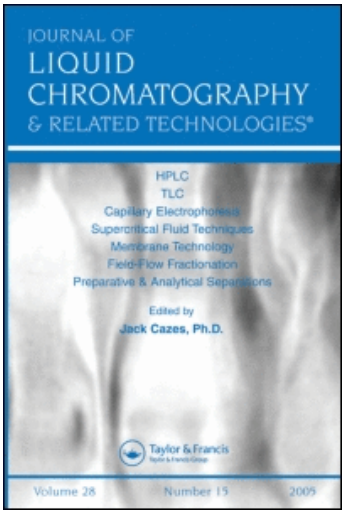

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COMPARISON OF METHYLXANTHINE, PHENOLICS AND SAPONIN CONTENTS IN LEAVES, BRANCHES AND UNRIPE FRUITS FROM *ILEX PARAGUARIENSIS* A. ST.-HIL (MATE)

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□ *The contents of the methylxanthines, saponins, and phenolics compounds were determined in different parts of Ilex paraguariensis A. St. Hil., in order to investigate the chemical differences among some well explored plant parts, leaves and branches, with an innovative raw source as the unripe fruits of the specie. Three specific LC methods were employed. In the plant raw material, the highest contents of methylxanthines (caffeine and theobromine) were found in leaves (0.86% and 0.15%), while the lowest content was observed in the fruits (0.04% and 0.04%). In the same manner the content of phenolic compounds (chlorogenic acid and rutin) was greater in leaves (0.14% and 1.09%) and lowest in unripe fruits (0.03% and not detectable). In contrast, the unripe fruits presented the highest saponin content (12.30%), followed by the leaves (4.14%), and the branches (0.94%). In a mate commercial beverage the high amounts of phenolic compounds (3.82% and 0.51%) and methylxanthines (0.79% and 0.30%) were observed.*

Keywords column liquid chromatography, *Ilex paraguariensis*, mate, methylxanthines, phenolics, saponins

INTRODUCTION

Ilex paraguariensis A. St.-Hil. (Aquifoliaceae) is a South American native tree commonly known as "mate." Its leaves and branches are widely consumed in South American countries as tea like beverages due to their stimulating properties. Mate dried leaves are also recognized by the medicinal proprieties in Europe countries.[1] The stimulant properties on CNS have been associated to the high methylxanthines content in the leaves, specially caffeine and theobromine.[1–4] A significant antioxidant activity through the "scavenger" effect was earlier reported and ascribed

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to the presence of polyphenols, especially caffeoylquinic derivatives and flavonoids.[2–6] Moreover, the mate leaves and especially its unripe fruits a contain high saponin content, consisting of mono- and di-glucosyltriterpenes.[7,8] Besides their anti-inflammatory, hipocholesterolemic, and diuretic activities, the mate saponins are surface active agents and foam formers, which are also able to complex steroids and to build intricate micelles.[9,10] Notwithstanding, mate fruits are still seen as a waste product resulting from the industrial processing of "erva-mate."

Due to its intensive use by the food, cosmetic, and pharmaceutical industries, the seasonal and environmental factors, cultivar types, and agricultural crop conditions and other natural variation factors affecting *I. paraguariensis* harvests have already been well established and in some cases standardized.[2,4,11,12] On the other hand, studies regarding the chemical differences in the plant parts require further investigation since different parts *I. paraguariensis* have a basic importance in the improvement of the derived products.

In this work, we compare the methylxanthine, phenolics, and saponin contents in leaves, branches, and unripe fruits of *I. paraguariensis* of a credited "erva-mate" producer by means of three LC methods already described in literature. For comparison purposes, the most traded soluble extract in Brazil and an enriched saponin fraction obtained from unripe fruits were also analyzed.

EXPERIMENTAL

Chemicals and Reagents

Acetonitrile, phosphoric acid, and methanol were HPLC grade (Merck, Germany). Ultra-pure water was obtained from a Milli-Q® system (Millipore, USA). Caffeine, theobromine, chlorogenic acid, and rutin were purchased from Sigma (Sigma, St. Louis, USA) and used as reference substances (RS). Matesaponin-3 (MS-3) was kindly gifted by Prof. Dr. Grace Gosmann (Universidade Federal do Rio Grande do Sul, Brazil) and used as the reference substance.

Plant Material

Aerial parts containing leaves, branches, and unripe fruits were harvested in January, 2006, in Barão de Cotegipe, RS, Brazil. The material was selected, air dried (*Memmert*, Germany) at 40°C, for three days and comminuted in a cutter mill (SK1 Retsch, Germany), provided with a 2 mm steel sieve. The powder fraction with a particle size of 180 μm was stored in glass containers, protected from light.

Extraction Procedures

Freeze Dried Extracts

The extractives solutions of leaves, branches, and unripe fruits were prepared by turbo extraction (IKA, Germany) for 15 min, at 10,000 rpm using a hydroethanolic solution 40% (v/v) and a drug solvent ratio of 1:10 (w/v). The extracts were filtered and concentrated under vacuum, at 50°C, up to a half of their original volumes. The concentrated extracts were then freeze dried (Modulyo 4 L, Edwards, USA).

Methylxanthines Extraction[3]

Samples of 2 g of each plant material were extracted with a 2 M H_2SO_4 solution by decoction during 10 min. The acidic extracts were neutralized with NH_4OH and fractionated successively four times with 50 mL of a mixture of chloroform: isopropanol (3:1; v/v). The organic phases were collected and concentrated to dryness, under vacuum at 50°C.

Saponin Enriched Fraction (SEF)

The freeze dried extract of 0,5 g from the unripe fruits was diluted in 10 mL of water and fractioned by a solid phase process using a solvent gradient of water and methanol as described in the Brazilian Pat. PI 0501510-3 (04/22/2005).

LC Analysis

LC analyses were performed using Shimadzu LC-10 Class equipment (Kyoto, Japan), provided with a FCV-10 AL system controller, a LC-10 AD pump system, a SIL-10A 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 (Phenomenex® RP-18, 250 × 3,9 mm i.d., 4 μm) was used.

Method I – Methylxanthines Separation[13]

An isocratic system using methanol: water 40% (v/v) was applied, using a flow rate of 1.1 $mL \cdot min^{-1}$ and detection at 280 nm.

Method II – Phenolic Compounds Separation[14]

A gradient system was applied using acetic acid 2% (v/v) (phase A) and methanol:water 85% (v/v) (phase B). The gradient steps were: 31% B (10 min), 31–56% B (10 min), 56% B (8 min), 56–77% B (12 min), 77–56% B (5 min), 56–31% B (5 min). The flow was 0.7 $mL \cdot min^{-1}$ and

the detection wavelength at 340 nm. The total phenolic content was calculated for each extract by means of the chlorogenic acid standard curve and expressed as the sum of the numbered peak areas.

Method III – Saponins Separation

The method consisted of a gradient system of phosphoric acid 0.1% (v/v) (phase A) and acetonitrile (phase B). The gradient steps were: 30–45% B (40 min), 45% B (5 min), 45–30% B (20 min), flow rate of $0.9\,mL \cdot min^{-1}$, and the detection was at 205 nm. The total saponin content was calculated for each extract by means of the MS-3 standard curve and expressed as the sum of the numbered peak areas.

Standard Curves

Standard solutions were freshly, daily prepared with purified water and protected from light. Methylxanthines: caffein and theobromin were dissolved in a mixture of methanol:water 40% (v/v) and diluted so as to obtain six standard solutions within a concentration range from 0.2 to $10\,\mu g \cdot mL^{-1}$. Phenolic compounds: chlorogenic acid and rutin were dissolved in methanol:water 50% (v/v) mixtures to yield concentrations of 2.0, 4.5, 6.0, 8.0, and $10\,\mu g \cdot mL^{-1}$. Saponins: The reference substance MS-3 was dissolved in a mixture of acetonitrile:water 25% (v/v) and diluted adequately to obtain standard solutions within a concentration range from 6.06 to $60.60\,\mu g \cdot mL^{-1}$.

Sample Preparation

Methylxanthines: The residues obtained in the concentration of the methylxanthine liquid–liquid extractions were dissolved in 500 mL of methanol. An aliquot of 1.0 mL of this solution was diluted up to 20.0 mL with a mixture of methanol:water 40% (v/v). Phenolic compounds: Sample of freeze dried extracts from each leaf, branch, and unripe fruit were dissolved in a mixture of methanol:water 50% (v/v) and diluted adequately to obtain concentration values of $0.15\,mg \cdot mL^{-1}$ Saponins: The three freeze dried extracts from each leaf, branch, and unriped fruit were dissolved in a mixture of acetonitrile:water 70% (v/v), and diluted to obtain final concentrations of $1.0\,mg \cdot mL^{-1}$. Saponin Enriched Fraction (SEF): It was dissolved in a mixture of acetonitrile:water 30% (v/v), yielding a concentration of $1.0\,mg \cdot mL^{-1}$. Mate Commercial Product (MCP): An amount of the granulated extract was diluted in methanol:water 50% (v/v) to obtain a concentration of $0.1\,mg \cdot mL^{-1}$.

All the liquid samples were filtered trough 0.4 μm pore size membranes (Millipore®) and then injected. In all cases, each peak area represents the mean value of at least three injections and each concentration refers to the particular substance content in 100 g of dried plant material, SEF or MCP (w/w %).

RESULTS AND DISCUSSION

Barão-de-Cotegipe is a credited cultivar of *Ilex paraguariensis* St. Hil., being a supplier for earlier studies[4,15] and assuring the raw material genuineness. The turbo-extraction and the solvent system were standardized in a previous work[16] and they led to a good yield considering the range of polarity of the mate compounds, which are the subject of this work. The LC methods were chosen because of the good selectivity and applicability to the different main compounds found in *I. paraguariensis*.

Methylxanthines Fraction

The methylxanthines are responsible for the mate stimulant properties, but its content can vary depending on the vegetal tissue, season, and environmental factors.[12,17] Due to that reason and because caffeine and theobromine have been used as mate reference substances,[1] the content assay of those compounds in different samples and different parts of the plant have become relevant regarding quality control parameters. The present LC method was previously developed and validated[13] and it has analytical advantages over similar methods related in the literature,[3,18,19] namely simplicity and a shorter time of analysis. The standard curves of caffeine and theobromine are shown in Table 1.

The advantages associated to the acid base extraction and the chosen wavelength in order to avoid interfering substances can be appraised by the comparison of the LC-chromatograms obtained from leaves, branches, and unripe fruits (Figure 1). Regarding caffeine and theobromine contents, the differences among the aerial parts are clearly evident (Table 2). The lowest contents of both methylxanthines were found in unripe fruits.

TABLE 1 Regression Analyses of the Caffeine and Theobromine Standard Curves Obtained by LC Method I, with Detection at 280 nm

Compound	Regression Equation	R^2
Caffeine	$y = 48957.40x + 76.3107$	0.9993
Theobromine	$y = 51814.03x + 61.9733$	0.9995

R^2 = Correlation coefficient.

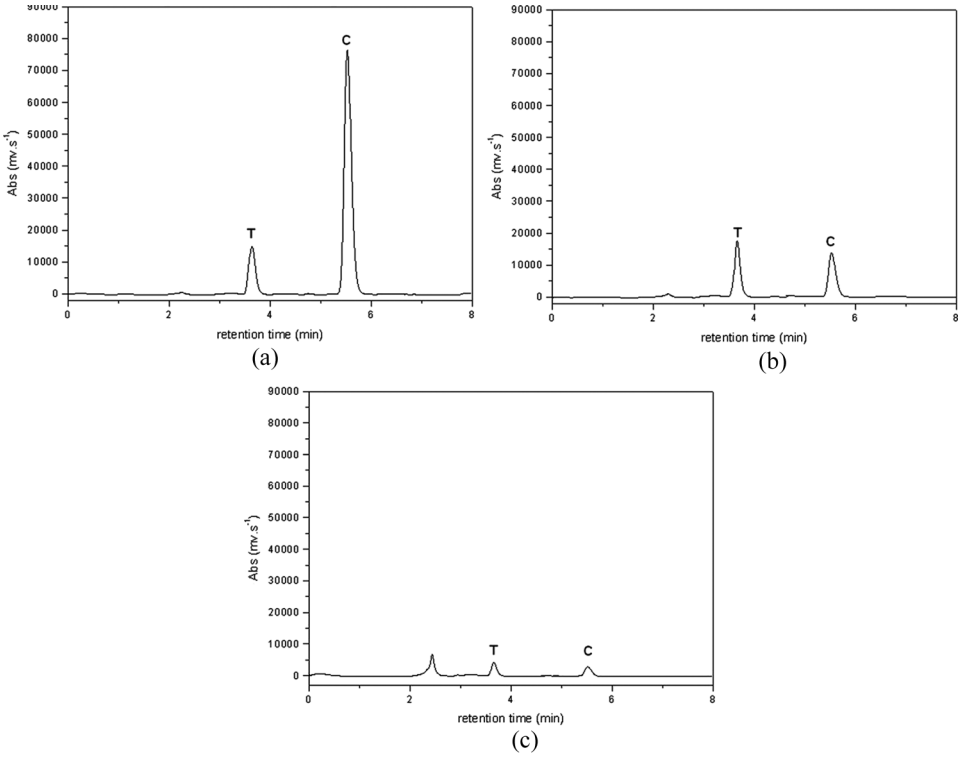

FIGURE 1 LC chromatograms of the methylxanthines in leaves (1), branches (2) and unripe fruits (3) obtained by Method I. T: theobromine and C: caffeine.

Caffeine was predominant over theobromine in leaves while it was less evident in branches. Moreover, the amounts of methylxanthines in mate leaves are in agreement with earlier works[2–4,19] and justify its use in the preparation of typical stimulant beverages.

Phenolic Compounds

The phenolics from mate include phenolic acids and flavonoids, here expressed by chlorogenic acid and rutin, in that order. The occurrence of both phenolic compounds in *I. paraguariensis* is thoroughly described

TABLE 2 Caffeine and Theobromine Contents (Mean ± Standard Deviation) Calculated by LC Method I and Expressed in g per 100 g of Dried Plant Material (w/w %)

Compound	Leaves	Branches	Fruits
Caffeine	0.86 ± 0.002	0.16 ± 0.002	0.04 ± 0.002
Theobromine	0.15 ± 0.002	0.17 ± 0.001	0.04 ± 0.002

in literature.[2,6,11,14] The LC standard curves of both phenolic compounds showed a good linearity (Table 3) and the phenolic constituents of the freeze dried extracts are well separated (Figure 2). The peaks 1 and 3 were previously identified by LC-MS as neo-chlorogenic acid and crypto-chlorogenic acid, respectively, and the other numbered signals were characterized by photodiode array (PDA) detection, showing typical absorption spectra of phenolics compounds.[14]

The large content of phenolic compounds in leaves and branches is noteworthy whereas the unriped fruits showed the lowest content of these compounds (Table 4). The occurrence of rutin was foremost evident in leaves, while branches contained only few amount of it and in unripe fruits it was not detectable. The chlorogenic acid was detected in all freeze dried extracts. The total phenolic contents were calculated through the chlorogenic acid standard curve as the sum of the numbered peak areas of each chromatogram and presented in Table 4.

Saponins

Only a few LC methods have been reported for separation and quantitation of saponins in *I. paraguariensis*. The have employed RI detection[20] or UV detection with previous sample acidic hydrolysis.[21] The method used in this work allows the qualitative analysis of saponins having a wider range of polarity than an isocratic method separation previously described.[16] The standard curve obtained with the MS-3 showed the regression equation of $y = 4613.768x + 6194.295$ ($R^2 = 0.9994$) allowed to estimate the total saponin content in each plant part.

Concerning the chromatographic analysis of saponins by UV detection specifically, it is critical due to the absence of practical absorption above 220 nm and an undesirable effect arose from analytical interferences. A concomitant analysis of mate extracts was carried out at higher wavelength, in order to segregate the peaks of interest, which have absorption only at 205 nm but not at 280 nm (Figure 3).

As it can be observed, there is a significant saponin accumulation in unripe fruits (C), when compared to leaves (A) and branches (B), confirming the results reported in earlier works.[8] The analysis of the corresponding

TABLE 3 Chlorogenic Acid and Rutin Standard Curves Obtained from LC Method II with Detection at 340 nm

Compound	Regression Equation	R^2
Chlorogenic acid	$y = 66505.76x - 5904.97$	0.9977
Rutin	$y = 36407.71x - 3515.25$	0.9986

R^2 = Correlation coefficient.

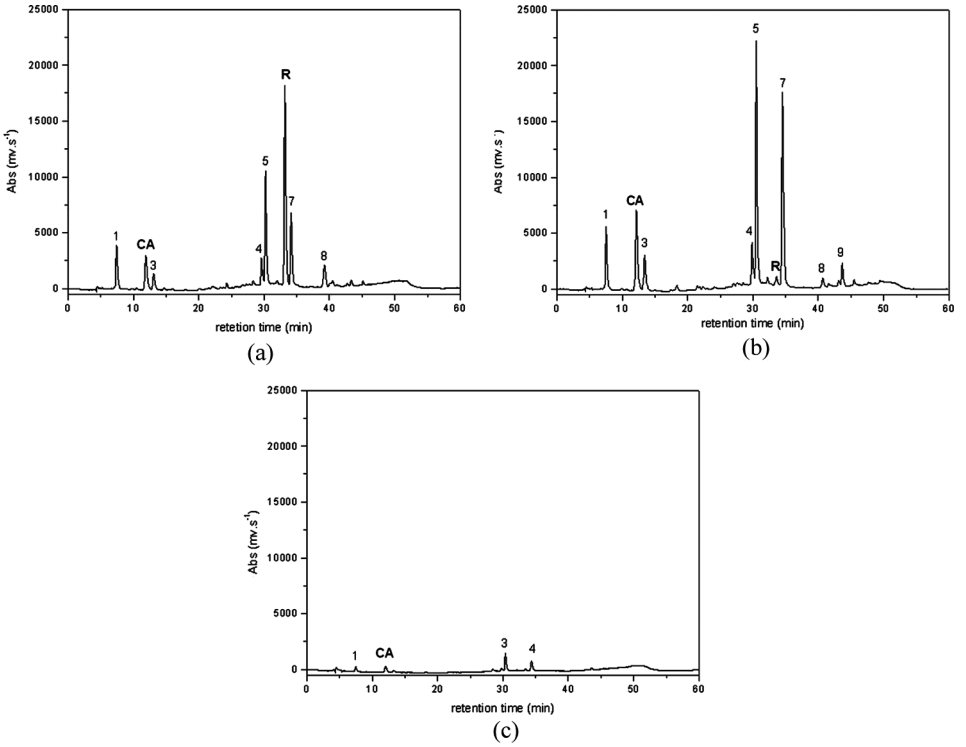

FIGURE 2 LC-chromatograms of the phenolics of freeze dried extracts from leaves (a), branches (b) and unripe fruits (c), obtained by LC Method II and detection at 340 nm. CA: chlorogenic acid and R: rutin.

saponin retention time reveal that the saponins from unripe fruits seems to be more hydrophilic than the leaves, suggesting the predominance of higher glycosylation patterns in unripe fruits triterpenes.

The total saponin contents calculated for leaves, branches, and fruits were 4.14 ± 0.08, 0.94 ± 0.08, and 12.30 ± 0.15 (w/w %), respectively, expressed as MS-3 in 100 of dried plant material.

TABLE 4 Chlorogenic Acid, Rutin and Total Phenolic Contents (Mean ± Standard Deviation) Calculated by LC Method II and Expressed in g per 100 g of Dried Plant Material (w/w %)

Compound	Leaves	Branches	Fruits
Chlorogenic acid	0.14 ± 0.002	0.33 ± 0.001	0.03 ± 0.001
Rutin	1.09 ± 0.003	0.06 ± 0.002	ND
Total Phenolics	1.60 ± 0.006	2.13 ± 0.005	0.11 ± 0.003

ND = Not detectable.

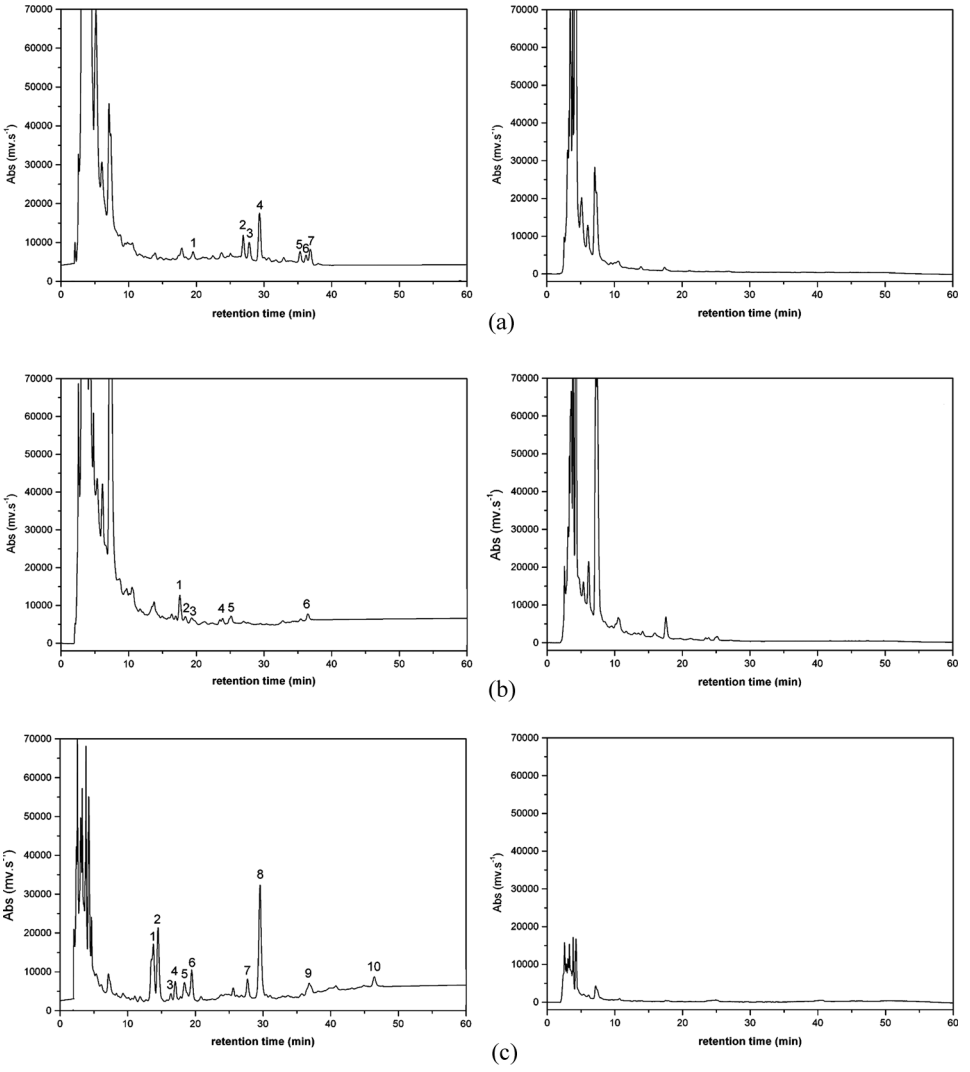

FIGURE 3 LC chromatograms of the saponin content of leaves (a), branches (b) and unripe fruits (c), obtained by Method III and detection at 205 nm (left column) and 280 nm (right column).

Mate Derivative Products

The LC-chromatograms from the unripe fruit extracts and SEF seems to be similar (Figures 3c and 4a), except for the initial peaks with retention time below 10 min. These peaks arose from undesired substances eliminated by the purification process applied for an SEF preparation. The same LC procedure, but with detection at 280 nm, confirms that those compounds, including phenolic acids and flavonoids, are not evident in

SEF (Figure 4B). The sum of the numbered peak areas was used to estimate the content of saponins, which was about 88.3 g in 100 g of the SEF.

The MCP is a commercial granulated extract of mate leaves in Brazil, extensively consumed for the preparation of tea like beverages and commercialized having high amounts of polyphenolic compounds specifically. From a quantitatively point of view, there are practical differences between the chromatograms for phenolics obtained from MCP and the leaf freeze dried extract (Figures 5A and 2A). The content of chlorogenic acid (CA) is higher than the rutin one (R), for example (Table 5). The differences

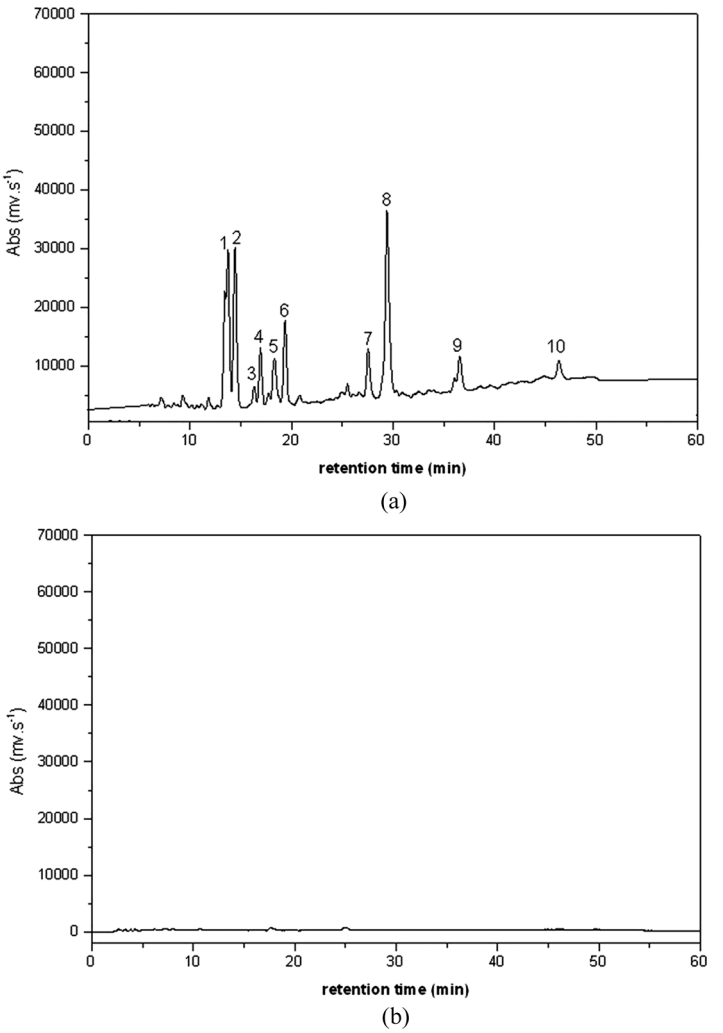

FIGURE 4 LC chromatogram of the saponin enriched fraction (SEF) from *Ilex paraguariensis* unripe fruits at 205 nm (a) and 280 nm (b).

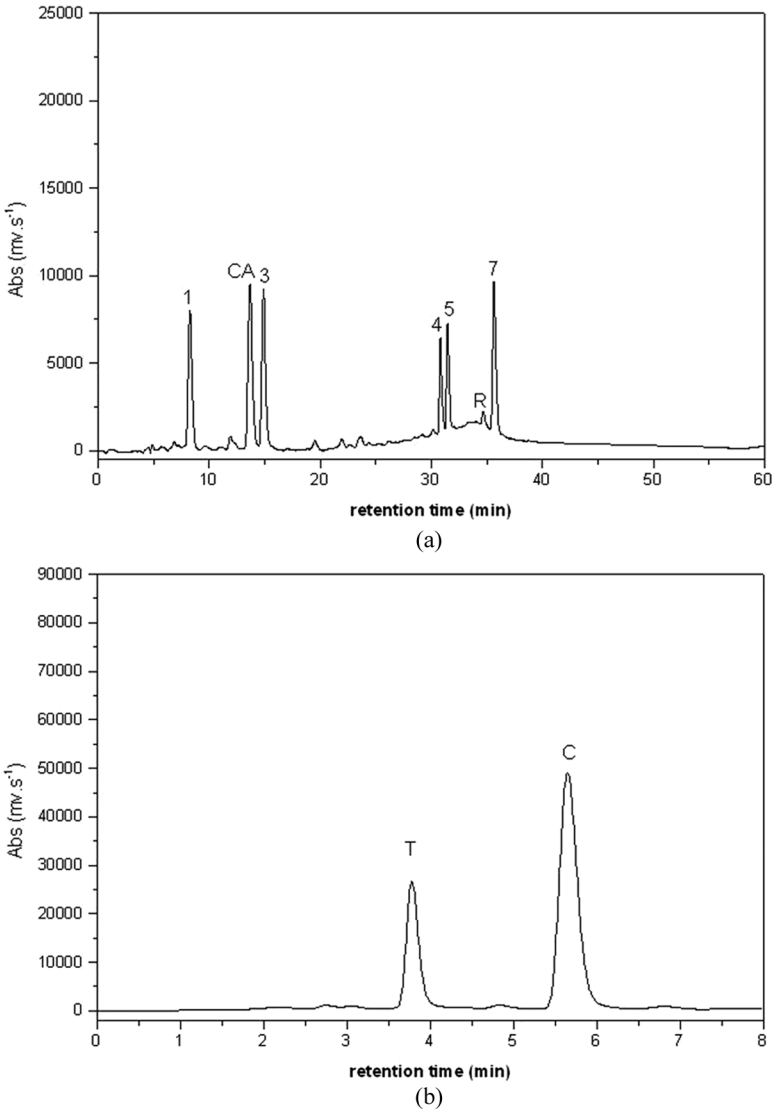

FIGURE 5 LC chromatograms of phenolics (a) and methylxanthines (b) from mate commercial product (MCP).

TABLE 5 Chlorogenic Acid, Rutin and Total Phenolic Contents (Mean ± Standard Deviation) Expressed in g per 100 g of Mate Commercial Product (w/w %)

Compound	g% MCP
Chlorogenic acid	3.82 ± 0.010
Rutin	0.51 ± 0.006
Total phenolic	15.8 ± 0.170

TABLE 6 Caffeine and Theobromine Contents (Mean ± Standard Deviation) Expressed in g per 100 g of Mate Commercial Product (w/w %)

Compound	g% MCP
Caffeine	0.79 ± 0.02
Theobromine	0.30 ± 0.02

observed can be attributed either to the origin of the raw material or to the influence of technological processing that was not reported for MCP. The LC analysis of mate leaves extracts prepared by aqueous decoction showed a very similar chromatographic profile,[14] supporting in part the later hypothesis. On the other hand, the saponin fraction present in MCP was negligible and its quantification was therefore unfeasible.

The LC analysis of the methylxanthines (caffeine and theobromine) in MCP showed similar results when compared to the plant leaves material (Table 6), despite the extraction procedures and different extraction procedures in both cases.

CONCLUSIONS

The set of analytical methods evidenced the different concentrations of the employed markers in accordance with the vegetal part analyzed. Guiding future actions in the use of the vegetal, aiming the application in the food and pharmaceutical issues, with better exploration of the available natural resources. The selected analytical methods were also suitable for the derivative products, allowing their application in the intermediary and final quality control.

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