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VALIDATION OF A HPLC METHOD FOR QUANTIFICATION OF LIMONENE IN NANOEMULSIONS CONTAINING SICILIAN LEMON ESSENTIAL OIL AND NANOEMULSIONS CONTAINING RED RED MANDARIN ESSENTIAL OIL

VALIDAÇÃO DE UM MÉTODO POR HPLC PARA QUANTIFICAÇÃO DE LIMONENO EM NANOEMULSÕES CONTENDO ÓLEO ESSENCIAL DE LIMÃO SICILIANO E NANOEMULSÕES COTENDO ÓLEO ESSENCIAL DE MANDARINA VERMELHA

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ABSTRACT

The aim of the present study was to validate a liquid chromatography method for the quantification of the limonene compound present in different citrus essential oils, namely nanoemulsified essential oils of Sicilian lemon and Red Mandarin. The chromatographic separation of limonene was performed on a Lichrospher® 250 RP-18 reverse phase column, Lichrospher® 250 RP-18 pre-column, with a mixture of methanol and water in the radio (90:10) as mobile phase. The flow rate used was 0.6 mL/min, the injection volume was 20 µL, the wavelength was 213 nm and the run time was 15 minutes. The method was specific, linear (R^2= 0.9978), precise (RSD: 0.6412 for nanoemulsion with lemon essential oil and RSD: 0.8585 for nanoemulsion with Red Mandarin essential oil), robust and accurate (from 83.5 to 92% recovery).

Keywords: HPLC, *citrus lemon*, *citrus delicious tenore*, chromatographic separation

RESUMO

O objetivo do presente estudo foi validar um método de cromatografia líquida para a quantificação do composto limoneno presente em diferentes óleos essenciais cítricos, nomeadamente óleo essencial de limão siciliano e mandarina vermelha nanoemulsionados. A separação cromatográfica do limoneno foi realizada em coluna de fase reversa Lichrospher® 250 RP-18, pré-coluna Lichrospher® 250 RP-18, com mistura de metanol e água na proporção (90:10) como fase móvel. A vazão utilizada foi de 0,6 mL/min, o volume de injeção foi de 20 µL, o comprimento de onda foi de 213 nm e o tempo de corrida de 15 minutos. O método foi específico, linear (R^2= 0,9978), preciso (DPR: 0,6412 para nanoemulsão com óleo essencial de limão e DPR: 0,8585 para nanoemulsão com óleo essencial de mandarina vermelha), robusto e exato (de 83,5 a 92% de recuperação).

Palavras-Chave: *CLAE, citrus lemon, citrus deliciosa tenore, separação cromatográfica*

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INTRODUCTION

Limonene, composed of the chemical formula C10H16, is a monoterpene, which constitutes 98% of the essential oils of citrus fruits, such as oranges, tangerines, lemons, grapefruits and limes (BORA *et al.,* 2020). It is a chiral molecule with two possible enantiomers: D-limonene (dextrorotatory, chiral form R) and L-limonene (levorotatory, chiral form S). The most common isomer has the (R) configuration, which corresponds to D-(+)-limonene (CAS 5989-27-5). Its full chemical nomenclature according to Chemical Abstract is (R)-1-methyl-4-(1-methylethenyl)-cyclohexene or (R)-(+)-para-mint-1,8-diene by the International Union of Purity and Applied (BATHAIE & TAMANOI, 2014).

Regarding the concentration of Limonene present in these fruits, it can vary from 32% to 98%, for example, in sweet orange (68-98%), bergamot (32-45%), lemon (45-76%) (MOUFIDA & MARZOUK, 2003). Other plants may also have the presence of Limonene, such as ginger, rosemary, lavender, sage, mint, lemongrass, not only found in citrus fruits (BORA *et al.,* 2020).

Limonene is widely used in food, pharmaceuticals, beverages and industrial products due to its taste and aroma (D'ALESSIO *et al,* 2014). Furthermore, it has properties such as antioxidant (AMINI, ASLA-ROUSTA, AGHAZADEH, 2020), antiviral (ASTANI & SCHNITZLER, 2014), immunomodulatory (TERÃO *et al.* 2019), antinociceptive (ARAUJO-FILHO *et al.* 2017), anticancer (MUKHTAR *et al.* 2018), analgesic (PICCINELLI *et al.* 2017), cardioprotective (DURCO *et al.* 2019). anticonvulsant (VIANA *et al.* 2000), gastroprotective (DESOUZA *et al.* 2019) and anxiolytic (LIMA *et al.* 2013).

The quantification of the main compounds present in essential oils, such as Limonene, is essential to determine the quality of a pharmaceutical, cosmetic or food product, as the content of essential oils varies according to geographic and seasonal variation. In addition, essential oils are subject to oxidation, hydrolysis or polymerization processes when stored for a long period (POREL, SANYAL, KUNOV, 2014).

Some liquid chromatography methods have been reported for the determination of Limonene alone or when present in different types of citrus essential oils. The isocratic PR elution method (UHPLC) using a superficially porous stationary phase layer and photodiode array detector (PDA) was developed to determine the content of limonene present in sweet orange (*Citrus sinensis*) oil (BERNART, 2015). Zoccali and collaborators (2017) evaluated the oxygen fraction of four types of tangerine essential oils by liquid chromatography and gas chromatography. Ferrarini and collaborators (2017) developed a liquid chromatography method for the quantification of limonene in nanoemulsions.

However, no liquid chromatography (LC) method is reported so far for the determination of Limonene present in nanoemulsions containing sicilian lemon essential oil and nanoemulsions containing Red Mandarin essential oil.

Thus, the aim of the present study was to validate an HPLC method for the quantification of Limonene in nanoemulsified Sicilian lemon and Red Mandarin essential oils according to official

guidelines. This developed and validated HPLC method can be considered an important quality control tool in formulation development.

METHODS

MATERIALS AND REAGENTS

Limonene (LNO) was purchased from SigmaAldrich®. Polysorbate 80 (TWEEN 80®) and sorbitan monooleate (SPAN 80®) were purchased from Synth®. Crodamol GTCC was obtained from Alpha Química LTDA. HPLC grade methanol was purchased from J.T. Sicilian lemon (*citrus limon*) essential oil (69,93% Limonene) and Red Mandarin (*Citrus deliciosa Tenore*) essential oil (78,32% Limonene) were purchased from Vimontti.

CHROMATOGRAPHIC CONDITIONS

HPLC analysis was performed on a Shimadzu Prominence (Tokyo, Japan) high performance liquid chromatograph equipped with a DGU-20A 5R degasser, an LC-20AT pump, CBM-20A system controller, a SIL 20A HT autosampler, SPD-M20A and a CTO-20AC Column Oven, with Lichrospher® 250 RP-18 reversed-phase column stationary phase, Lichrospher® 250 RP-18 pre-column. The chromatographic conditions were: mobile phase flow rate of 0.6 mL/min; 213 nm wavelength; injection volume of 20 µL and the mobile phase composed of methanol: water (90:10 v/v).

SAMPLE PREPARATION

For the preparation of nanoemulsions, the emulsification method under high agitation using the Ultra Turrax® equipment was used, as described by Godoy *et al.* (2017) with the modifications. Nanoemulsions containing essential oil of Sicilian lemon (NEOL) and nanoemulsions containing essential oil of Red Mandarin (NEOMV) were prepared with 5% essential oil or inert Crodamol oil for white nanoemulsions (NEBR). The nanoemulsions were subjected to an extraction process, initially a stock solution (0.5 mg/mL) was prepared with a nanoemulsion containing essential oil of Red Mandarin, diluted in methanol (25 mL flask) and taken to the ultrasonic bath for 30 minutes. Then, a volume of the stock solution corresponding to the midpoint of the analytical curve (30 µg/mL) was added to a 10 mL volumetric flask, completed with methanol and taken to the ultrasound bath again for 20 minutes. After the described procedure, the sample was filtered using a 0.22 µm cellulose membrane.

STANDARD SOLUTION

To validate the method, a standard stock solution (0.5 mg/mL) was used by solubilizing 14.84 µL of Limonene in 24.985 mL of Methanol. Then, the first stock solution was diluted in methanol to obtain different concentrations (10, 20, 30, 40 and 50 µg/mL) that were later used in the linearity study. A second stock solution was placed in the ultrasonic bath for 30 minutes, the solutions remained in the ultrasonic bath for 20 minutes and all solutions were filtered (0.22 µm Membrane - Millex) before being injected (n = 3) into the HPLC system.

VALIDATION METHOD

The method was validated according to the 2017 RDC 166 Resolution and the International Conference on Harmonization guidelines for the validation of analytical procedures (ICH, 2017). The analytical parameters for method validation were: specificity, linearity, limits of quantification and detection, intermediate, precision and repeatability, robustness and precision.

Specificity

One of the parameters required for the validation of a chromatographic method is the specificity test, in this case, it serves to show that there is no interference of other compounds present in essential oils or nanoemulsions in the quantification of limonene. The specificity of the method was determined by comparing the analysis of the solution with Sicilian lemon oil or free Red Mandarin oil, white nanoemulsions, nanoemulsions with Sicilian lemon essential oil and nanoemulsions containing Red Mandarin oil at a concentration of 30 µg/mL. The specificity was also confirmed by analyzing the purity of the peaks as recommended by the international guide (ICH, 2017).

Linearity, detection and quantification limits

The linearity of the method was evaluated by injecting samples of the standard solution prepared at 5 different concentrations, namely 10, 20, 30, 40 and 50 µg/mL, obtained from a stock solution prepared at a concentration of 0.5 mg /mL. Three calibration curves were prepared and linearity was assessed by the least squares regression method, which was used to calculate the correlation coefficient, y-intercept and slope of the regression line ($p < 0.05$). The limits of detection (LOD) and quantification (LOQ) were calculated directly from the calibration graph. LOD and LOQ were calculated based on 3.3 σ/S and 10/σ, respectively, where σ is the standard deviation of the intercept and S is the slope of the calibration graph (ICH, 2017).

Precision

The precision of the method was determined by intermediate precision (inter-day precision) and repeatability (intra-day precision). Initially, intermediate precision was verified, samples were prepared at the same concentration (30 µg/mL) and were injected on three consecutive days. While repeatability was assessed, in triplicate, six solutions at the concentration of 30 µg/mL were injected on the same day. The results were expressed as relative standard deviation (RSD%).

Robustness

To evaluate the robustness of the method, a sample (30 µg/mL) containing a nanoemulsion with Sicilian lemon essential oil or a nanoemulsion containing essential oil of Red Mandarin was prepared. And the following variation of the method parameters was performed: flow rate (0.5 and 0.7 mL/min), Mobile phase [Methanol only] and [Methanol: Water (95:05, v/v)], wavelength (211 and 214 nm). Samples were evaluated with an n=3 for each variation tested.

Accuracy

The accuracy of the method was determined, in triplicate, from a solution with a nanoemulsion containing Sicilian lemon essential oil and a nanoemulsion containing Red Mandarin essential oil prepared at a concentration of 20 µg/mL and enriched with three different concentrations (lower - 25% , medium - 50% , and higher concentration - 75%) of the standard solution containing Limonene. In the end, we obtained concentrations of 25, 30 and 35 µg/mL. The recovery percentage (%) was calculated from the differences between the concentration obtained for enriched and non-enriched solutions and expressed as the relative standard deviation (RSD%) of the triplicate.

RESULTS AND DISCUSSIONS

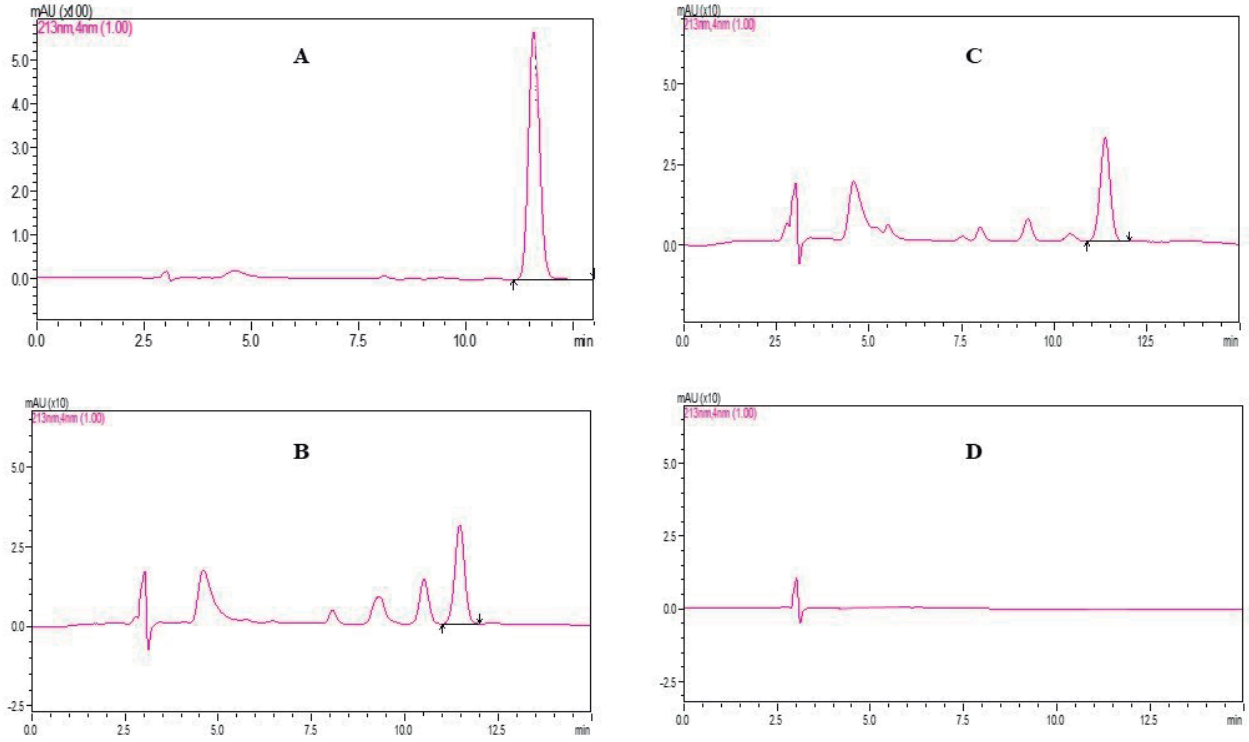
The most widely used method for the quantification of essential oils is gas chromatography (GC), but it presents some limitations for analyzing the volatile components, as structural changes may occur in the thermally labile compounds during the analysis due to the high temperature of the inlet or columns (POREL;SANYAL;KUNDU, 2014). In a study, Mondello *et al*. (2005), on the analysis of lemon essential oil by gas chromatography, the authors showed that the method has a separation power, however, when dealing with highly concentrated compounds (such as limonene), severe modulator overload can occur during analysis. Furthermore, nanoemulsions are water-containing preparations and require sample preparation steps, which are often time consuming for GC analysis.

The validation of an analytical method is a dynamic process and essential to demonstrate its suitability for future application (BRASIL,2017). In this study, the chromatographic conditions were based on the method by Ferrarini *et al*. (2013), in which some changes had to be made.

Initially, we tested a mobile phase composed of acetonitrile and water, in different proportions (90:10, 80:20, 70:30 and 50:50), but we could not obtain a single peak with purity equal to 99.9% as is recommended. Thus, we arrived at the ideal mobile phase by testing methanol and water in the ratio (90:10 v/v).

The specificity of the method was evaluated from the injection of nanoemulsions containing Sicilian lemon essential oil, nanoemulsions with Red Mandarin essential oil and white nanoemulsions (with the absence of the active). These analyzes demonstrate the specificity of the method, considering that only samples containing essential oil (majority compound: limonene) showed optical deviation. As can be seen in figure 1:

Figure 1 - Chromatogram of the standard solution with Limonene (A), nanoemulsions containing (B) Sicilian lemon essential oil (C) red tangerine essential oil and nanoemulsions in white (D) (in the absence of Sicilian lemon essential oil and Red Red Mandarin essential oil)



From the analysis of the solution with Limonene, three independent analytical curves were prepared in the range of 10-50 µg/mL, and the method was sensitive enough to detect and quantify all the selected concentrations. The correlation coefficient for the average curve was 0.9978, which is the

value recommended by Brazilian legislation to demonstrate the linearity method in the determination of an active (BRASIL, 2017). In addition, the statistical analysis showed that there was linear regression and no deviation from linearity ($p<0.05$). The analytical limonene curve was obtained from the average of the three standard curves as can be seen in the figure below. The values obtained for the LOD and LOQ were 5.97 and 18.10 $\mu g.mL^{-1}$, demonstrating a good sensitivity of the method for the determination of limonene present in the nanoemulsion containing Sicilian lemon essential oil and nanoemulsion containing Red Mandarin essential oil.

Intermediate precision (inter-day precision) and repeatability (intra-day precision) results were expressed in relative standard deviation (RSD), the values are presented in Table 1.

Table 1 - Results obtained in the intermediate precision and repeatability test.

	Parameters	N	RSD (%)
NEOMV	1º day	6	0.4182
	2º day	3	0.1885
	3º day	3	0.2722
	Average between days	9	0.8585
NEOL	1º day	6	0.4857
	2º day	3	0.7180
	3º day	3	0.7209
	Average between days	9	0.6412

Legend: NEOMV - Nanoemulsion with essential oil of Red Mandarin.
NEOL - Nanoemulsion containing essential oil of Sicilian lemon.

It is observed in Table 1 that the intermediate precision and repeatability of the values expressed in relative standard deviation were all less than 5%. Thus, the results indicate that the method demonstrates precision for the quantification of limonene present in citrus essential oils, specifically Sicilian lemon essential oil and Red Mandarin essential oil. Ferrarini et al. developed a method to quantify limonene in nanoemulsions by HPLC. The method developed by obtained a standard deviation of 1.1, 0.9 and 1.9 % for repeatability and intermediate precision. The values found for repeatability and intermediate precision in our study showed similarity with the aforementioned study.

The robustness tests were carried out by varying the method conditions regarding the mobile phase proportion (only Methanol and Methanol: water (95:05 v/v), flow rate (0.5 and 0.7 mL/min) and wavelength (211 and 214 nm) and as can be seen in Table 2, even after the modifications, the Limonene Content (%) values obtained were in the range of 90 and 105%, indicating that the method developed is robust.

Table 2 - Robustness of the Limonene quantification method in nanoemulsions with Sicilian lemon essential oil and nanoemulsions with Red Mandarin essential oil (sample solutions with theoretical concentration of 30.0 µg.mL^{-1}).

Formulation	Condition	Limonene Content (%)	Relative Standard Deviation (%)
NEOMV	Mobile Phase		
	Methanol (100 v/v)	97.66	0.823
	Methanol:water (95:05 v/v)	102.5	2.019
	Flow rate		
	0.7 mL/ minutes	105.0	0.791
	0.5 mL/minutes	95.37	2.040
	Λ (nm)		
	211 nm	93.12	2.044
	214 nm	94.21	2.115
	Content	98.62	0.098
NEOL	Mobile Phase		
	Methanol (100 v/v)	90.81	4.374
	Methanol:Water (95:05 v/v)	97.29	2.641
	Flow rate		
	0.7 mL/minutes	96.86	5.003
	0.5 mL/minutes	97.02	4.088
	Λ (nm)		
	211 nm	97.30	3.053
	214 nm	94.75	1.809
	Content	101.2	2.828

Legend: NEOMV - Nanoemulsion with essential oil of Red Mandarin.
NEOL - Nanoemulsion essential oil of Sicilian lemon.

The developed method proved to be suitable for the quantification of limonene present in nanoemulsified Sicilian lemon essential oil and Red Mandarin essential oil.

The accuracy of the method was expressed in percentage of recovery (%), the results are described in Table 3.

Table 3 - Accuracy of the method for quantifying limonene in nanoemulsions containing Red Mandarin or Sicilian lemon essential oils.

Formulation	Know sample (µg.mL^{-1})	Added (µg.mL^{-1})	Found (µg. mL^{-1})	Recovery (%)	Relative Standard Deviation (%)
NEOMV	20	5	25	85.5	1.96
	20	10	30	88.8	1.26
	20	15	35	92.0	1.99
NEOL	20	5	25	87.3	0,20
	20	10	30	83.5	0.19
	20	15	35	90.6	1.34

Legend: NEOMV - Nanoemulsion with essential oil of Red Mandarin.
NEOL - Nanoemulsion containing essential oil of Sicilian lemon.

The results obtained for the accuracy, as can be seen in Table 3, showed a good recovery with values higher than 80% for Limonene, thus indicating that it was possible to recover the concentrations tested for the developed method.

CONCLUSION

The high performance liquid chromatography analytical method used to quantify the Limonene present in the nanoemulsioned essential oils of Sicilian lemon and Mandarin Red, represented an alternative analytical method, and was shown in this study to be specific, linear, precise, accurate and robust in the range of 10 to 50 µg/mL. Therefore, the method developed in this study can be used to meet the need for an easy analytical method in laboratories and by the pharmaceutical, perfumery and food industries for the quantification of Limonene, which is the major compound present in most citrus oils, like Bergamot, Lemon, Orange and Tangerine oil.

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