Original article

UHPLC–MS quantification of coumarin and chlorogenic acid in extracts of the medicinal plants known as guaco (Mikania glomerata and Mikania laevigata)

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A B S T R A C T

In Brazil, Mikania glomerata Spreng. and M. laevigata Sch. Bip. ex Baker, Asteraceae, known popularly as guaco, are widely used for colds and asthma. Although coumarin is adopted as the chemical marker of both species, it was not always detected in M. glomerata, for which chlorogenic acid was identified and quantified instead. The purpose of this study was to develop and validate a method to quantify both coumarin and chlorogenic acid and apply it to extracts of plants identified as M. glomerata, M. laevigata, or as guaco, to determine the pattern of composition of these two species and to observe differences between oven-dried and lyophilized leaves. A method using ultra-high resolution liquid chromatography–mass spectrometry (UHPLC–MS) in the full scan mode was validated for selectivity, matrix effect, linearity, limits of detection and quantification, precision and accuracy. The concentration of coumarin varied between species and samples, therefore these two species should not be used interchangeably. The concentration of chlorogenic acid was also determined for all samples. The UHPLC–MS method permitted the quantification of coumarin and chlorogenic acid in 16 samples of guaco and several commercial samples were possibly misidentified.

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Introduction

The therapeutic use of medicinal plants is part of the history of humanity. Frequently the population of underdeveloped countries still depends strongly on medicinal plants for spiritual, cultural or economic reasons (Quiroz et al., 2014). Furthermore, even in developed countries, the use of traditional herbal remedies is widespread. Herbal remedies are even crossing borders, for example the widespread use of traditional Chinese Medicine in Europe (Hook, 2014).

In Brazil, two species of the Mikania genus, Asteraceae, popularly known as guaco, are used in syrup or prepared as tea for colds and other respiratory problems due to their bronchodilator effect (Silva et al., 2008). Mikania glomerata Spreng. and M. laevigata Sch. Bip. ex Baker may be found in the Atlantic Coast forest, ranging from the state of Bahia in the Northeast of Brazil, to the southern states of Brazil (Gasparetto et al., 2010) and even in Paraguay and Argentina (Lima et al., 2003). M. glomerata was included in the first Brazilian Pharmacopeia (Brasil, 1929) while M. laevigata was only included in the fourth edition of the Brazilian Pharmacopeia (Brasil, 2005). Both species are morphologically similar and may be easily mistaken; they are often commercialized and prepared indistinctly (Anvisa, 2011).

Studies of their composition have led to the identification of phenolic compounds, di- and tri-terpenes, tannins and other components (Gasparetto et al., 2010). Although coumarin has been adopted as the chemical marker of both species (Anvisa, 2008, 2011) and their pharmacological properties are often attributed to this substance, several studies present conflicting results on the concentration of coumarin. For example: Santos et al. (2006) reported that the extract of M. glomerata presented twice as much coumarin as that M. laevigata, whereas Bolina et al. (2009) concluded that M. laevigata presented a slightly higher coumarin content (0.43%) than M. glomerata (0.30%). A third study reported that coumarin was not present in M. glomerata, only in M. laevigata (Bertolucci et al., 2009) which is consistent with our results. These contradictory results may be due to misidentification of the plant species (due to their morphological similarity) or to the diverse analytical methods employed. The use of fresh leaves, oven dried leaves or lyophilized material may also have affected the results (Santos et al., 2006). Further possibilities are variations in secondary metabolites due to seasonal or environmental factors.
(Gobbo-Neto and Lopes, 2007). Preliminary studies by our group detected the presence of chlorogenic acid in *M. glomerata* leaves, which was a surprising as only one study was found relating the presence of chlorogenic acid in *guaco* leaves (Silva et al., 2006). Therefore it was necessary to begin this investigation by defining the plant samples, the drying method and the analytical method to be used. This method should be selective enough to quantify coumarin and chlorogenic acid correctly, but should also detect other, still unidentified, components in the extracts. Several methods have been found in literature for the quantification of coumarins: from simple thin layer chromatography (Alvarenga et al., 2009) to modern electrochemical methods (Miyano et al., 2014). Celeghini et al. (2001) quantified coumarin in extracts of *M. glomerata* leaves by high resolution liquid chromatography with UV detection (HPLC–UV), while Muceneeki et al. (2009) quantified o-coumaric acid, coumarin and syringaldehyde by HPLC–UV. Park et al. (2009) and Chen et al. (2012) used HPLC coupled to mass spectrometry (HPLC–MS) in the MRM mode to quantify coumarins. While this method is highly selective and sensitive, it only permits the analysis of the selected components. In fact, all the methods cited above would not have allowed us to observe the presence of chlorogenic acid in *M. glomerata* samples, if they had been developed for coumarin. The same can be said of the HPLC–MS (MRM) method used to quantify dicafeoylquinic acids (Clifford et al., 2008) and chlorogenic acid metabolites (Santos et al., 2005). However, by using MS detection in full scan mode it is possible to quantify selected ions as well as detect other components of the extract.

In order to evaluate the concentration of coumarin and chlorogenic acid in leaves of *M. glomerata* and *M. laeavigata*, while allowing the detection of the other sample components, a method using ultra-high resolution liquid chromatography–mass spectrometry (UHPLC–MS) was developed and validated. This method was used to analyze the ethanolic extracts of oven dried and lyophilized leaves of both species, as well as leaves commercialized simply as *guaco*. The method was developed for ethanolic extracts of leaves of two plants identified by specialists as *M. laeavigata* and *M. glomerata*; and then applied to other samples of fresh and dry leaves (commercialized as *guaco*).

### Materials and methods

#### Plant samples

*Mikania glomerata* Spreng. and *M. laeavigata* Sch. Bip. ex Baker, Asteraceae, plants were donated and identified by CPQBA, Unicamp (Paulinia, SP) and voucher specimens deposited at the State University of Campinas Herbarium (UEC) number 102046 for *M. laeavigata* and number 102047 for *M. glomerata*. These plants are growing in the Experimental Field of the Institute of Biology. For this study, leaves of both species were collected the same morning and dried by two different methods before grinding and extraction.

Other commercial samples fresh and dry leaves labeled as *guaco*, *M. laeavigata* or *M. glomerata* were bought from local markets or collected from institutional (CPQBA-Unicamp) or home gardens (Table 2). All samples were extracted within days of their acquisition. The dry leaves were extracted in the same way as the identified plant samples; the fresh leaves were lyophilized and then extracted. One plant which was collected in the state of São Paulo and identified as *M. glomerata* by Prof. George Y. Tamashiro of the Biology Institute of Unicamp, presented concentrations of coumarin and chlorogenic acids below quantification level and was therefore dried and extracted to be used as a blank plant matrix (BPM) for calibration curves.

#### Drying and extraction

Approximately half of the leaves collected from each plant were dried in an oven with air circulation at 40 °C for 50 h; the other half was dried by lyophilization for 50 h. The material was then ground in a mortar, passed through a sieve with 0.84 mm spacing, and placed in 67% ethanol (Ecibra, Brazil) to extract, following the proportion of 200 g of leaves to a total of 1.01 of solvent (Brasil, 1929). After filtering, a 10 ml aliquot was taken to evaluate the total solids extracted by drying the solvent in an oven at 105 °C until constant weight.

#### Chromatographic method

A chromatographic method was developed and validated using an ultra-high performance liquid chromatographer coupled to a triple quadrupole mass spectrometer. The equipment used was an Acuity UPLC-TQD (Micromass, Waters, Manchester, England) and the column was a C18 BEH Acuity Waters (1.7 µm × 2.1 mm × 50 mm), oven temperature of 30 °C. The elution was carried out with a flow of 200 µl/min, Solvent A – purified water (Milli-Q) with 0.1% formic acid and Solvent B – HPLC grade acetonitrile (JT Baker, PA, USA), under a gradient starting with 10% B, ramping to 25% B in 4 min, then to 100% B in 8 min, held at 100% B until 8.5 min the returning to the initial conditions and stabilizing until 10 min.

MS detection was performed with electrospray ionization in both positive and negative ion modes, under the following conditions: capillary ± 3000 V, cone ± 35 V, source temperature 150 °C and desolvation temperature of 300 °C. Due to their structure, chlorogenic acid ionized well in negative ion mode and coumarin in the positive ion mode.

Prior to injection the extracts were further diluted in purified water (Milli-Q) in the proportion of 1 part extract to 2 parts water, 2 µl of each sample were injected. The concentration of coumarin and chlorogenic acid in the plant extracts was quantified by comparison to external calibration curves of coumarin (Sigma–Alrich) and chlorogenic acid (Sigma–Aldrich) in solutions of 70% ethanol and in BPM. The method was validated according to the parameters described below.

**Selectivity.** Solutions of the standards of coumarin and chlorogenic acid, plant extracts and plant extracts spiked with the standards were injected, evaluating retention times and fragmentation spectra (MS/MS) of the ion of *m/z* 147 in the positive ion mode (coumarin) and or *m/z* 353 in the negative ion mode (chlorogenic acid).

**Matrix effect.** This effect was calculated according to Economou et al. (2009) using the formula: %C = 100 × (1 − Sm/5S); where %C is the percentage of increase or suppression of the signal, Sm is the angular coefficient of the calibration curve using BPM and Ss is the angular coefficient of the calibration curve using a solution of 70% ethanol.

**Linearity.** This parameter was evaluated by the correlation coefficient of the curves of coumarin and chlorogenic acid in BPM.

**Limit of detection (LD) and quantification (LQ).** These parameters were determined by the injection of a series of dilutions of coumarin and chlorogenic acid in BPM, with LD determined as the concentration that resulted in a peak area three times greater than the noise level and LQ a concentration that resulted in a peak area ten times greater than the noise level.

**Precision.** This parameter was evaluated for five injections of extracts of *M. laeavigata* and *M. glomerata* leaves along one day for the concentration of coumarin and chlorogenic acid.

**Accuracy.** As no certified material was available, this parameter was evaluated by the addition (fortification) of coumarin and chlorogenic acid in three levels of concentration: low (30 µg/ml), medium (150 µg/ml) and high (400 µg/ml), in a procedure similar.
to the preparation of the calibration curve. Recovery (R%) was calculated according to the following equation: 

$$R\% = \left( \frac{C1 - C2}{C3} \times 100 \right)$$

where C1 is the concentration which was determined for the fortified sample, C2 is the concentration of the non-fortified sample or matrix and C3 is the concentration of standard which was added (fortification).

**Results and discussion**

**Drying method**

Leaf extracts of both species (dried in the oven or by lyophilization) presented similar amounts of solid residue: 1.2% (m/v) for oven dried *M. laevigata* and 1.3% for lyophilized *M. laevigata*; 1.3% for oven dried *M. glomerata* and 1.4% for lyophilized *M. glomerata*. The UHPLC–MS profile of the leaf extracts of each species was different (Fig. 1) but the drying procedure did not affect the general profile of the species. However the amount of coumarin and chlorogenic acid was lower for the oven dried leaves of both species (Table 1), showing that heat affected the contents of the bioactive compounds evaluated. For this reason, all the other fresh leaves collected during this study (Table 2) were lyophilized, rather than oven dried, before extraction.

**Validation of UHPLC–MS method**

Analytical curves of coumarin and chlorogenic acid standards with concentrations between 1 ng/ml and 800 µg/ml were built in 70% ethanol/water and in BPM to validate the method. This range of concentrations was necessary due to the variable content of coumarin and chlorogenic acid in both plant species and in the extracts that were analyzed. Furthermore all parameters were successfully validated for this range of concentrations.

**Selectivity.** This parameter was determined comparing the retention time (RT) and the fragmentation of coumarin standard (positive ion mode m/z 147) and chlorogenic acid standard (negative ion mode m/z 353) with the same ions in the samples. Fig. 1A shows the selected ion chromatogram (m/z 147 positive ion mode) of the coumarin standard and Fig. 1B shows the selected ion chromatogram (m/z 147) of the *M. laevigata* extract, the retention times are practically identical and both present the same MS/MS (Fig. 1E). Fig. 1F shows the selected ion chromatogram of m/z 353 negative ion mode. Although three peaks are present, the first retention time (2.45 min) corresponds to chlorogenic acid and the others are isomers, present as impurities in the standard. Fig. 1G shows the selected ion chromatogram (m/z 353) of the *M. glomerata* extract, the peak at retention time of 2.51 showed the same MS/MS as chlorogenic acid (Fig. 1J). Small variations in the retention time were due to the complex matrix of the plant extracts. The solvent did not present peaks of these compounds and in BPM (Fig. 2) the areas of peaks of these compounds were below the LD.

**Matrix effect.** This parameter was evaluated comparing curves of both standards in solvent with curves in BPM. The results showed that the plant matrix resulted in a reduction of peak area of 4.37% for coumarin and of 19.20% for chlorogenic acid in relation to the same concentrations in solvent. Therefore all subsequent analytical curves used in this study were built using BPM.

**Linearity.** Analytical curves of both standards in BPM were built between 1 ng/ml and 800 µg/ml, with triplicate injections of each point. The ideal parameter of linearity ($R^2 > 0.99$) could not be attained due to the interference of the matrix and the wide range of concentrations used. The analytical curve for coumarin (m/z 147 in the positive ion mode) was linear ($R^2 = 0.9718$) for concentrations between 1.5 µg/ml and 730.0 µg/ml, and the curve for chlorogenic acid (m/z 353 in the negative ion mode) was linear ($R^2 = 0.9831$) for concentrations between 10.0 µg/ml and 550.0 µg/ml. Only one sample of dry leaves (sample 8, Table 2) fell outside the range of these curves and the concentration of coumarin was obtained by extrapolation of the curve.

**Limit of detection (LD) and quantification (LQ).** For coumarin the LD was 0.32 µg/ml and the LQ was 3.30 µg/ml in BPM. For chlorogenic acid the LD was 4.18 µg/ml and the LQ was 20.38 µg/ml in the blank plant matrix. The LD and LQ were higher for chlorogenic acid, possibly because of less ionization in the negative ion mode.

**Precision.** The area of the peaks of five replicate injections of the extracts of *M. glomerata* presented variation of 5% for coumarin (m/z 147 in the positive ion mode) and 4% for chlorogenic acid (m/z 353 in the negative ion mode). For the extracts of *M. laevigata* the variation was of 1% for coumarin and 4% for chlorogenic acid.

**Accuracy.** The recovery (R) values for the three levels of fortification of coumarin were in the extract of *M. laevigata* 75.15% (low), 84.60% (medium) and 67.04% (high). For the fortification with chlorogenic acid in the extract of *M. glomerata* the R was: 106.42% (low), 88.56% (medium) and 95.74% (high). As only the result for the highest concentration of coumarin fell slightly outside the accepted recovery parameter (70–120%) (Ribani et al., 2004).

**Analytical results for plant samples** One advantage of the method presented herein is that acquisition in both positive and negative modes is obtained in the same short chromatographic run. This chromatographic method was first used to analyze the extracts of the oven dried and lyophilized leaves of *M. glomerata* and *M. laevigata*. The extracts were prepared using 200 g of leaves/l of solvent; therefore the results shown in Table 1 in µg/ml correspond to 200 mg of dried leaves. In this manner we observe that the leaves of the identified *M. glomerata* plant contained practically no coumarin. Chlorogenic acid content was between 0.67% (m/m) for oven dried leaves and 0.82% (m/m) for lyophilized leaves. In comparison, *M. laevigata* oven dried leaves contained 0.37% (m/m) coumarin and lyophilized leaves contained 0.57% (m/m) coumarin. This result is in agreement with the coumarin content presented by Bolina et al. (2009) for *M. laevigata* leaves, and also with the results presented by Bertolucci et al. (2009) who stated that no coumarin was detected in *M. glomerata*. Furthermore, the UHPLC–MS chromatograms show that the two species of guaco present distinct chromatographic profiles in both positive (Fig. 1C and D) and negative (Fig. 1H and I) ion modes.

The peak of coumarin (RT 4.06) is clearly seen in the chromatogram of the *M. laevigata* extract (Fig. 1C) but absent in the chromatogram of the *M. glomerata* extract (Fig. 1D). Inversely, the peak of chlorogenic acid (RT 2.51) is absent in the chromatogram of the *M. laevigata* extract (Fig. 1H) but is clearly seen in the chromatogram of the *M. glomerata* extract (Fig. 1I). Furthermore, the chromatographic profiles of both species are clearly different. Although climatic and seasonal variations could affect these results, both species are planted side-by side in the Experimental Field in Unicamp and collected at the same time, so subject to the same influences.

In order to check if other guaco samples would behave in the same manner, eight samples of fresh leaves and eight samples of dry leaves were acquired, extracted and analyzed using the same validated chromatographic method. The results are shown in Table 2. Fresh leaves, samples 9–12, identified as *M. glomerata* and collected at CPQBA-Unicamp, presented the same pattern as our original *M. glomerata* leaves, with varying amounts of chlorogenic acid and no detectable levels of coumarin. Sample 13, identified as *M. laevigata* and collected at CPQBA-Unicamp, presented approximately the same amount of coumarin as our original sample (Table 1) and chlorogenic acid below the LQ. This confirmed the expected pattern of compounds expected for these species. The fresh leaves of both species were collected several months after the voucher specimens. These results confirmed the pattern of more...
coumarin than chlorogenic acid for *M. laevigata* and the opposite, more chlorogenic acid than coumarin, for *M. glomerata*.

Dried leaf samples 2, 3, 5, 6, 7 and 8, presented a pattern similar to *M. laevigata* leaves, with varying concentrations of coumarin and less or no chlorogenic acid. The way these commercial samples were dried is unknown, but possibly affected the contents of coumarin/chlorogenic acid. These samples were labeled simply as guaco (sample 2) or *M. glomerata* and may have been misidentified.

Dried leaf sample 4, labeled as guaco and acquired in the Amazon, presented an approximately equal concentration of coumarin and chlorogenic acid, which is different from the patterns previously encountered and could even belong to a different species of Mikania. Sample 1, also labeled as guaco, presented several moldy leaves and stems in the package and no detectable levels of coumarin or chlorogenic acid. Therefore it is impossible to ascertain if this sample was mislabeled or if the original components degraded due to
bad conservation. It is worrying that this sample was being sold at a market and could have been consumed by children or adults wishing to alleviate the symptoms of a cold!

Fresh leaf samples 14–16, known simply as guaco by their users, presented varying concentrations of coumarin but chlorogenic acid contents below LD or LQ, which is similar to the results of M. laevigata voucher plant and fresh plant from CPQBA.

The only samples which presented concentrations of coumarin and chlorogenic acid compatible with the identified sample of M. glomerata were those fresh leaves identified and collected at CPQBA–Unicamp. Most other samples, regardless of their labels, had concentrations of coumarin and chlorogenic acid which were similar to those found in leaves of the M. laevigata voucher plant. The only two guaco samples which did not follow this pattern were possibly degraded (sample 1) or belonged to another species of Mikania (sample 4).

The variation in the concentration of coumarin and chlorogenic acid found in identified specimens of M. glomerata and M. laevigata demonstrates that these two species do not contain similar amounts of coumarin and therefore should not be used interchangeably. Furthermore commercial samples also contain variable contents of coumarin and chlorogenic acid and may be misidentified or degraded. Further studies regarding the morphological description of plants known as guaco throughout Brazil are underway to ascertain which species is being used regionally and if environmental factors affect the concentration of coumarin and chlorogenic acid in these plants.

By using UHPLC–MS in the full scan mode, not only were coumarin and chlorogenic acid identified and quantified, but also other compounds, which make up the complex chemical profile of these plant extracts, were detected. Although other HPLC–MS methods in the MRM mode maybe more sensitive, they do not provide us with a panoramic view of sample composition, which leads to new discoveries, such as the importance of chlorogenic acid in M. glomerata samples. This chromatographic method allowed us to distinguish between samples of leaves from two morphologically similar species and can be used for the quality control of the dry leaves and extracts of these species. This information is paramount for the correct use of these medicinal plants by the population.

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**Table 1**

Concentration of coumarin and chlorogenic acid in hydro-ethanolic extracts of oven dried and lyophilized leaves of M. glomerata and M. laevigata planted in the experimental field (IB, UNICAMP), triplicate extractions.

<table>
<thead>
<tr>
<th>Leaves of</th>
<th>Coumarin content in extract (μg/ml)</th>
<th>CV (%)</th>
<th>Chlorogenic acid content in extract (μg/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven dried M. glomerata</td>
<td>15</td>
<td>–</td>
<td>1348</td>
<td>9.94</td>
</tr>
<tr>
<td>Lyophilized M. glomerata</td>
<td></td>
<td>–</td>
<td>1634</td>
<td>5.79</td>
</tr>
<tr>
<td>Oven dried M. laevigata</td>
<td>775</td>
<td>3.26</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lyophilized M. laevigata</td>
<td>1131</td>
<td>0.61</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Below LD.

**Table 2**

Concentration of coumarin and chlorogenic acid in hydro-ethanolic extracts of dry and fresh leaves labeled as M. glomerata, M. laevigata or guaco.

<table>
<thead>
<tr>
<th>Sample labeled as</th>
<th>Form</th>
<th>Bought or collected in</th>
<th>Coumarin (μg/ml)</th>
<th>Chlorogenic acid (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Guaco</td>
<td>Dry leaves</td>
<td>Porto Alegre – RS</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>2. Guaco/Mikania</td>
<td>Dry leaves</td>
<td>Porto Alegre – RS</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>3. M. glomerata</td>
<td>Dry leaves</td>
<td>Florianópolis – SC</td>
<td>170</td>
<td>64</td>
</tr>
<tr>
<td>4. Guaco</td>
<td>Dry leaves</td>
<td>Manaus – AM</td>
<td>128</td>
<td>156</td>
</tr>
<tr>
<td>5. M. glomerata</td>
<td>Dry leaves</td>
<td>Brasília – DF</td>
<td>375</td>
<td>b</td>
</tr>
<tr>
<td>6. M. glomerata</td>
<td>Dry leaves</td>
<td>Paulínia – SP</td>
<td>254</td>
<td>a</td>
</tr>
<tr>
<td>7. M. glomerata</td>
<td>Dry leaves</td>
<td>Paulínia – SP</td>
<td>316</td>
<td>a</td>
</tr>
<tr>
<td>8. M. glomerata</td>
<td>Dry leaves</td>
<td>Paulínia – SP</td>
<td>2794</td>
<td>a</td>
</tr>
<tr>
<td>9. M. glomerata (1)</td>
<td>Fresh leaves</td>
<td>CPQBA, UNICAMP Paulínia – SP</td>
<td>a</td>
<td>529/86</td>
</tr>
<tr>
<td>10. M. glomerata (2)</td>
<td>Fresh leaves</td>
<td>CPQBA, UNICAMP Paulínia – SP</td>
<td>a</td>
<td>211/86</td>
</tr>
<tr>
<td>11. M. glomerata (3)</td>
<td>Fresh leaves</td>
<td>CPQBA, UNICAMP Paulínia – SP</td>
<td>a</td>
<td>786/260</td>
</tr>
<tr>
<td>12. M. glomerata (4)</td>
<td>Fresh leaves</td>
<td>CPQBA, UNICAMP Paulínia – SP</td>
<td>a</td>
<td>260/1024</td>
</tr>
<tr>
<td>13. M. laevigata (1)</td>
<td>Fresh leaves</td>
<td>CPQBA, UNICAMP Paulínia – SP</td>
<td>b</td>
<td>354/1024</td>
</tr>
<tr>
<td>14. Guaco</td>
<td>Fresh leaves</td>
<td>Guarapuava – PR</td>
<td>172</td>
<td>b</td>
</tr>
<tr>
<td>15. Guaco</td>
<td>Fresh leaves</td>
<td>João Pessoa – PB</td>
<td>365</td>
<td>b</td>
</tr>
<tr>
<td>16. Guaco</td>
<td>Fresh leaves</td>
<td>São Paulo – SP</td>
<td>354</td>
<td>a</td>
</tr>
</tbody>
</table>

a: Below LD, b: Below LQ, Id – botanical identification by CPQBA-UNICAMP.

Fig. 2. Selected ion chromatograms of (A) ion m/z 147 in the positive ion mode and (B) m/z 353 in the negative ion mode of BPM, showing that the contents of coumarin and chlorogenic acid are below the LQ in the blank plant matrix.
Conflict of interest

The authors have none to declare.

Authors' contributions

ACHFS planned the research, LVM performed the experiments validated the method. Both authors helped with writing this paper.

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