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LC determination of citral in *Cymbopogon citratus* volatile oil

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Abstract

It was the aim of this study to develop and validate a HPLC method for the quantitative determination of citral in *Cymbopogon citratus* volatile oil. The HPLC assay was performed using a Spherisorb® CN column (250 mm × 4.6 mm, 5 μm), a *n*-hexane:ethanol (85:15) mobile phase and an UV detector (set at 233 nm). The following parameters were evaluated: linearity, precision, accuracy, specificity, quantification and detection limits. The method showed linearity in the range of 10.0–30.0 μg ml^{-1}. Precision and accuracy were determined at the concentration of 20 μg ml^{-1}. The concentration of citral in *C. citratus* volatile oil obtained with this assay was 75%. The HPLC method developed in this study showed an excellent performance (linearity, precision, accuracy and specificity) and can be applied to assay citral in volatile oil.

Keywords: *Cymbopogon citratus*; Volatile oil; Citral; Normal-phase chromatography

1. Introduction

Cymbopogon citratus (DC) Stapf (Gramineae) is an herb worldwide known as lemongrass. The tea made from its leaves is popularly used in Brazil as antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative [1]. The volatile oil (VO) obtained from fresh leaves of this plant is widely used by the perfume and cosmetics industries. It has also been used in chemical synthesis, due its high contents of citral, a natural mixture of two isomeric aldehydes, neral and geranial [2]. The antibacterial and antifungal activities of lemongrass VO and its components have been reported in the literature [3–9]. Moreover, the literature points that the VO properties are mainly due to its major component, citral [10]. Kurita et al. [11] have shown that citral act as a fungicidal agent because it is able to form a charge transfer complex with an electron donor of fungal cells, which result in fungal death.

Previous studies have reported a great antifungal activity of the *C. citratus* VO against *Candida albicans* [12]. Besides, semi-solid formulations containing VO were prepared and characterized [13].

According to various pharmacopoeias [14,15], the quality control of essential oils and formulations containing vegetable oils or extracts from medicinal plants is very important and it should involve analytical methods for the quantification of the constituents present in the sample, in particular the main substance.

Gas chromatography (GC), or in combination with other techniques, such as mass spectrometry – GC/MS; headspace, on-line liquid chromatography–GC (LC–GC), has been used for separation, identification and quantification of several volatile compounds. The GC features are used in the quality control of natural materials and products, as well as in the characterization of new volatile oils and biotechnological investigations [16].

An alternative to GC analysis is high performance liquid chromatography (HPLC) with UV detection, because of its selectivity, sensitivity and overall versatility. It is also used

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routinely in pharmaceutical industry as a quality control technique for many dosage forms, including natural products. HPLC is the method of choice in the analysis of less volatile constituents of essential oils [16]. Moreover, the development of quantitative assays, for terpenoid compounds and their metabolites in biological matrices and pharmaceutical products is important [17].

Although GC methods have been reported in the literature as the main analytical method for VO analysis, some examples of the use of HPLC as an alternative method for assaying terpenoids and their metabolites in biological matrices and pharmaceutical products can also be found. The composition of essential oils from healthy and infected *S. sclarea* plants was performed using GC–MS and HPLC analysis, as reported by Hudaib et al. [18].

Therefore, it was the objective of this work to develop and to validate a liquid chromatography method to quantify the citral contents in *C. citratus* VO.

2. Experimental

2.1. Chemical and reagents

Citral (assigned purity of 95%) was obtained from Sigma–Aldrich (Darmstadt, Germany) while *C. citratus* VO was obtained commercially (Destilaria Maripá, Paraná, Brazil). Ethanol and *n*-hexane LC grade (Merck, Darmstadt, Germany) were used for preparation of mobile phase. *n*-Hexane was used as oil diluent. Geraniol was obtained from Sigma–Aldrich (Darmstadt, Germany).

2.2. Apparatus and chromatographic conditions

The liquid chromatography system consisted of a Schimadzu LC-10A with a SPD-10A variable-wavelength UV detector (set at 233 nm), a SCL-10A system controller, a C-R6A integrator, a LC-10AS pump, and a Rheodyne injection valve with a 20 μl loop (Shimadzu, Kyoto, Japan). Separation was achieved using a Spherisorb® CN column (250 mm × 4.6 mm, 5 μm) and Spherisorb® CN guard column (10 mm × 4.6 mm, 5 μm). The mobile phase used consisted of *n*-hexane:ethanol (85:15, v/v), flowing at a rate of 0.3 ml min^{-1}. The instrument was operated at room temperature and the detector sensitivity was set to 1.0 AUFS.

2.3. GC analysis

GC analysis was performed on a Perkin-Elmer chromatograph (AutoSystem XL, Shelton, USA) using a fused silica capillary column (30 m × 0.25 mm, coated with DB-525 μm film thickness) and nitrogen as carrier gas (1 ml min^{-1}). The temperature programming ranged from 60 to 300 °C at 15 °C min^{-1} increments. The FID injector and detector temperatures were 220 and 250 °C, respectively. The compositions, in percentage, were obtained from electronic integration measurements using flame ionization detection without taking into account response factors. GC–MS analysis was carried out in a capillary GC–quadrupole MS system (QP 5000 – Shimadzu, Kyoto, Japan) operating at 70 eV, and helium as carrier gas (2 ml min^{-1}), in the same conditions as described above. The identification of the compounds was performed by comparing their retention indexes (determined relatively to the retention times of a series of *n*-alkanes) and mass spectra to those obtained from authentic samples.

3. Methods

3.1. Calibration curves

Aliquots of 2.0, 3.0, 4.0, 5.0 and 6.0 ml of a 125 μg ml^{-1} citral standard solution were transferred to 25 ml volumetric flasks and diluted to volume with *n*-hexane. The final concentrations obtained were 10.0, 15.0, 20.0, 25.0 and 30.0 μg ml^{-1}, respectively. Each solution was prepared three times and triplicate injections of each solution were made into the HPLC system.

3.2. Sample preparation

A 25.0 mg of *C. citratus* VO was weighted and transferred to a 200 ml volumetric flask and diluted with *n*-hexane (theoretical concentration of 125 μg ml^{-1}). Further dilutions were made with *n*-hexane to give a final theoretical concentration of 20.0 μg ml^{-1}.

3.3. Method validation

The method was validated by determining the parameters: linearity, precision, accuracy, specificity, detection limit (DL) and quantitation limit (QL), according to ICH guidelines and USP 27 recommendations [14,19].

The linearity of the method was determined using citral as a reference substance at five concentration levels. Three calibration curves were prepared as described above. The slopes and the statistical analysis of the calibration curves were calculated by linear regression. The DL and QL were calculated based on the S.D. and the slope (S) of the calibration curves [14,19].

Method's precision was studied by repeatability and intermediary precision. The repeatability of the method was evaluated by eight repeated assays of the VO at same concentrations, in triplicate injections, during the same day under the same experimental conditions. The intermediary precision was demonstrated by assaying six samples of VO, at same concentrations, during three consecutive days. The S.D. and R.S.D. were calculated. The citral content of the *C. citratus* VO was determined by referring to the calibration curve or by sample/equivalent reference substance direct matching. The results were compared to the GC analysis.

Accuracy was determined by recovery, in which known amounts of citral were added to VO solutions. The recovery test was performed at three concentration levels. Aliquots of 1.0, 2.0 and 2.5 ml of a 125.0 μg ml^{-1} citral standard solution were added to three VO samples solutions, prepared as cited in Section 3.2 (corresponding to 5.0, 10.0 and 12.5 μg ml^{-1} added, respectively). The amount recovered was determined by HPLC. Each solution was prepared in triplicate and injected three times.

The specificity of the method in the presence of other compounds of the VO was evaluated by assaying an amount of geraniol (about 5% contents in VO). The solution was made with *n*-hexane to give a final concentration of 2 μg ml^{-1}.

4. Results and discussion

The chemical composition of the *C. citratus* VO consists of monoterpenes compounds, hydrocarbons, ketones, aldehydes and esters. The GC method was used to identify and quantify these compounds. The oil is characterized by high percentages of citral (70–85%) according to the geographical area [2].

In this work, a method based on normal-phase HPLC combined to UV spectroscopic detection for assaying citral in *C. citratus* VO was developed. The aim of this study was to develop and to validate a simple HPLC assay for the analysis of this substance in VO. A HPLC method to determine citral in *C. citratus* VO and dosage forms has not been reported in the literature yet. The content of citral in VO is usually determined by GC analysis and the HPLC method could be an alternative technique to quantify this drug in vegetable samples, for example, essentials oils as proposed in this study.

The choice of the chromatographic conditions was influenced by the nature of the drug, such as solubility and UV absorption. The mobile phase, a mixture of *n*-hexane:ethanol (85:15, v/v), as well as the other chromatographic conditions, showed high resolution of the citral peak, indicating that the proposed method could be applied for the determination of citral in the *C. citratus* VO. The UV absorption spectrum of citral shows intense absorption and good selectivity at 233 nm. The Fig. 1(A and B) show the identical HPLC profiles at 233 nm for the citral (reference substance) and *C. citratus* VO.

For drug analysis in quality control, the validation of the analytic methods is necessary and the main objective is to demonstrate that it is suitable for its intended purpose. According to the ICH guidelines and USP 27 [14,19], some validation parameters must be evaluated, such as linearity, specificity, precision, accuracy, detection and quantitation limits, and robustness. However, there is no need to evaluate all these parameters, and the analyst should select those considered relevant for each test procedure.

The linearity of an analytical method is its ability to elicit tests results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [14,19]. In this study, the linearity of HPLC method was investigated for citral in the range of 10–30 μg ml^{-1} at five concentration levels. The citral retention time was about 13.8 min. The calibration curves were constructed by plotting concentrations versus peak area and showed good linearity in the range of 10–30 μg ml^{-1}, with excellent correlation coefficients (r). The representative linear equation for citral was: $y = 191154x + 78863$ ($n = 6$, $r = 0.9991$). The data were validated by means of the analysis of variance, which demonstrated significant linear regression

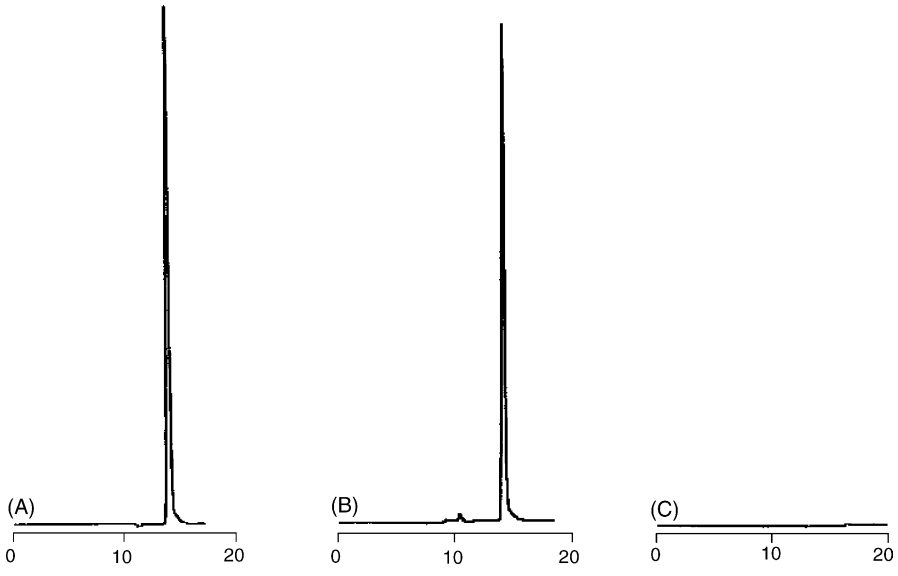

Fig. 1. Chromatogram of citral at 20 μg ml^{-1}: (A) reference substance; (B) *C. citratus* VO; (C) geraniol at 2 μg ml^{-1}. Chromatographic conditions – column: Spherisorb® CN (250 mm × 4.6 mm, 5 μm) and Spherisorb® CN guard column (10 mm × 4.6 mm, 5 μm); mobile phase: *n*-hexane:ethanol (85:15, v/v); flow rate 0.3 ml min^{-1}; injection volume: 20 μl; detection UV 233 nm (1.0 AUFS); retention time about 13.8 min.

Table 1
Results of quantitative determination of citral in *C. citratus* VO by HPLC – repeatability test

Sample	Concentration of citral in VO ($\mu g\,ml^{-1}$)	Content (%)	Average (%)	R.S.D. (%)
1	14.737	73.69		
2	14.998	74.99		
3	15.185	75.92		
4	14.769	73.85	75.20	1.37
5	15.323	76.62		
6	15.083	75.41		
7	15.012	75.06		
8	15.205	76.02		

and not-significant linearity deviation ($P < 0.01$). The R.S.D. of the slope of the three lines was 1.78%. The detection limit (DL) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The quantitation limit (QL) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The DL and QL are usually expressed as the concentration of analyte in the sample [14,19]. The DL and QL determined were 0.36 and 0.12 $\mu g\,ml^{-1}$, respectively. The low values indicated the sensitivity of the HPLC method.

The method was validated by evaluating the precision (repeatability – intra-day) and intermediary precision (inter-day). The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample [14,19]. In this study, the repeatability was studied by assaying eight samples of VO, at same concentration (20 $\mu g\,ml^{-1}$), during the same day. The R.S.D. obtained was 1.37%. The concentration of citral in VO obtained in this assay was 75.2% (Table 1). The intermediary precision was demonstrated by assaying six samples of VO, during three successive days (Table 2). The R.S.D. obtained was 1.12% for citral in the concentration of 20 $\mu g\,ml^{-1}$. The lows R.S.D.s values obtained showed the precision of the method, especially for complex matrices, as VO. The retention time obtained for the samples was about 13.8 min.

This method was applied for the determination of citral content in *C. citratus* VO, showing a content of 75% of citral (Table 1). In addition, the GC/FID and GC/MS analysis of the *C. citratus* VO revealed a similar citral content (76%), demonstrating the suitability of proposed HPLC method.

Table 2
Data obtained from intermediary precision of VO samples by HPLC analysis

Sample	Citral content (%)				
	1 Day	2 Day	3 Day	Average	R.S.D. (%)
1	74.52	74.72	73.69		
2	74.68	74.89	74.99		
3	74.46	74.99	75.92		
4	75.49	74.67	73.85	75.01	1.12
5	75.75	75.43	76.62		
6	76.38	73.74	75.41		

Table 3
Recovery of standard solution added to *C. citratus* VO samples

Amount added ($\mu g\,ml^{-1}$)	Amount found ($\mu g\,ml^{-1}$)	Percentage of recovery[a]	R.S.D. (%)
5	5.013	100.27	
10	9.811	98.11	1.11
12.5	12.351	98.81	

[a] Each value is the mean of three determinations.

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. It can be determined by application of the analytical procedure to an analyte of known purity or recovery studies, where known amount of standard is added [14,19]. The recovery test resulted in 99.06% of mean recovery with low R.S.D. (less than 2.0%) (Table 3), which show the accuracy of the method.

Specificity is define as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components [14,19]. The specificity test was evaluated by assaying an amount of geraniol (2 $\mu g\,ml^{-1}$), which is also present in VO (in concentrations about 5%). No interference was observed at the detection wavelength (233 nm). The chromatogram obtained showed no interference in the drug peak (Fig. 1C).

5. Conclusion

The HPLC method developed in this study showed excellent performance and showed to be simple, linear, precision, accurate and sensitive. It can be used to quantify citral in *C. citratus* VO, giving concentration similar to that obtained by GC/FID analysis. Moreover, it is the first report on the validation of citral in VO by HPLC method.

The proposed method can also be applied to assay citral in semi-solid formulations containing *C. citratus* VO in and stability studies of these natural products, as according to research development in our laboratory.

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