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Article

HPLC Method to Assay Total Saponins in *Ilex paraguariensis* Aqueous Extract

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Ilex paraguariensis St. Hilaire é uma espécie sul-americana da qual ramos e folhas são utilizados para o preparo de uma bebida de grande consumo em alguns países da América do Sul. A planta é conhecida como "erva-mate" em português ou "yerba-mate" em espanhol. Tendo em vista o potencial uso das saponinas como tensoativo bem como o seu potencial terapêutico, o presente trabalho propõe um método de extração e quantificação para as saponinas presentes em *Ilex paraguariensis*. As saponinas foram extraídas por decocção, hidrolisadas e quantificadas por CLAE e detecção em UV. A concentração de saponinas foi expressa em ácido ursólico (saponinas totais). O método cromatográfico mostrou linearidade na concentração de 13,5 a 135 μg mL^{-1}. O extrato aquoso apresentou uma concentração de saponinas totais de 352 μg mL^{-1}. Os resultados sugerem a possibilidade de adaptação do método para doseamento de saponinas com núcleo triterpênico em extratos de outras plantas.

Ilex paraguariensis St. Hilaire is a South American tree from which leaves and twigs are used to prepare a commonly consumed tea in several South American countries. The plant is known as "erva-mate" in Portuguese or "yerba mate" in Spanish. Considering the potential use of the saponins as surfactant as well as its therapeutic potential, the present work was designed to propose extraction and quantitation methods for the saponins present in *Ilex paraguariensis.* The saponins were extracted by decoction, hydrolyzed and quantified by a HPLC method with UV detection. The saponins concentration was expressed in ursolic acid (total saponins). The method showed linearity for ursolic acid in the range of 13.5 to 135 μg mL^{-1}. The aqueous extract presented total saponins concentration of 352 μg mL^{-1}. The results also suggest the possibility of use of similar method for assaying triterpenoid saponins in other plants.

Keywords: HPLC, saponins, *Ilex paraguariensis*, "erva-mate", ursolic acid, saponin quantitation

Introduction

Ilex paraguariensis St. Hilarie is a South American tree from which leaves and twigs are used to prepare a tea (known as "erva-mate" in Portuguese or "yerba mate" in Spanish), being one of the most commonly consumed beverages in several South American countries, including Brazil (especially in the South states), Uruguay, Paraguay and Argentina. In South America, approximately 30% of the population drinks more than 1 L/day of this beverage. It represents an important crop, with more than 1,400 ton/year.[1]

Besides the substantial amounts of purine alkaloids[2] and caffeoyl-quinic acid derivatives,[3] the leaves of *Ilex paraguariensis* contain also a significant amount of triterpenoid saponins. Monodesmosidic and bidesmosidic saponins have been isolated from the aerial parts of *Ilex paraguariensis,*[4-8] all compounds containing the ursolic or oleanolic moieties (Figure 1). These bitter and highly water-soluble compounds are likely to be partially responsible for the taste of the beverage[9] and also for foaming observed in the "mate". Additionally, *Ilex paraguariensis* is also used in folk medicine for treating several diseases, e.g. arthritis, slow digestions, liver diseases, headache, rheumatism, and obesity. Some of the therapeutic properties of this plant are possibly due to its saponin content, like the potential antiinflamatory[7] and hypocholesterolemic[10] uses. The biological applications of saponins are, usually, based on their membrane-disrupting properties,[11] and formation of large mixed micelles with steroids and bile acids.[12]

The surfactant and biological properties of saponins justify the interest of extraction and quantify these constituents of "erva-mate". At the present time, no method

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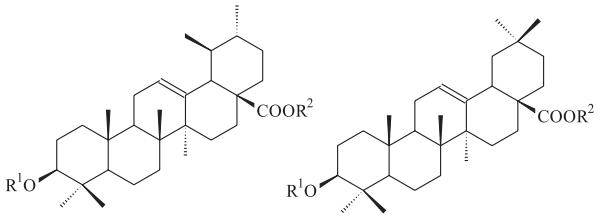

ursolic acid oleanolic acid

	R^1	R^2
Matesaponin 1	β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl	β-D-glucopyranosyl
Matesaponin 2	β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]arabinopyranosyl	β-D-glucopyranosyl
Matesaponin 3	β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl	β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl
Matesaponin 4	β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]arabinopyranosyl	β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl
Matesaponin 5	β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]arabinopyranosyl	β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl
J1a	α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	H
J1b	α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	H
J2a	β-D-glucopyranosyl-(1→3)-arabinopyranosyl	H
J2b	β-D-glucopyranosyl-(1→3)-arabinopyranosyl	H
J3a	α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	β-D-glucopyranosyl
J3b	α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	β-D-glucopyranosyl

Figure 1. Saponins from *Ilex paraguariensis*.

has been described for assaying saponins in *Ilex paraguariensis* raw material or extracts. These compounds are poor chromophores, what explains the difficulties in assay them. The present report describes an HPLC method for quantify the total saponin content (ursolic and oleanolic derivatives) in *Ilex paraguariensis* aqueous extracts, expressed as ursolic acid with UV detection.

Experimental

Plant material

Aerial parts of *Ilex paraguariensis* were collected in Mato Leitão, RS, Brazil. An herbarium specimen is on deposit in the Botany Department Herbarium of Rio Grande do Sul Federal University (ICN-7787), Porto Alegre, Brazil.

Chemicals and reagents

Acetonitrile (HPLC grade, Merck, Darmstadt, Germany) and HPLC-grade water (Milli-Q system, Millipore, Bedford, MA, USA) were used for the mobile phase preparation. Ursolic and oleanolic acids were used as external standards and Matesaponin 1 was used for validation of the analytical method. These substances were previously isolated from *Ilex paraguariensis* leaves.[4] They were pure substances according to the ^{1}H and ^{13}C NMR spectra.

Apparatus and chromatographic conditions

HPLC analysis were performed using a liquid chromatograph (Waters, model 600E), a Rheodyne 7125 injection valve with a 20 mL loop, a 486 UV variable-wavelength detector (set at 203 nm) and a Waters 747 integrator. Ursolic acid was analyzed using a Novapack® column C-18, 4 μm, 150 mm x 3.9 mm i.d. The mobile phase consisted of a mixture, acetonitrile:water (70:30, v/v). The solution was degased in an ultrasound bath and filtered under vacuum through a membrane (Millipore, PVDF). The flow was of 1.0 mL min^{-1} and the sensitivity was 0.001 AUFS. The HPLC system was operated at room temperature (23 ± 1 °C).

Ursolic acid calibration curve

Ursolic acid standard was dissolved in acetonitrile yielding concentrations of 13.5; 27.0; 54.0; 108.0 and 135.0 μg mL^{-1}. The solutions were filtered through a 0.45 μm membrane (Millipore, HVHP). Evaluation of each point was repeated 3 times and the calibration curve was fitted by linear regression.

Preparation and analysis of the Ilex aqueous extract (IAE)

An aqueous extract was prepared in triplicate and submitted to the corresponding hydrolysis procedure.

The *Ilex* aqueous extract was prepared by decoction. Fifteen grams of the dried ground leaves were boiled for 10 min with water (plant:solvent ratio of 1.5:10 m/v). The extractive solution was filtered through filter paper (grade 1: 11 μm, Whatman, UK) and the volume made up to 100 mL with the solvent.

Hydrolysis of the saponins

One hundred milliliters of the *Ilex* aqueous extract were treated with 15 mL chloridric acid in view to yield an acid concentration of 4 mol L^{-1}. The mixture was refluxed for 2 h. The sapogenins were extracted with 50 mL chloroform. The extraction was repeated four times. The whole chloroform fraction was evaporated to dryness and the residue dissolved and made up to 50 mL with acetonitrile. From this solution, designed saponin fraction (SF), 1 mL was diluted to 10 mL with acetonitrile. This solution was filtered through a 0.45 μm membrane (Millipore, HVHP)

and analyzed by HPLC. The evaluation was repeated three times.

Validation

Linearity was determined by the calibration curves obtained by HPLC analysis of the standard solution of ursolic acid. 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 standard deviation (SD) and the slope (S) of the calibration curves.[14] Precision of the method was determined following International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guideline[14] with a determination covering the specified range 13.5 μg mL^{-1} to 135 μg mL^{-1}. For evaluation of repeatability, the SD, and the relative standard deviation (RSD, %), a number of nine injections were considered. The intermediate precision was evaluated by preparing the extrative solutions in triplicate, in five different days.

Efficiency of matesaponin 1 hydrolisys and recovery of the corresponding ursolic acid was determined by adding of three different exact amounts of matesaponin 1 to the IEA before hydrolysis. The recovery of the corresponding amounts of ursolic acid was performed at three concentration levels (60, 90, and 120%). The recovery were determined following the equation (1):[14]

$$R = (IAEad - IAE)/y\ 100 \quad (1)$$

where: R = recovery (%); $IAEad$ = amount (mg) of matesaponin 1 in IAE added of the standard; IAE = amount (μg) of matesaponin 1 in IAE; y = the amount (μg) of matesaponin 1 standard added to the IEA.

Results and Discussion

The absence of chromophores moieties in saponin molecular structure and, consequently, its poor UV absorption is the major limitation to analyze this class of compounds using UV detection. However, we can find reports of saponin detection, in other plants, in low wavelength range as 210 nm.[15] Derivatization with 4-bromophenacyl bromide has been used, allowing detection at 254 nm,[16] but the additional step required in this techinique involves potential addition of error. Nowadays, no analytical method to quantify saponins has been reported for *Ilex paraguariensis* and its preparations.

This work was designed to develop a saponin extraction method, as well as a method, based on reverse-phase HPLC separation combined with UV detection, for saponin assay in *Ilex paraguariensis* extracts. An isocratic system was chosen to minimize the variation of the baseline and also considering the simplicity, precision and neggdness. The Figures 2a and 2b show, respectively, the ursolic and oleanolic acids HPLC profile, detected at 203 nm. Figure 2c shows the peaks of the sapogenins in the saponin fraction, at 15 min. Therefore, all saponins were hydrolyzed and the total their concentration expressed as ursolic acid.

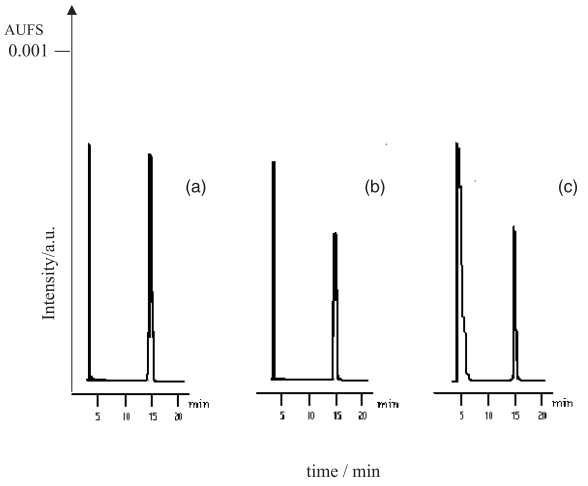

Figure 2. (a) Ursolic acid HPLC profile at 203 nm. (b) Oleanolic acid HPLC profile. (c) *Ilex paraguariensis* saponin fraction (SF) HPLC profile.

For validation of analytical methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH)[14] and USP 25[17] have recommended the accomplishment of accuracy tests, precision, specificity, linearity, work strip and robustness of the method. The type of method and its respective use determine the parameters to be evaluated, especially, when the samples are complex biologic matrices, as the case of herbal extracts. Ursolic acid presented retention time of 15 min. The calibration curve for ursolic acid was linear in the range of 13.5 to 135.0 μg mL^{-1}. The representative linear equation for ursolic acid was $y = 24395x + 46401$ ($n = 5$; $r = 0.9996$) where y represents the peak area (mV s^{-1}) and x the ursolic acid concentration (μg mL^{-1}). The detection limit, taken as the lowest absolute concentration of analyte in a sample, which can be detected but not necessary quantified under the stated experimental condition, was, 1.35 μg mL^{-1}. The limit of quantitation, taken as the lowest concentration of analyte in a sample,

which can be determined with acceptable precision and accuracy, was, 4.1 μg mL^{-1}.

Precision (repeatability and intermediary precision) and accuracy (recovery) were determined for this solution. The repeatability was demonstrated with RSD of 2.38% in the concentration of 100 μL mL^{-1}. This variation can be considered satisfactory since the majority of phytochemicals shows a range from 3% to 6%. [18] The intermediary precision of this sample showed a RSD of the 3.95%. These results demonstrated high reprodutibility between peak areas for the sapogenins.

The accuracy of the HPLC method was determined by recovery analysis in extrative solution after adding of three amounts of matesaponin 1, corresponding to 60, 90 and 120% of the ursolic acid concentration in IAE. The recovery analysis included, therefore, the hydrolysis plus HPLC steps. The recovery of ursolic acid was, respectively, 94.5, 99.2 and 96.4%, what values can be considered satisfactory for complex matrices as an *Ilex paraguariensis* extract.

The concentration of total saponins in the *Ilex paraguariensis* saponin fraction (SF) was 704 μg mL^{-1}, corresponding to 352 μg mL^{-1} in the aqueous extract (IAE) (prepared with a plant:solvent ratio of 1.5:10 m/v).

Conclusions

The method employed for saponin extraction *Ilex paraguariensis* yielded a product with high saponin content. Moreover, for the first time a HPLC method with UV detection for assaying the total saponin in *Ilex paraguariensis* extracts is reported where high precision and adequate accuracy were reached. The results suggest that similar method could be used in the saponin assay of other preparations of this abundant raw material. Moreover, the results also point to the possibility of use of similar method for assaying triterpenoid saponins in chemical composition of plants.

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