05)}$	D-W test	LOD (μg/mL)	LOQ (μg/mL)
Peak 1	$237.44\ x + 3295.01$	0.9994	5346.71	2.04	1.09	0.362
SPA	$1653.89\ x - 54293.07$	0.9982	1661.56	2.09	N.C.	N.C.

Note: D-W: Durbin-Watson; LOD: Limits of Detection; LOQ: Limits of Quantification; SPA: sum of the peak areas; N.C.: not calculated.

Table 3. Method MP-II reproducibility and intermediate precision tests

Parameter	Reproducibility R.S.D. (%)	Intermediate precision R.S.D. (%)
Peak 1	1.03	4.99
SPA	3.86	7.63

Note: R.S.D.: relative standard deviation.

As expected, the variation coefficient of the intermediate precision was greater than the reproducibility one (Table 3).

All critical values associated to the test reproducibility and intermediate precision were lower than 5%, with the exception of the intermediate precision of the saponin total content (7.63%). In this case, the baseline instability and lack of a better peak resolution, especially between peaks 2 and 3, had certainly played an important role.

With regard to the freeze-dried extract, the peak 1 content was 5.13% and the peaks sum (*total saponin*) was 30.48 g%. Both results were expressed as matessaponin-3 content in 100 g of the dried drug. The latter result is comparable to the spectrophotometric total saponin content of 36.93 g% (expressed as ursolic acid), which was earlier reported for green fruits of *Ilex paraguariensis*.[10]

CONCLUSIONS

Two efficient and robust HPLC methods intended for the characterization and separation of the main hydrophilic saponins of green fruits of ervamate were developed and successfully validated. Besides matesaponin-3, ten other peaks were characterized as saponins by HPLC analysis at different detection wavelengths. In spite of a baseline instability and lack of resolution, the main validation requirements were fulfilled for the MP-II method.

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