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Short communication

Quantification of catechin in the spray-dried extract of *Pimenta pseudocaryophyllus*

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ABSTRACT

This paper describes the quantification of catechin in the spray-dried extract of *Pimenta pseudocaryophyllus* (Gomes) Landrum, Myrtaceae, citral chemotype using a validated HPLC-PDA method. The method employs a RP-18 column with acetonitrile:water-orthophosphoric acid 0.05% (gradient system) and UV detection at 210 nm. The method was demonstrated to be simple, sensitive, specific, linear, precise, accurate and robust. The response was linear over a range of 5–200 μg/ml ($r > 0.999$). The range of recoveries was 92.27–102.54%. The relative standard deviation values for intra- and inter-day precision studies were 4.30 and 3.78%, respectively. This assay can be readily utilized as quality control method for catechin in the dried extract of *P. pseudocaryophyllus*.

Introduction

Pimenta pseudocaryophyllus (Gomes) Landrum, Myrtaceae, known as "pau-cravo", "louro-cravo", "louro", "craveiro", and "chá-de-bugre" is the only species of the genus native to Brazil. In folk medicine, teas and refreshing drinks prepared with its leaves are used as tranquilizers, diuretics, digestive regulators, and for the relief of cold symptoms, fatigue, fevers, and menstrual problems. They have also been used to treat arthritis, gonorrhea and syphilis (Landrum, 1986, D'angelis and Negrelle, 2014).

Pimenta pseudocaryophyllus is an aromatic plant with essential oils of variable chemical constitution in its leaves. Intraspecific variability studies have identified three chemotypes for this species based on its essential oil chemical composition: citral, caryophyllene and (*E*)-methyl isoeugenol (Paula et al., 2011).

Many studies have shown the biological and pharmacological activities of this plant: anti-nociceptive, anti-inflammatory, antifungal, anti-depressive-like, against UVB-induced oxidative stress (Paula et al., 2012; Campanini et al., 2013; Fajemiroye et al., 2013; Campanini et al., 2014; Silva et al., 2014).

The compounds lupeol, α-amyrin, β-amyrin, quercetin, quercitrin, catechin and afzelin isolated from the fractions of the crude extracts of the leaves of *P. pseudocaryophyllus* citral chemotype may be related to the anti-inflammatory, antinociceptive, antioxidant and antimicrobial activities previously mentioned (Paula et al., 2012; Silva et al., 2014). Catechin can be considered one of the main compounds because of its considerable concentration in the extract and its therapeutic importance already registered for other plants (Bansal et al., 2013; Sharma and Goyal, 2015; Rana et al., 2016).

Traditional HPLC coupled with ultraviolet (UV), photodiode array (PDA), electrochemical detection (ECD), and mass spectrometry (MS) is among the most frequently employed methods in the identification of catechins in plant materials (Novak et al., 2010; Cuevas-Valenzuela et al., 2014; Bae et al., 2015; Tao et al., 2016). Most recently, ultrahighpressure liquid chromatography (UHPLC) has shown considerable potential in terms of speed and separation efficiency for the analysis of catechins (Rahim et al., 2014). HPLC-MS and HPLC-MS/MS are considered more useful than other methods because they provide information about the molecular mass and structural features of components. However, since UHPLC and HPLC-MS methods are quite costly to purchase and maintain, many laboratories prefer to use HPLC-UV or PDA detection, which are found to be less costly, comparably convenient to operate, and remain suitable for providing routine analysis (Bae et al., 2015).

Therefore, considering the pharmacological potential of *P. pseudocaryophyllus*, the goal of the present work was to quantify catechin in the spray-dried extract of the citral chemotype from *P. pseudocaryophyllus* leaves, using a validated HPLC-PDA method, to

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Table 1
Summary of parameters of catechin and spray-dried extract calibration curves.

	Catechin	Spray-dried extract
Linear range (μg/ml)	200–5	4000–100 (219.32–4.36 of catechin)
LOD (μg/ml)	0.7825	21.3463
LOQ (μg/ml)	2.6085	71.1545
Linear regression data[a]		
n	7	7
Slope (*a*)	91 367.0161	5064.4
Standard deviation of the slope	1658.363	126.1257
Standard deviation of the slope (%)	1.8150	2.4904
y-Intercept (*b*)	32 641.3373	−78 592
Linear correlation coefficient (*r*)	0.9998	0.9999

[a] $y = ax + b$, where x is the concentration of the analyte and y is the peak area.

contribute to the choice of a chemical marker and of the analytical method that is most useful in its quality control.

Material and methods

All reagents and solvents were HPLC grade (J.T. Baker®, USA), except orthophosphoric acid (Impex®, P.A. grade). The water was purified using a Milli-Q system (Millipore®, USA). Catechin (Sigma®, USA) of the highest grade (purity > 98.0%) was used as external standard.

Leaves of *Pimenta pseudocaryophyllus* (Gomes) Landrum, Myrtaceae, citral chemotype were collected in São Gonçalo do Abaeté (Minas Gerais, Brazil) 18°20′58.4″ South; 45°55′23.4″ West, 864 m, in February 2014. The plant material was identified by Carolyn E. Barnes Proença, from the Universidade de Brasília. A voucher specimen was deposited in the herbarium of the Universidade Federal de Goiás under the number UFG-27.159.

Leaves were dried in a circulating air oven at 40 °C, and powdered. The milled plant material was submitted to percolation with 95% (v/v) ethanol. The fluid extract was concentrated in a rotary evaporator until plant:solvent ratio of 1:2.

The spray-drying of the concentrated extract was performed using a mini spray-dryer model LM-MSD 1.0 (Labmaq®, Brazil) with a concurrent flow regime. The drying conditions were: set at drying air inlet temperature of 125 °C, outlet temperature of 65 °C, the atomizing air flow rate was maintained at 40 l/min with a pressure of 4 kgf, drying air flow rate at 3 m^3/min and flow rate of the concentrated extract at 4 ml/min. The temperature in the drying room was maintained at 25 °C and the relative humidity at 25%. No drying adjuvants were used.

For quantification of catechin in the spray-dried extract, a Waters® HPLC Alliance system (Waters Corp., USA) consisting of a separation module e2695, a 2998 photo diode array detector, and software Empower 2.0 were used. The chromatographic runs with injection volume of 10 μl were carried out on a Zorbax Eclipse Plus (Agilent®) C-18 5 μm (250 mm × 4.6 mm) column in an oven equilibrated at 25 °C. During development of the method, different solvent systems were tried out and the analyses were monitored from 190 to 400 nm. The mobile phase that demonstrated the best results consisted of a previously sonicated mixture of acetonitrile (A) and water-orthophosphoric acid 0.05% (B); 12:88 (0–9 min); 30:70 (9–17 min); 12:88 (17–30 min), in gradient condition and flow rate of 1 ml/min. Wavelengths between 200 and 280 nm are ordinarily applied to the detection of catechins (Santagati et al., 2008; Yu et al., 2009; Dhanani et al., 2016). In this work the chromatograms was recorded at 210 nm. At this wavelength, the best base-line separations with maximum absorbance in gradient conditions were achieved (data not showed).

For preparation of the sample, 1 mg of the spray-dried extract was dissolved in methanol (10 ml) by sonication for 10 min and filtered through a 0.45 μm Millipore® membrane filter.

The catechin standard (1 mg) was dissolved in methanol (10 ml) by sonication for 10 min in order to prepare a working solution, which was used for the subsequent dilutions for validation of the method.

The validation of the method was performed according to the International Conference on the Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005), and Brazilian Resolution RE 899/2003 from the Brazilian National Surveillance Agency (Anvisa, 2003).

The chromatographic system was evaluated before the method validation in order to assess the suitability parameters (US-FDA, 2001), including Resolution (Rs), tailing factor (TF) and the number of theoretical plates (n). The results were expressed as the average (±SD) of six determinations.

The selectivity of the method was evaluated through the comparison of the chromatograms of the blank (methanol), the sample solution and the catechin standard in order to detect if there were any co-elution interferents. The spectral similarity of the catechin peak in the standard and sample was also evaluated by comparing the UV spectra over a range of 190–400 nm.

The linearity was determined by the analytical curve of seven solutions with concentration of the catechin standard ranging between 5.0 and 200 μg/ml. These solutions were prepared in triplicate and injected three times, after filtration through a 0.45 mm Milipore® membrane. The analytical curve and linearity equations were calculated by linear regression analysis from the correlation between the peak areas and standard concentrations, using Excel software. The linear correlation coefficient (r) was calculated.

Based on Anvisa's Normative Instruction n° 4, published on June 18th 2014 (Anvisa, 2014), the linearity was also determined for the spray-dried extract in concentration solutions ranging from 4.36 to 219.32 μg/ml of catechin.

The limit of detection (LOD) and limit of quantification (LOQ) were mathematically determined based on the standard deviation (SDb) of the y-intercept and the slope (S) of the calibration curve, according to Eqs. (1) and (2).

$$\text{LOD} = \frac{\text{SDb} \times 3}{S} \quad (1)$$

$$\text{LOQ} = \frac{\text{SDb} \times 10}{S} \quad (2)$$

The precision was evaluated at two levels: repeatability (intra-day) and intermediate precision (inter-day), using the relative standard deviation (RSD) as the criterion.

The repeatability was determined through analysis of the sample solution by performing three injections at three levels (5, 50 and 200 μg/ml) of the marker in triplicate.

The intermediate precision was assessed by the same procedure over a period of two days and by different analysts.

The accuracy was determined through the analyte recovery test. Sample solutions were prepared at low, medium and high level concentrations (80%, 100%, and 120%) of the marker in the liner range, in triplicate. Standard concentration of 25 μg/ml of catechin was added to three sample solutions.

The accuracy was calculated for each level through the ratio between the average experimental concentration and theoretical concentration of the added standard according to Eq. (3).

$$\text{Accuracy} = \frac{\text{sample con. with standard} - \text{sample conc. without standard}}{\text{standard theoretical concentration}} \times 100 \quad (3)$$

The robustness of the method was evaluated through the analysis of the catechin content in the SDEC in the original and modified

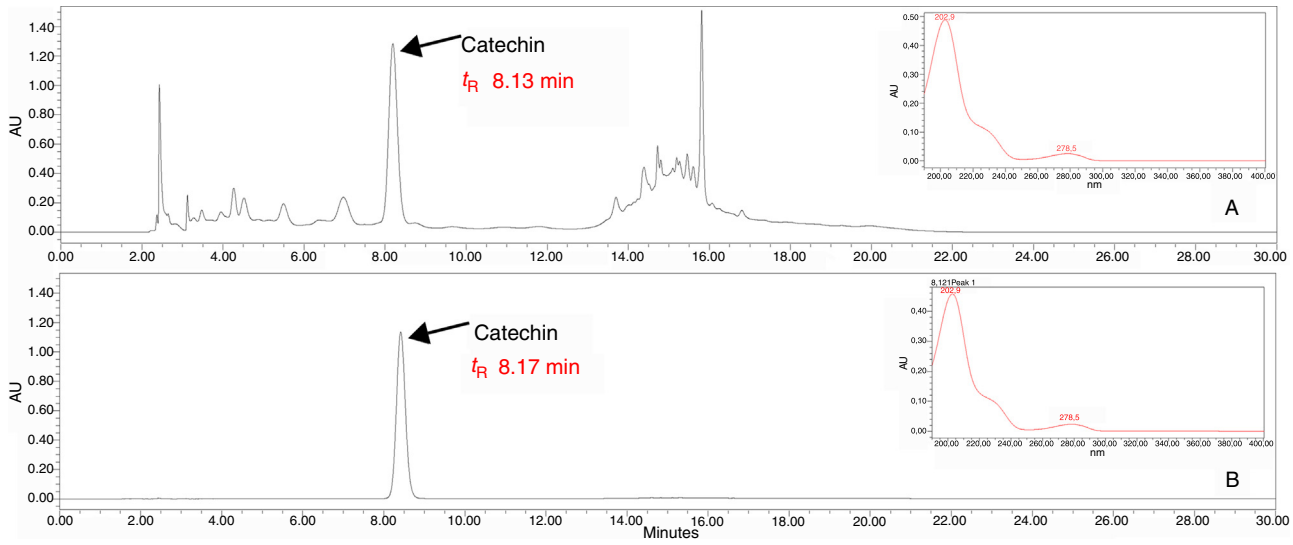

Fig. 1. Chromatogram of *Pimenta pseudocaryophyllus* citral chemotype spray-dried extract (A), catechin standard (B). For chromatographic conditions, see Material and Methods section.

Table 2
ANOVA data to the linearity of catechin and spray-dried extract.

	DF	SS	MS	*F*	*F*tab
Catechin					
Model	1	8.6741×10^{14}	8.6741×10^{14}	19706.89984	4.38
Residual	19	9.04547×10^{11}	$4.7607741360 \times 10^{10}$	Linear	
Lack of fit	5	2.8833×10^{11}	$5.7665921163 \times 10^{10}$	1.3101265	2.95
Error	14	6.16217×10^{11}	$4.4015534288 \times 10^{10}$	No lack of fit	
Spray-dried extract					
Model	1	1.03912×10^{15}	1.03912×10^{15}	9056.30325	4.38
Residual	19	1.75672×10^{12}	$9.2458980207 \times 10^{10}$	Linear	
Lack of fit	5	1.50361×10^{11}	$3.0072203206 \times 10^{10}$	0.26209	2.95
Error	14	1.60636×10^{12}	1.1474×10^{11}	No lack of fit	

DF, degrees of freedom; SS, sum of squares; MS, mean sum of squares; *F*, calculated *F* value; *F*tab, tabulated *F* value.

conditions. The following conditions were changed: the batch number of the column, the oven temperature of the column (25 °C, 23 °C, and 27 °C) and the manufacturer of acetonitrile used in the mobile phase. For each of the above conditions, the sample solutions were injected in triplicate. The data were evaluated using the relative standard deviation (RSD).

The quantification of catechin in the spray-dried extract was performed through the linear regression of the external standard, in six replicates. The catechin content was determined by Eq. (4).

$$\text{Content} \quad (\%) \frac{C \times 100}{Ca} \tag{4}$$

C = catechin concentration (μg/ml) in the extract, calculated through the linear regression equation.

Ca = extract concentration in the sample solution (μg/ml).

Results and discussion

The analytical separations were optimized by evaluating different parameters and solvent systems consisting of methanol, acetonitrile, orthophosphoric acid solution (0.05%) and acetic acid (1–2%) as mobile phase, in isocratic and gradient conditions. Amongst the tested mobile phases, the best peak resolution for catechin and the best base-line separations with maximum absorbance were achieved employing a gradient method with mobile phase that has consisted of a mixture of acetonitrile (A) and water-orthophosphoric acid 0.05% (B): 12(A):88(B) (0–9 min); 30(A):70(B) (9–17 min); 12(A):88(B) (17–30 min), monitored at 210 nm at flow rate of 1 ml/min. It is opportune to note that even though the retention time of catechin is about 8.10 min, the run time of the method was proposed to be 30 min so that the chromatographic system is set to its first condition slowly. Besides, there are other compounds in the extract with longer retention times that need to be removed from the column before the next injection.

Fig. 1 shows the chromatograms of the sample solution, marker (catechin), and blank (methanol) as well as the UV spectra (190–400 nm) of catechin in the chosen conditions described above. The developed method proved to be selective and specific. No co-elution interferents were detected and there was no interference in the retention time of catechin.

The average values (±SD) of system suitability parameters of catechin in the sample were: 1.066 (±0.004) for tailing factor; 2.799 (±0.045) for resolution; and 7195.577 (±74.155) for number of theoretical plates. The system suitability tests showed results according to FDA specifications and proved that the system was adequate for the catechin quantification in the *P. pseudocaryophyllus* spray-dried extract.

The linearity results for catechin and sample in the developed method are presented in Table 1. The catechin and sample calibration curves proved to be linear over the proposed range, 5–200 μg/ml and 100–4000 μg/ml respectively, and the data show a good linear correlation coefficient (r), which demonstrates an acceptable data fit to the regression curve.

In accordance with the Analytical Methods Committee (AMC), a value of linear correlation coefficient close to the unit is not enough data to support linearity, and, consequently, the test for the lack of fit should be applied. This test assesses the variance of the residual values (Hadad et al., 2009). According to Table 2, the calculated F values for lack of fit were lower than the tabulated F values at 95% confidence level ($p = 0.05$); therefore, the linear regression did not show lack of fit for the marker and sample, confirming the linearity of the methods. The relative standard deviation (%RSD) regarding the slope of the calibration curve of catechin was 1.815%. This value is within the limits set by ICH (2005) and Anvisa (2003), which should not exceed 5%.

The LOD values were 0.7825 μg/ml and 21.3463 μg/ml for catechin standard and catechin in the sample, respectively. The LOQ values were 2.6085 μg/ml and 71.1545 μg/ml for catechin standard and catechin in the sample, respectively.

The results of the method's precision, at the levels of repeatability and intermediate precision, both exhibited RSD values lower than 5%. The RSD for day 1, analyst 1 was 4.30%; the RSD for day 2, analyst 2 was 2.41%; and the RSD inter-day was 3.78%.

For the accuracy results, the recovery interval ranged from 92.276% to 102.547%, with RSD% of 3.16%. The recovery test measures the amount of the analyte, present or added in the analytic portion of the test material that is recovered and could be quantified. The acceptable recovery intervals depend on the analytical complexity and the sample, and can range from 50 to 120% with precision up to ±15% (Ribani et al., 2004).

The modified conditions in the column oven temperature, the acetonitrile manufacturer and column batch resulted in %RSD values of 3.71, 3.90, and 4.31 for the peak area, and 3.30, 3.44, and 3.61 for the catechin content, respectively, demonstrating the robustness of the proposed method, since there was no significant effect on chromatographic resolution in the method.

The catechin content in the spray-dried extract, calculated from linear regression equation was 5.44% (±0.18).

Conclusion

For the analysis of catechin in *P. pseudocaryophyllus*, a sensitive and accurate HPLC-PDA method was developed. The method was validated according to the ICH guidelines and Brazilian regulations. The results proved that this method is suitable for its purpose, and thus can be used in the quality control of *P. pseudocaryophyllus* citral chemotype spray-dried extract.

Authors' contributions

LCS contributed by running the laboratory work, chromatographic analysis, and analysis of the data and also drafted the paper. RDM contributed to the laboratory work and chromatographic analysis. DRS contributed to chromatographic analysis. VCSA contributed to critical reading of the manuscript. JRP and ECC contributed by providing the standard catechin and with the critical reading of the manuscript. JAMP contributed by running laboratory work, and designed the study, supervised the laboratory work and contributed to the drafting and critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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