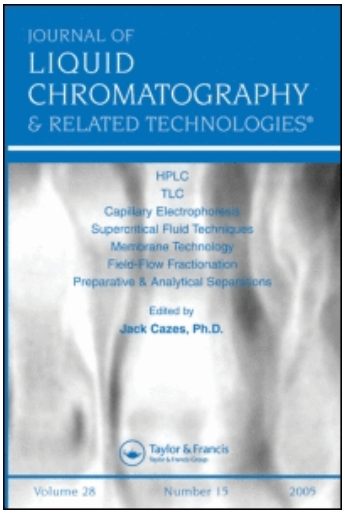


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# Validation of an LC Method for Polyphenol Assay in Extractive Solutions from *Ilex paraguariensis* (Mate)

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**Abstract:** A liquid chromatography (LC) method was developed and validated for identification and quantification of polyphenols in aqueous extractive solution from *Ilex paraguariensis* (erva-mate), in agreement with the ICH requirements for analytical methods. The analysis was performed using a RP 18 column, in gradient solvent. Chlorogenic acid (CLOA) and rutin (RU) were used as external standards. The standard curves for CLOA and RU were linear with correlation coefficients higher than 0.9980. The LC method showed excellent performance in separating seven peaks. The method showed excellent repeatability (R.S.D. $< 2.0\%$) and accuracy (CLOA $= 97.4$ and RU $= 104.0\%$). Besides CLOA and RU, six other constituents

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were detected, which were identified by LC-MS/MS based on their mass spectra in full scan mode and retention times compared with those available in the literature data. The analytical method was successfully applied for quantifying polyphenols in four different extractive solutions from erva-mate, a decoction (ES), an infusion (ESI), and an extractive solution obtained by turbo extraction with water ($TE_1$) or ethanol 40% (v/v) ($TE_2$). The last one presented the highest polyphenol concentration.

**Keywords:** LC, Polyphenols, ICH validation, *Ilex paraguariensis*, Chlorogenic acid, Rutin



## INTRODUCTION

*Ilex paraguariensis* A. St. Hil. is a popular species known as "Mate". The product available in the market, which is prepared by stabilization, drying, and grinding of leaves and stems is also called "Mate" or "erva-mate".

The species is economically and socially of great interest in South America where it is consumed as the traditional beverage called "chimarrão" or "Mate", which is prepared by infusion of "erva-mate".

Concerning the chemical composition, the main constituents reported for *I. paraguariensis* are methylxanthines,[1] saponins,[2–4] and polyphenols,[5,6] all of them presenting potential therapeutic interest. In the polyphenols class, the presence of flavonoids such as quercetin, rutin, and caffeoyl derivatives such as chlorogenic acid have already been reported,[6] being that its presence is related to the antioxidant activity of this species.[7–9]

Regarding the analysis of these compounds, Filip et al.[6] have previously reported a LC quantitative method to separate and to identify the phenolic compounds in South America *Ilex* species. Carini et al.[10] have developed an analytical method for the caffeoyl derivatives in *I. paraguariensis* extracts using liquid chromatography associated with mass spectroscopy detection to identify ten compounds. Recently, Bravo et al.[11] reported a LC/MS method for determining the polyphenol composition of the mate extracts. However, as far as we know, no method regarding polyphenol quantification has been described taking into account the ICH requirements in order to validate it.

In this context, the present work was designed to develop an easy, reproducible, and accurate LC method for quantifying the polyphenols in *I. paraguariensis* extractive solutions, determining the main ICH validation parameters for this complex matrix. Further, the method was applied to four extractives solutions from erva-mate, in view to compare its polyphenol contents.

## EXPERIMENTAL

### Plant Material

*I. paraguariensis* leaves and stems were supplied by "Fino Mate" (Mato Leitão – RS, Brazil). The specimen was identified and deposited at the

Herbarium of the Universidade Federal do Rio Grande do Sul (ICN 133726). The traditional method of "erva-mate" production was employed, briefly, the raw material was stabilized by roasting, then dried and ground.

## Chemicals and Reagents

Methanol (HPLC grade, Merck, Darmstadt, Germany), glacial acetic acid (Synth, São Paulo, Brazil), and HPLC grade water (Milli-Q system, Millipore, Bedford, MA, USA) were used for the mobile phase preparation. Chrologenic acid (Sigma, St. Louis, MO, USA) and Rutin (Sigma, St. Louis, MO, USA) were used as external standards.



## Apparatus and Chromatographic Conditions

LC analysis was performed using a Shimadzu liquid chromatograph (LC-10 AD) equipped with a gradient controller (FCV-10 AL), an autosampler (SIL-10 A), a UV/VIS detector (SPD-10 A), and a CLASS LC-10 software (Shimadzu, Kioto, Japan) and photodiode array detector, PDA, (Waters Millenium, Milford, MA, USA). A Shim-pack CLC-ODS (M) RP-18 (5 μm, 250 mm × 4 mm i.d) column coupled to a pre-column with Lichrosorb RP-18 (Waters Millenium, Milford, MA, USA) was employed. The UV spectra of the non identified peaks, obtained by PDA detection, were additionally analyzed. Mass spectrometric analysis was performed on a Varian 1200 mass spectrometer (Walnut Creek, CA, USA) fitted with an electrospray ionization source (ESI). The negative ion mode [m/z M-H] was used for all compounds. Preliminary analysis was carried out using full scan, data dependent MS/MS scanning from m/z 150–800. Identities of the compounds were obtained by matching their molecular ions (m/z) obtained by LC-MS/MS with the literature data.[10]

The analytical method employed a linear gradient system, which consisted of (A) acetic acid 2.0% (v/v); (B) methanol:water 85.0% (w/w). The gradient elution for the first 10 min was 31% B, 31–56% B during 10 min, then in 8 min 56% B, 12 min of 56–77% B, 77–56% B during 5 min, and 56–31% B for the last 5 min. The flow rate was adjusted to 0.70 mL/min, the detection wavelength to 340 nm, and 20 μL were injected. The sensitivity was 0.05 AUFS. The LC analysis was carried out at 23 ± 1°C.

The polyphenols were identified, comparing its retention time and the corresponding diode array spectra to those obtained from the standards.

Calibration curves of standard compounds were used for quantification purposes, as usual.

## Standard Curve

CLOA and RU standards were dissolved in methanol-water (50:50, v/v) yielding concentrations of 2.0; 4.5; 6.0; 8.0, and 10.0 μg/mL. The solutions were filtered through a 0.45 μm membrane filter (Millipore, HVHP). The results were expressed by the mean of peak areas obtained from three injections.

## Extractive Solution (ES)

### Preparation of Extractive Solution

For validation of the method, an aqueous extractive solution (ES) was prepared by decoction (ES) of the erva-mate, for 15 min, in a plant:solvent ratio of 1.5:10. The ES was filtered and the volume was made up to 100.0 mL with distilled water. The loss on drying assay of ES, following the procedure described in the USP 27,[12] yielded 3.8% (w/v).

In order to compare their polyphenol content, three other extractive solutions were prepared by infusion or turbo-extraction method. The infusion (ESI) was prepared by pouring boiling water on the aerial parts. The mixture was left to stand for 15 min and then filtered. For preparing the two extractive solutions by turbo-extraction, water ($TE_1$) or ethanol 40% (v/v) ($TE_2$) were employed as solvent; the extraction was carried out at 10,000 rpm for 15 min. The drug:solvent ratio employed for all extractive solutions was 1.5:10 (w/w). All the extractive solutions were filtered through filter paper and the volume was made up to 100.0 mL with the solvent.

### Extractive Solution Curve

Samples of 0.5; 1.0; 2.0; 4.0, and 5.0 mL of the ES were diluted in methanol-water (50:50 v/v) to 10 mL, yielding concentrations of 50.0, 100.0, 200.0, 400.0, and 500.0 μL/mL. The samples were filtered through a 0.45 μm membrane filter (Millipore, HVHP) prior to injection. Evaluation of each point was repeated three times.

## Validation

The validation of the analytical method included the determination of linearity, repeatability, intermediary precision, and accuracy according to the International Conference on the Harmonization (ICH) guideline.[13]

The linearity of the method was determined by the calibration curves obtained by LC analysis of the standards CLOA and RU. The linearity of the method was also evaluated by the calibration curves of the corresponding

peaks when different volumes of ES samples were employed. The calibration curves were fitted by linear regression and the results expressed by regression coefficient and other statistical parameters, as follows.

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated based on the standard deviation (S.D.) and the slope (S) of the standard calibration curves.[13]

The precision of the method was evaluated through the determination of the repeatability and intermediary precision. The repeatability experiment was performed analyzing the R.S.D. values, obtained from nine samples evaluated in the same day. The intermediary precision was evaluated in triplicate for three consecutive days and the results were expressed as the R.S.D.

The accuracy was evaluated through recovery studies by adding known amounts of CLOA and RU to ES. The ES without standard addition was analyzed as control. The assay was performed at three concentration levels (50, 100, and 150%) of the standard CLOA and RU, three times each. The recovery was determined by subtracting the values obtained for the control samples from those samples that were prepared with the added standards, divided by the amount added and multiplied by 100%.[13]



### Analysis of the Extractive Solutions

Samples of 5.0 mL of ES, ESI, $TE_1$, and $TE_2$ were diluted in methanol-water (50:50 v/v) to 10 mL, yielding concentrations of 200.0 μL/mL. The samples were filtered through a 0.45 μm membrane filter (Millipore, HVHP) prior to injection. Evaluation of each point was repeated three times.

## RESULTS AND DISCUSSION

The recognized antioxidant potential of *I. paraguariensis* has motivated the development of methods for identifying and quantifying polyphenols in the plant or in the corresponding extracts. The method, reported by Carini et al.[10] using LC associated to MS detection was developed with the aim of identifying the phenolic constituents from the species. The method reported by Filip et al.[6] involves a LC method with UV detection in order to compare the profile of the phenolic compounds from several American species. Recently, Bravo et al.[11] reported a LC/MS method for characterization of phenolic constituents, identifying more than twenty quinic acid or hydroxycinnamates derivatives. Caffeoylquinic acid and dicaffeoylquinic acid isomers were referred to as the major components of the phenolic fraction of erva-mate.

Validation of analytical methods has the aim of demonstrating that the employed analytical procedures are suitable for their intended use. The guidelines, as ICH, describe the procedure for carrying out the validation of the

analytical procedures included as part of an application for approval and registration of a pharmaceutical product, including those used in storage stability. In the case of *Ilex paraguariensis*, since this raw material is abundant in South America and considering its therapeutic potential properties, the development of new products from the plant seems to be interesting and economically feasible. In this way, the first step is the development of extractive solutions as an intermediary product for the pharmaceutical or food industry for producing solid or semi-solid products. Consequently, besides the development, the validation of a simple method to determine the main polyphenol concentration in extractive solutions is necessary.

In the choice of the LC conditions, the complex composition of the *I. paraguariensis* ES required the use of a gradient elution system. This system also allowed shortening the analysis time. The mobile phase consisting of methanol:water showed suitable resolution and peaks separation, allowing the quantification of the standards in the ES. The analysis time was also appropriate, since all the compounds were eluted in less than 40 min.



Figure 1 shows the chromatographic separation of the standards CLOA and RU at 340 nm (Fig. 1a) and also the corresponding peaks in the ES (Figure 1b), with the respective diode array spectra. Among the eight peaks, CLOA and RU are observed at the retention time of 10.6 and 30.0 min, respectively. Among the others peaks, P1, P3, P4; P5, and P6 exhibited UV spectra pattern of caffeoylquinic acid derivatives with UV-max absorption bands at 328 nm, indicating that this class of polyphenols are the major constituents in the ES polyphenol fraction.

The analysis by LC MS/MS reveals that the structures of peaks P1 and P3 show identical $[M-H^+]^-$ ions at $m/z$ 353 and also the ions corresponding to the caffeic acid (m/z 179) and quinic acid (m/z 191) (Fig. 2), which can be attributed to the chlorogenic acid (5-*O*-caffeoylquinic acid) isomers. Hence, the proposed structures for P1 and P3 on the basis of elution order,[10] are as neochlorogenic (3-*O*-caffeoylquinic acid) (NEO) and crypto-chlorogenic (4-*O*-caffeoylquinic acid) (CPC) acids. The peaks P4, P5, and P6 show the same molecular ion m/z 515 and fragments ions at $m/z$ 179, 191, and 353 $[M-H\text{-Caffeoyl}]^-$, corresponding, probably to isomeric dicaffeoyl esters of quinic acid (i.e., 3,4-*O*-dicaffeoyl, 4,5-*O*-dicaffeoyl, 3,5-*O*-dicaffeoyl or 1,5-*O*-dicaffeoyl esters). Although, with the lack of reference standards, no definitive structure assignment can be done; these results are in agreement with those reported by Carini et al.[10] and Bravo et al.[11]

In this first report on validation of a LC-UV method for *I. paraguariensis* polyphenol assay, the concentration of the substances corresponding to the major peaks (P1, P3, P4, P5, and P6) were determined by the CLOA calibration curve due to their similar UV spectra.

The validation of the analytical method included the determination of linearity, repeatability, intermediary precision and accuracy, according to the ICH requirements.[13]

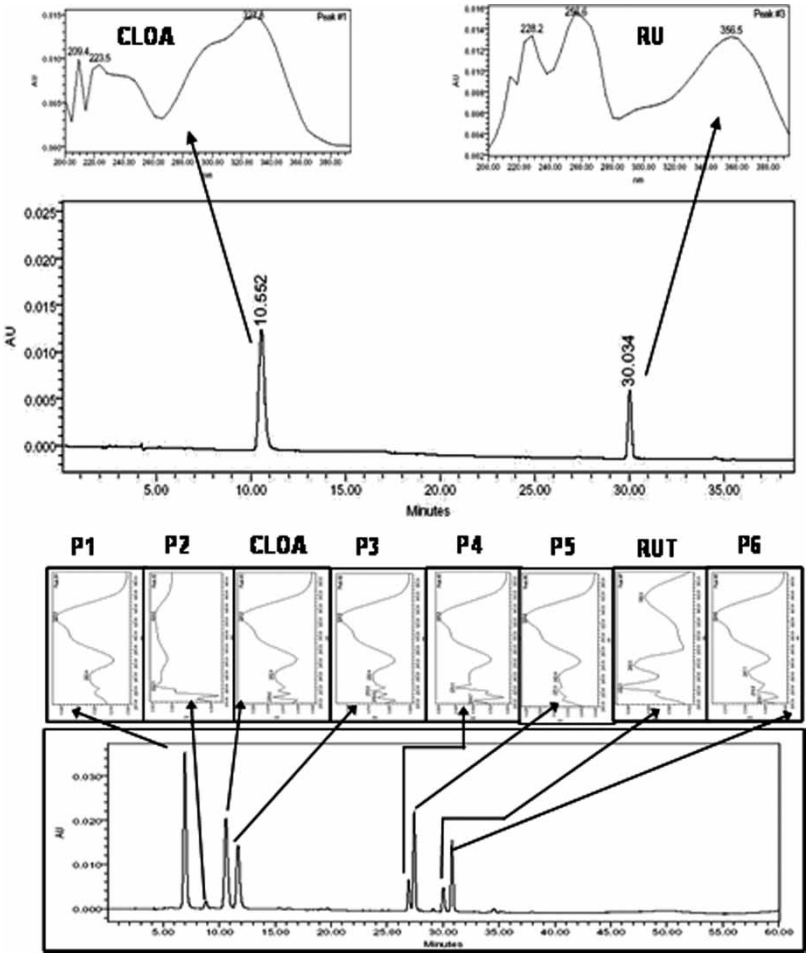


***Figure 1.*** LC profile at 340 nm and diode array spectra 200–400 nm of (A) chlorogenic acid (CLOA) and rutin (RU); (B) extractive solution from *Ilex paraguariensi* (Mate).

The linearity evaluation was performed in three different days by calibration curves analysis for the two standards, CLOA and RU and also for the ES curve. The results are presented in the Tables 1 and 2.

The CLOA and RU calibration curves were linear in the concentration range from 2.0 to 10.0 $\mu g/mL$ with regression coefficients ($r^2$) higher than 0.9980. Therefore, these results show the absence of deviation from linearity for the tested concentration range. The confidence limit, calculated for the intercept, included zero, demonstrating the absence of constant

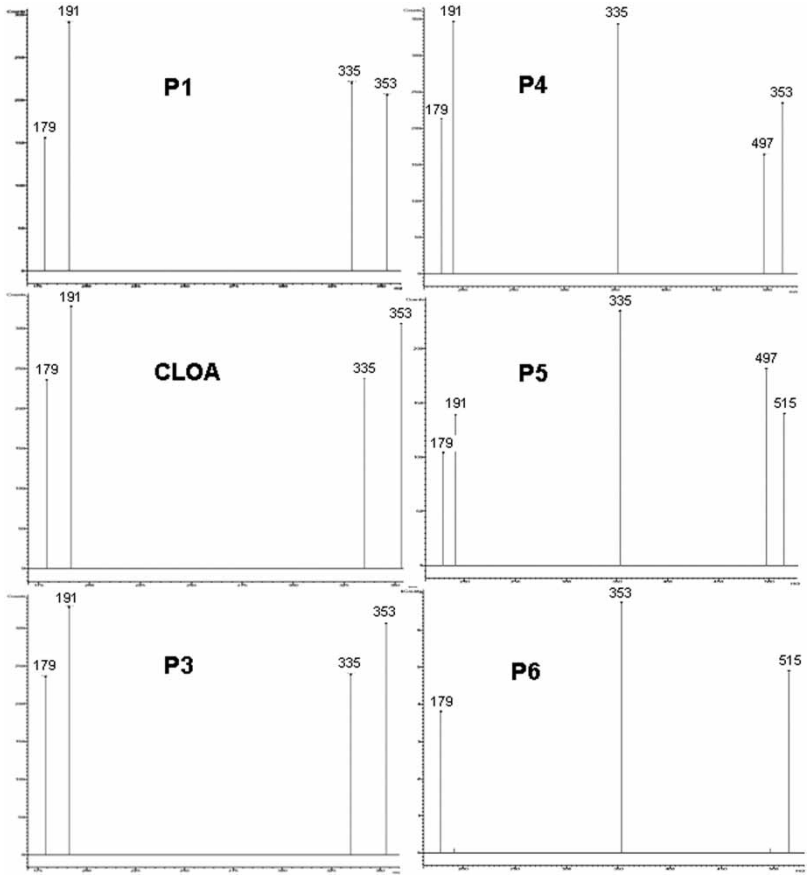


***Figure 2.*** LC MS/MS spectra of the peaks P1, ACLO, P3, P4, P5, RUT, and P6 in extractive solution obtained by decoction of *Ilex paraguariensis* (Mate).

systematic errors. The LOD for CLOA and RU were, respectively, 0.29 and 0.32 μg/mL and the LOQ found were, respectively, 0.89 and 0.97 μg/mL. Therefore, the results show that the method presents good sensitivity for the standards analyzed.

***Table 1.*** Linear regression data for chlorogenic acid and rutin standard curves

| Peak | $r^2$ | a | b | R.S.D. (%) |
|---|---|---|---|---|
| Chlorogenic acid | 0.9980 | −5904.98 | 66505.77 | 2.98 |
| Rutin | 0.9992 | −3515.26 | 36407.72 | 1.53 |

$r^2$ = regression coefficient; a = slope; b = intercept; R.S.D. = relative standard deviation.

***Table 2.*** Linear regression data for both chlorogenic acid and rutin in extractive solution curve

| Peak | $r^2$ | a | b | R.S.D. (%) |
|---|---|---|---|---|
| Chlorogenic acid | 0.9978 | −1290.63 | 712700.52 | 4.69 |
| Rutin | 0.9971 | −634.10 | 125150.42 | 6.08 |

$r^2$: regression coefficient; a: slope; b: intercept; R.S.D. = relative standard deviation.

Table 2 shows the linear regression data obtained for CLOA and RU in the ES curve. The absence of linearity deviation in the range from 50.0 to 500.0 μL/mL is demonstrated. The $r^2$ values of 0.9978 for CLOA and 0.9971 for RU can be considered suitable, considering the complex composition of this herbal sample.

Precision of the LC method was evaluated through the intermediary precision and repeatability tests (Tables 3 and 4). The assay of intermediary precision was performed during three consecutive days, in order to determine the accumulation of the random errors between different ES samples and days. The R.S.D. lower than 2.5% demonstrate the reproducibility and, thus, the low interference of the sample preparation step. All R.S.D. values observed in the repeatability test were lower than 1.5% (Table 4). These values can be considered excellent for complex matrices.[14]

The accuracy of the method was evaluated by the recovery test. Concentrations of CLOA and RU (50, 100, and 150%) were added to ES.



***Table 3.*** Intermediary precision for the standards chlorogenic acid and rutin and for the corresponding polyphenols in the *Ilex paraguariensis* extractive solution

| Compound | Standard concentration (mg/mL) | R.S.D. (%) | Extractive solution concentration (mg/mL) | R.S.D. (%) |
|---|---|---|---|---|
| Neo-chlorogenic acid | — | — | 2.216[a] | 0.44 |
| Chlorogenic acid | 0.098 | 2.14 | 1.669 | 0.82 |
| Crypto-chlorogenic acid | — | — | 1.222[a] | 0.54 |
| P4 | — | — | 0.359[a] | 0.43 |
| P5 | — | — | 1.173[a] | 0.91 |
| Rutin | 0.101 | 2.24 | 0.563 | 0.90 |
| P6 | — | — | 0.933[a] | 0.80 |

[a]Calculated as chlorogenic acid.

R.S.D. = relative standard deviation.

***Table 4.*** Repeatability test for the reference substances and for the corresponding polyphenols in the *Ilex paraguariensis* extractive solution

| Compound | Standard concentration (mg/mL) | R.S.D. (%) | Extractive solution concentration (mg/mL) | R.S.D. (%) |
|---|---|---|---|---|
| Neo-chlorogenic acid | — | — | 2.058[a] | 0.91 |
| Chlorogenic acid | 0.107 | 0.30 | 1.628 | 0.95 |
| Crypto-chlorogenic acid | — | — | 1.288[a] | 1.11 |
| P4 | — | — | 0.388[a] | 1.13 |
| P5 | — | — | 1.081[a] | 1.33 |
| Rutin | 0.103 | 0.25 | 0.563 | 1.02 |
| P6 | — | — | 1.009[a] | 0.75 |

[a]Calculated as chlorogenic acid.



The results (Table 5) indicate recovery rates for CLOA and RU of 97.4 and 103.9%, respectively, with R.S.D. lower than 1.0% in all concentrations analyzed.

Finally, the LC method was applied to compare the polyphenol content in four extractive solutions. For this purpose, besides ES, which was prepared by decoction, three other extractive solutions were analyzed. The first one was prepared by infusion (ESI) and the other one was prepared by turbo extraction ($TE_1$) using water as solvent. The last one was also prepared by turbo extraction, but using ethanol 40% (v/v) as solvent. The LC analysis showed similar polyphenol chromatographic profiles for all extractive solutions. Table 6 shows the content of CLOA, RU, NEO, CPC, P4, P5, and P6) in these extractive solutions (mg/mL). CLOA and its derivatives are present in higher concentration than RU in all extractive solutions. The aqueous extracts prepared using hot water as solvent, decoction, or infusion (ES and ESI) compared to that obtained by turbo extraction in water ($TE_1$), showed higher amounts of the total polyphenol examined (ES: 7.47 mg/mL; ESI: 8.45 mg/mL; $TE_1$: 5.71 mg/mL). However, the highest total polyphenol content was found in the extractive solution prepared by turbo extraction using ethanol 40% (v/v) as solvent ($TE_2$: 11.83 mg/mL).

Regarding the main peaks, the results also demonstrate that the most polar constituents NEO, CLOA, and CPC are present in higher concentration when water is used as solvent (ES: 4.69; ESI: 5.31; $TE_1$: 3.59 mg/mL) than that of less polar P5, RU and P6 (ES: 2.78; ESI: 3.14; $TE_1$: 2.12 mg/mL). However, when ethanol 40% was employed as solvent, the difference is significantly lower assuming almost similar

***Table 5.*** Recovery test for both chlorogenic acid and rutin in *Ilex paraguariensis* extractive solution

| Chlorogenic acid | | | | Rutin | | | |
|---|---|---|---|---|---|---|---|
| Theoretical concentration (μg/mL) | Experimental concentration (μg/mL) | Recovery (%) $\bar{X}$; R.S.D) | Avcerage of the recovery (%) $\bar{X}$; R.S.D) | Theoretical concentration (μg/mL) | Experimental concentration (μg/mL) | Recovery (%) $\bar{X}$; R.S.D) | Total recovery (%) $\bar{X}$; R.S.D) |
| 10.60 | 10.41 | 98.19; 0.89 | | 3.20 | 3.43 | 107.19; 0.87 | |
| 12.60 | 11.96 | 94.92; 0.65 | 97.36; 2.09 | 4.20 | 4.40 | 104.84; 0.24 | 103.95; 3.91 |
| 14.60 | 14.45 | 98.96; 0.33 | | 5.20 | 5.19 | 99.83; 0.19 | |

R.S.D. = relative standard deviation.

***Table 6.*** Polyphenol content in *Ilex paraguariensis* extractive solutions

| Peaks | ES (mg/mL) ($\bar{X}$; S.D.) | ESI (mg/mL) ($\bar{X}$; S.D.) | $TE_1$ (mg/mL) ($\bar{X}$; S.D.) | $TE_2$ (mg/mL) ($\bar{X}$; S.D.) |
|---|---|---|---|---|
| Neo-chlorogenic acid | 2.15[a]; 0.014 | 2,43[a]; 0,007 | 1,72[a]; 0,005 | 2,90[a]; 0,008 |
| Chlorogenic acid | 1.53[a]; 0.008 | 1.75[a]; 0,005 | 1,18[a]; 0,001 | 2,06[a]; 0,006 |
| Crypto-chlorogenic acid | 1.01[a]; 0.007 | 1,13[a]; 0,004 | 0,69[a]; 0,000 | 1,17[a]; 0,004 |
| Peak 4 | 0.41[a]; 0.001 | 0.41[a]; 0.002 | 0.38[a]; 0.001 | 0.58[a]; 0.003 |
| Peak 5 | 1.13[a]; 0.010 | 1,29[a]; 0,003 | 0,87[a]; 0,001 | 2,75[a]; 0,004 |
| Rutin | 0.49; 0.016 | 0,55[a]; 0,002 | 0,36[a]; 0,003 | 0,74[a]; 0,002 |
| Peak 6 | 0.75[a]; 0.015 | 0,89[a]; 0,002 | 0,51[a]; 0,004 | 1,63[a]; 0,005 |
| Total | 7.47 | 8.45 | 5.71 | 11.8 |

ES. Aqueous extractive solution obtained by decoction; ESI. Aqueous extractive solution obtained by infusion; $TE_1$. Extractive solution obtained by turbo-extraction with water. $TE_2$. Extractive solution obtained by turbo-extraction with ethanol 40% (v/v).

[a]calculated by chlorogenic acid standard curve; S.D. = standard deviation.

All the extractive solution were prepared using a plant:solvent ratio of 1.5:10 and 10 minutes of extraction time.



concentration. The interest of separating these two groups of polyphenols is being verified in our laboratory.

## CONCLUSIONS

This first report on validation of a LC analytical method for quantifying polyphenols in *Ilex paraguariensis* extractive solution demonstrated to be simple, specific, precise, rapid, and reproducible, therefore, appropriate for the separation and quantification of this relevant compound class. Applied to the polyphenol assay in four extractive solutions, the results revealed that ethanol 40% (v/v) was more suitable than hot water in the extraction of the polyphenols from the mate. Finally, the proposed LC method can be useful for the quality control of *I. paraguariensis* extractive solutions in phytopharmaceutical or food industries.

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