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Quantification of the components in commercial essential oil of *Eucalyptus globulus* labill. by gas chromatography – GC-FID and GC-MS

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Leaves of Eucalytus globulus Labill are characterized by the presence of essential oil, in which 1,8-cineole is the main component. The oil is used as an expectorant for symptomatic treatment of mild inflammation of the respiratory tract and bronchitis. This work addresses the chemical quantification of the constituents of E. globulus essential oil by gas chromatography. Commercial samples were diluted and analyzes by gas chromatograph coupled to flame ionization detector (GC-FID). The chromatographic separation was performed on a fused silica capillary (LM-20, 30 m x 0.25 mm x 0.25 μm film thickness) column. The proposed GC-FID method has demonstrated to have specificity and high sensitivity. The quantitation by the normalization technique demonstrated to be adequate for the analysis. Thus, the proposed method is effective for the quantification of E. globulus oil constituents, which may help in the quality control of commercial formulations.

Keywords: *Eucalyptus globulus*; 1,8-cineole; GC-FID; Quality Control.

Introduction

Eucalyptus essential oil is obtained from leaves of *Eucalyptus globulus* Labill., Myrtaceae family, a plant of Australian origin, and used to treat respiratory tract disorders and infections (1,2). The main component of the essential oil is the monoterpene 1,8-cineole (60-85%) (= eucalyptol) (Figure 1) (1). It is also reported the presence of other constituents such as α-pinene, β-pinene, limonene, terpinen-4-ol, among others, varying according to the origin of the plant material used to obtain the oil (3,4). Commercial eucalyptus oil is obtained by steam distillation and rectification from the fresh leaves in order to improve the 1,8-cineole concentration (5).

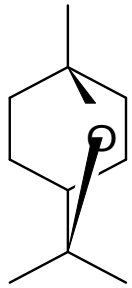

Figure 1 Chemical structure of 1,8-cineole.

In general, a major component is reported in an essential oil of a medicinal plant. However, the presence of the other constituents in the essential oil must be considered since they can also contribute with the pharmacological activity of the oil, although they are in smaller amounts when compared to the main component. Thus, it is possible to assign maximum or minimum values or even

ranges for these compounds. In the case of eucalyptus oil, six other compounds are considered and quantified besides 1,8-cineole (minimum of 70%): α-pinene (traces to 9.0%), β-pinene (less than 1.5%), sabinene (less than 0.3%), α-phellandrene (less than 1.5%), limonene (traces to 12.0%) and camphor (less than 0.1%). This values are defined by international pharmacopoeias, such as British Pharmacopoeia (5), Spanish Pharmacopoeia (6) and Portuguese Pharmacopoeia (7).

The methodology employed by the official compendia (pharmacopoeias) in the quality control of essential oils have focused in the gas chromatography technic, using the normalization procedure to determine the percentage of the oil content. The normalization technique is employed to provide a quantitative analysis of the separation of a mixture and requires no reference standards or calibration solutions to be prepared. This determination is directly linked to the processing of the area under the peaks of the mixture. In this way, the results are obtained by expressing the area of a certain peak as a part of the percentage of the sum of the areas of all the peaks (5-8). If all components respond equally in the detector and are eluted, then Area% provides a suitable approximation of the relative amounts of components (peak area integration with total area detected normalized to 100%, using electronic integrators). Thus, the technique usually employs Gas Chromatograph with a Flame Ionization Detector (GC-FID). The FID is the part of the apparatus that quantifies the result of the chromatographic effluent. In GC-FID, the appropriate resolution of the data and the measurement of the qualitative selectivity (separation) must be demonstrated before quantification (9-10).

In this context, the validation of analytical methods for quantification of chemical substances considered active principles of plants used in traditional medicine, as well as in the formulation of herbal medicines, is essential to guarantee their quality. The validation aims the development of methodologies that allow the determination of the concentrations of the substances responsible for the pharmacological activity in a secure and reproducible way (10). The optimization of experimental procedures allows the qualitative and quantitative analysis of the bioactive compounds, resulting in methods that can contribute in the quality control by the inspection agencies for the commercialization of these products (8-13).

Thus, a quantitative method to determine 1,8-cineole and the minor constituents of the essential of *Eucalyptus globulus* is essential to guaranty the quality of commercial essential oil.

Experimental

Chemical and Standard

Standards of α-pinene and β-pinene were purchase from Merck® (USA), sabinene from Fluka, limonene, 1,8-cineole, α-terpineol, α-phellandrene were purchased from Sigma-Aldrich (São Paulo, Brazil). The other analytical grade reagents were purchased from F. Maia (Cotia, SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil).

Preparation of Reference Solution and Sample Solutions

Only five commercial eucalyptus oils could be found in the Brazilian commerce. All of them were purchased. So, five samples the *E. globulus* essential oils were commercially-available and named Eg-01, Eg-02, Eg-03, Eg-4 and Eg-05. Each sample was solubilized with hexane into a 1 mL vial in a proportion of 4:200 (v/v). For the reference solution, 1 µL of α-pinene, 0.5 µL of β-pinene, 0.5 mg of sabinene, 0.5 µL of α-phellandrene, 1 µL of limonene, 5 µL of cineole and 0.5 mg of camphor were dissolved in 1 mL of hexane R.

Instrumentation and GC Conditions

Gas chromatography analysis

The analytical parameters were based in the British Pharmacopoeia (5). Analysis of the oils was performed using a PerkinElmer XL CG Auto System model coupled to a flame ionization detector and equipped with Total

Chrom Navigator – Autosystem_XL®, fitted with a LM-20 (L & M) fused silica capillary column (30 m, 0.25 mm; film thickness 0.25 µm). The oven temperature was programmed to go from 60 °C (5 min) to 200 °C (5 min) at 5 °C/min. Helium was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were set at 220 ºC and 250 ºC, respectively. The samples were injected in a volume of 1 µl, using split sampling technique, ratio 1:50. The percentage compositions were obtained from electronic integration measurements using the normalisation technique.

For the establishment of the analyses conditions, sample Eg-02 was used.

System suitability

The reference solution was considered for system suitability determination, where a minimum resolution of 1.5 between the peaks due to limonene and cineole is required. The following formula was considered for determination:

R= 1.18*(tr2 – tr1 / Wh1 + Wh2)

Where:

Tr2, tr1: retention time for each peak (tr1 < tr2);

Wh1, Wh2: peak width at half height (in unit of time) of each peak

Interlaboratory reproducibility

Interlaboratory reproducibility was performed in collaboration with the pharmacognosy laboratories of the Federal Universities of Campinas (UNICAMP) and of Pernambuco (UFPE). The sample and the reference solution were sent for analysis along with protocol describing the methodology. The analyses were performed in triplicate and the results found were compared and the DPR was calculated among the means of the determinations in the different laboratories. 1,8-Cineole levels and the relative standard deviation (RSD) of the means were evaluated. The results were based in the commercial sample Eg-02.

Specificity / Selectivity

The specificity was demonstrated by gas chromatography – mass spectrometry where the data were obtained by comparing the mass spectra profile to demonstrated the peak purity of the 1,8-cineole obtained from the injection of the standard 1,8-cineole solution (5 µg/mL) and the sample solution. For this purpose a gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP5000) was used and the analyses were performed under the same conditions as described above for GC-FID. Selectivity was performed by comparison of the obtained 1,8-cineole areas of the sample solution with and without addition of standard 1,8-cineole solution.

Commercial Samples Analysis

Samples Eg-01, Eg-02, Eg-03, Eg-04 and Eg-05 were prepared as described in item "Preparation of Reference Solution and Sample Solutions" and analyzed in the developed method, always in triplicate.

Results and Discussion

Method Development

In the development of the method, the reference solution and the sample produced the chromatograms shown in Figure 2. It is possible to identify each compound of the reference solution (Table 1), with the main compound, 1,8-cineole, peak at the retention time of approximately 9.182 minutes.

The system suitability test is used to check if the sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. Values greater than 1.5 are expected. For *E. globulus* essential oils, it was considerate the resolution between limonene and 1,8-cineole peaks. The resolution found for this system was 2.574, demonstrating a good separation between these two peaks.

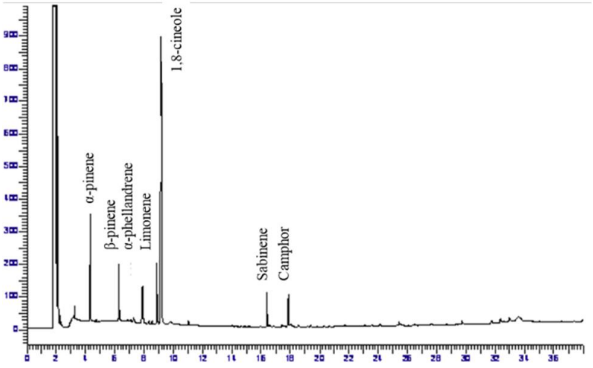

Figure 2 GC-FID chromatogram profile of the reference solution.

Table 1 Retention time of the substances from the standard solution.

Substances	Retention Time (min.)
α-Pinene	4.321
β-Pinene	6.299
α-Phellandrene	7.903
Limonene	8.894
1,8-Cineole	9.182
Sabinene	16.417
Camphor	17.856

The purity of the 1,8-cineole has been established by comparing mass ion fragmentation pattern with the 1,8-cineole reference standard using GC-MS. It was considered the fragmentation profile of this compound by monitoring the ions (m/z) 43, 81, 108, 111, 139 and 154 (Figure 3a). It was not observed any kind of impurity in the peak. Also, limonene is a compound with retention time of 8.894 min, and considering the proximity of this peak to 1,8-cineole, its mass spectrum (Figure 3b) was also monitored (ions (m/z) 41, 68, 93, 107, 121 and 136) in order to exclude any chance of peak overlap in the chromatogram. In this sense, it was observed that in the fragmentation of 1,8-cineole there was no evidence of interference with limonene.

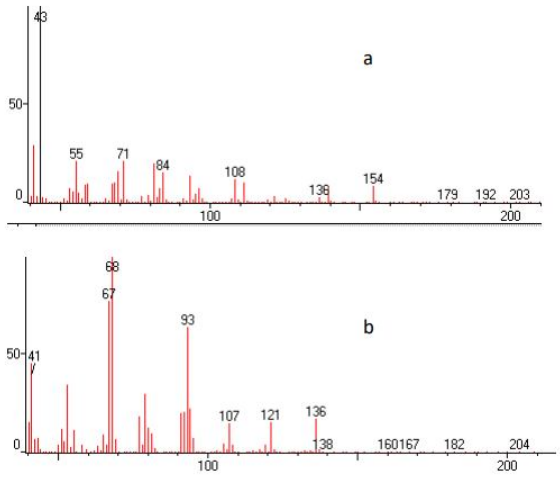

Figure 3 Typical GC-MS spectra of 1,8-cineole (a), and limonene (b).

The selectivity was confirmed by the addition of a reference substance of limonene and 1,8-cineole in separate in the sample solution, which produced an increase only in the peak area of the respective peak in the sample, without changing the area of the adjacent peaks (Figure 4 a, b and c). The areas measured before and after standard addition were 901827 and 7056854 for the limonene peak and 6757017 and 11269620 for 1,8-cineole peak. It was not observed changes in the other areas of the chromatogram. It was possible through the tests indicate that the method is specific and selective for determination of related substances in eucalyptus oil.

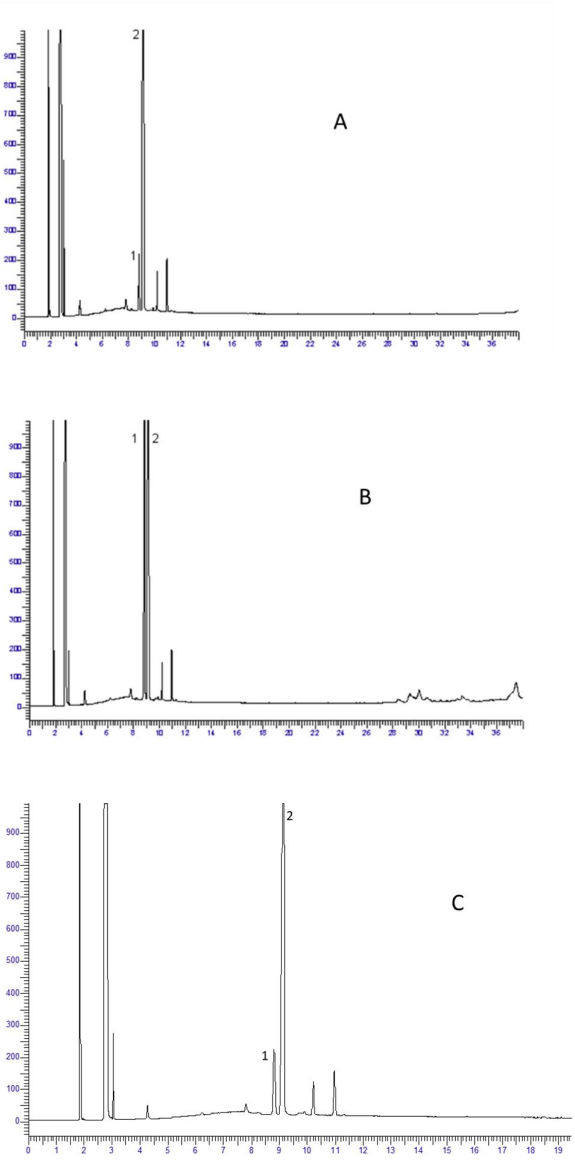

Figure 4 Typical GC-FID chromatograms of (A) eucalyptus oil solution, (B) eucalyptus oil solution with addition of limonene - 1 and (C) 1,8-cineole - 2.

In the comparison between the results of the 1.8-cineole content in the sample EG-02 of the three laboratories, the content ranged from 78.21 to 92.66% (RSD of 0.13%), which indicates, in most situations, appropriate reproducibility.

Commercial Samples Analysis

The analysis of commercial samples revealed levels varying from 66.02 to 78.21% of 1,8-cineole (Table 2), indicating marked variability among the different samples. Considering the limits imposed by the official codes, with the exception of sample Eg-01, all samples were within the desired 1,8-cineole content (minimum of 70%). Regarding the other constituents of the eucalyptus oil, all the samples presented levels within the recommended limits. The proposal of the use of the normalization technique for the quantification of commercial essential oils is adequate, once the step of extracting the oil from the plant material is eliminated. In this way, it is based on the direct dilution of the oil for analysis.

Table 2 Results of analysis of commercial samples of eucalyptus oil.

	Eg-01	Eg-02	Eg-03	Eg-04	Eg-05
α–Pinene	1.42 ± 0.08	1.42 ± 0.05	1.5 ± 0.06	1.38 ± 0.06	1.66±0.0
β–Pinene	0.47± 0.02	1.98 ± 0.10	0.16 ± 0.10	n.d.	n.d.
α–Phellandrene	0.31 ± 0.02	0.2 ± 0.02	2.05 ± 0.03	1.92 ± 0.02	1.54 ± 0.06
Limonene	11.18 ± 0.46	11.98 ± 0.24	10.82 ± 0.09	10.14 ± 0.16	9.71 ± 0.05
1,8-Cineole	66.02 ±0.66	78.21 ± 0.29	72.17 ±0.38	74.17 ± 0.97	76.10 ± 0.06
Sabinene	0.05 ± 0.00	0.48 ± 0.02	n.d.	n.d.	n.d.
Camphor	0.04 ± 0.01	0.04 ± 0.01	n.d.	n.d.	n.d.

"n.d.": not detected

Conclusions

The GC-FID method demonstrated to be adequate for determination of the components in the *E. globulus* essential oil. The gas chromatographic system demonstrated a good specificity and selectivity. The normalization method for quantification of the constituents of the oil is an efficient tool for quantitative determination of the constituents in the oil. The system suitability criteria of the study was within the acceptance limit. This approach may potentially be applied to routine for quality control of eucalyptus oil.

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

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