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Article

Determination of Thymol and Carvacrol in Plasma and Milk of Dairy Cows using Solid-Phase Microextraction

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Desenvolveu-se um método para análise de timol e carvacrol em plasma e leite de bovinos usando microextração em fase sólida no modo *headspace* (HS-SPME) e cromatografia gasosa acoplada à espectrometria de massas (GC-MS). Os limites de quantificação obtidos foram de 0,5 e 2,0 ng mL^{-1} para ambos analitos no plasma e no leite, respectivamente, com precisão e exatidão adequadas. Para a quantificação do timol, os resultados obtidos no presente trabalho foram superiores em comparação com a literatura existente, obtendo-se menor limite de quantificação. Em relação ao carvacrol, o método é o primeiro descrito na literatura para análise deste analito em plasma e leite de bovinos. O método analítico tem aplicação na identificação e quantificação do timol e carvacrol em plasma e leite de bovinos após a administração de uma formulação intramamária veterinária contendo óleos essenciais de plantas medicinais ricas em timol e carvacrol.

A method for determination of thymol and carvacrol in milk and plasma of dairy cows was developed by gas chromatography-mass spectrometry (GC-MS) and headspace solid-phase microextraction (HS-SPME). The limits of quantification were set at 0.5 and 2.0 ng mL^{-1} for both analytes in plasma and milk, respectively, with appropriate precision and accuracy. For quantification of thymol, the results obtained with the established methodology were superior compared with others reported in the literature, achieving lower limit of quantification. Concerning to carvacrol, this is the first methodology described in the literature for the analysis of this compound in plasma and milk of cows. The analytical methodology enabled the identification and quantification of thymol and carvacrol in the plasma and milk of cows after administration of an intra-mammary phytoformulation of veterinary use containing plant essential oils rich in thymol and carvacrol.

Keywords: carvacrol, GC-MS, cow milk and plasma, SPME, thymol

Introduction

Thymol and carvacrol are phenolic volatile monoterpenes present in essential oils of various herbs at concentrations ranging between 20 and 98%.[1,2] These substances presenting validated antimicrobial activity, mainly against Gram-positive microorganisms,[2-4] have been extensively used by food and veterinary industries to combat pathogenic microorganisms resistant to conventionally used antibiotics.[5]

Baskaran *et al.*[6] and McPhee *et al.*[7] validated the antimicrobial action of thymol and carvacrol against etiologic agents causative of mastitis, and based on their findings and experiments, our group developed a veterinary formulation containing essential oils rich in thymol and carvacrol for treatment of bovine mastitis, according to patent number Br PI 1002020-9.[8]

The search for more effective and safer antimicrobials than the existing ones, especially innovative drugs from natural sources (e.g., mainly medicinal plants), has motivated the veterinary industry. The presence of antimicrobial residues in milk is one of the biggest challenges of the food and veterinary industries worldwide, because they interfere with the production of dairy products and may cause hypersensitivity and resistance to microorganisms in humans, especially if the concentrations

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of these residues are above the maximum residue limit (MRL) as permitted by specific legislation.[9-11]

In order to ensure food excellence and consumer safety, the quality control for milk, dairy by-products and veterinary products requires the use of reliable and sensitive analytical methods to determine drug residues in foodstuff and biological matrices (e.g., plasma).[12]

Currently, gas chromatography-mass spectrometry (GC-MS) is the most reliable and most appropriate technique to detect and quantify active essential oils in biological matrices, including milk and plasma,[13] particularly when combined with solid-phase microextraction (SPME).[14] for sample preparation. This extraction technique was successfully used by Kohlert *et al.*[15] for determining the occurrence of thymol and its metabolites in human plasma and urine, after administration of a phytomedicine containing thymol. Moreover, McPhee *et al.*[7] also used the SPME technique to identify and quantify thymol in milk of goats treated with an intra-mammary formulation containing essential oils.

Considering that up to now SPME has not been used to determine the presence of carvacrol in biological matrices, the aim of this work was to validate the efficiency of such a method for identifying thymol and carvacrol in plasma and milk from dairy cow. After defining the ideal chromatographic and extraction conditions, the method was used to quantify both compounds in milk and plasma following administration of an intra-mammary formulation containing essential oils.

Experimental

Chemicals

Thymol (99.9%) and carvacrol (98%) were obtained from Riedel de Haen (Germany) and Sigma-Aldrich (USA), respectively. Propofol (≥ 97%), used as internal standard during optimization of the extraction procedure and in the step of method validation, was obtained from Sigma-Aldrich. Methanol used for preparation of standard solutions was also obtained from Sigma-Aldrich (USA), whereas the sodium chloride (Synth, Brazil) used in the extraction procedure was p.a. grade.

Thymol and carvacrol were extracted by means of headspace solid-phase microextraction (HS-SPME) employing fibers for manual use. Two types of fibers were evaluated, both obtained from Supelco (USA) as follows: 65 μm polydimethylsiloxane/divinylbenzene (PMDS/DVB) and 85 μm polyacrylate (PA).

Before the first use, fibers were conditioned in a chromatographic injector according to the manufacturer instructions: for PMDS/DVB, injector temperature of 250 °C for 30 min, and for PA, injector temperature of 280 °C for 60 min.

Standard solutions

The stock standard solutions of thymol, carvacrol and *o*-cresol were prepared in methanol at a concentration of 2 mg mL^{-1}, whereas the stock standard solution of propofol was also prepared in methanol at a concentration of 0.1 mg mL^{-1}.

To obtain the calibration curves, working solutions were daily prepared in the ranges of 0.4 to 40.0 and 0.01 to 1.5 μg mL^{-1} for milk and plasma analyses, respectively. The propofol solutions were used at concentrations of 5.0 and 0.5 μg mL^{-1} for milk and plasma analyses, respectively.

Apparatus and chromatographic conditions

A gas chromatograph (Shimadzu, model 17-A, Japan) interfaced with a mass spectrometer (GC-MS) (Shimadzu, model QP 5000) was used for analysis of thymol and carvacrol. Equipment control and data acquisition were performed by using the Class-500 software (Shimadzu). Electron impact ionization was performed at 70 eV. Selected ion monitoring (SIM) was employed with the following ions selected for quantification: *m/z* 135 for thymol and carvacrol and *m/z* 163 for propofol (internal standard).

Chromatographic conditions were optimized by analyzing 5 μL aliquots of standard solutions prepared in methanol at a concentration of 2 mg mL^{-1} (split ratio 1:10). Thymol, carvacrol and internal standard were separated by using a fused-silica capillary column (RTX-5ms®, 30 m × 0.25 mm i.d. and 0.25 μm film thicknesses, Restek Corporation, USA). The analyses were carried out by using helium as carrier gas at a flow rate of 1 mL min^{-1}, injector, detector interface temperature set at 250 °C, and column temperature starting at 100 °C (1 min) and programmed to increase 10 °C min^{-1} to 160 °C (1 min), resulting in a total running time of 8 min.

Optimization of the solid-phase microextraction procedure for milk

The volatile analytes (thymol and carvacrol) were extracted by HS-SPME. The extraction procedure was optimized by examining the influence of the following variables: fiber type (PMDS/DVB, PA), extraction temperature (40, 60, 80, 90 and 100 °C), stirring speed (0, 180, 360 and 540 rpm), amount of sodium chloride (0.0, 0.5, 1.5 and 3.0 g), extraction (10, 25, 40 and 55 min) and

desorption times (1, 2, 4 and 5 min), heating (90 °C), sample agitation (540 rpm) before extraction (without heating and stirring, heating and stirring for 2.5, 5.0 and 7.5 min), and dilution of the milk sample in water (0, 25, 50 and 75%). The overall procedure is described below.

Aliquots of 10 mL of UHT cow milk were placed in 20 mL vials containing 25 µL of thymol and carvacrol standard solutions (2 mg mL^{-1}) and 25 µL of *o*-cresol internal standard solution (2 mg mL^{-1}). The vials were sealed with PTFE/silicone septa (Supelco, USA) and kept in water bath at 90 °C. Next, the needle of the SPME device was injected into the vial and the fiber was exposed to the headspace. After the extraction time, the fiber was repositioned inside the SPME device and was immediately introduced into the chromatographic system for desorption of the analytes. The experiments were performed in triplicate and a stopwatch (BSH-200 OX, China) controlled all time intervals.

The obtained data were statistically analyzed and means compared by Scoot-Knott test ($p < 0.05$) by using the software Sisvar (Federal University of Lavras, Brazil).

Solid-phase microextraction procedure for plasma

Blood samples (10 mL) from health cows, drawn from the coccygeal vein of each animal with a 20 gauge Vacutainer needle, were placed into glass tubes containing K2-EDTA (BD Vacutainer®). Blood samples were centrifuged at 1800 g and 4 °C for 20 min and the plasma samples obtained were stored at −20 °C until analysis. Aliquots of 1 mL drug-free plasma were placed into vials containing 1.5 g NaCl and 9 mL of pure water (obtained from a Milli-Q Plus system, Millipore, USA), added with 50 µL of thymol, carvacrol and propofol solutions. Extraction was conducted according to the conditions optimized for milk samples.

Validation of the bio-analytical method

The analytical method for analysis of thymol and carvacrol in bovine milk and plasma was validated according to official recommendations set by ANVISA (Brazilian National Health Surveillance Agency)[16] and US FDA (US Food and Drug Administration).[17]

Analyses for evaluation of the method selectivity were carried out in triplicate by using plasma samples from six untreated cows and from six different brands of commercial ultra-high temperature (UHT) Brazilian milk. The results were compared to those obtained from water spiked with analytes at concentrations near the limit of quantification.

Linearity was evaluated by performing the calibration curve data obtained from analyses of plasma and milk samples spiked with analytes at six concentration levels: 0.5, 2.5, 5.0, 10.0, 25.0 and 75.0, and 2.0, 10.0, 20.0, 50.0, 100.0 and 200.0 ng mL^{-1} for plasma and milk, respectively (n = 2 for each concentration). Propofol was used as internal standard at concentrations of 2.5 and 25 ng mL^{-1} for plasma and milk, respectively.

The precision of the method was evaluated by intra-day (n = 5) and inter-day (n = 3) assays and expressed as RSD (relative standard deviation) value. Three concentration levels: 0.5, 5.0 and 2.0, and 50.0, 20.0 and 200.00 ng mL^{-1} for plasma and milk, respectively, were analyzed. The RSD values of intra-day and inter-day studies lower than 15% showed that the precision of the method was satisfactory.

The limit of quantification (LOQ) was established by analyzing milk and plasma samples (n = 5) spiked with decreasing concentrations of thymol and carvacrol. LOQ was calculated when precision and accuracy obtained were lower than 20%.

The limit of detection (LOD) was found by analyzing milk and plasma samples added with known and decreasing concentrations of thymol and carvacrol to the lowest level detected, but not quantified as an exact value.

Milk and plasma samples spiked with carvacrol and thymol at concentrations of 0.5 and 50 ng mL^{-1} for plasma and 2.0 and 200 ng mL^{-1} for milk were used to determine the stability of the analytes in the biological matrices after freeze-thaw cycles (3 cycles of freezing, −20 °C for 24 h and thawing, and long-term storage, −20 °C for 30 and 60 days), short-term storage (room temperature, 2 and 4 h). Data obtained for the samples submitted to stability assays were compared to those obtained in stability tests carried out with newly-prepared samples. Standard solutions (0.01 and 1.0 µg mL^{-1} for plasma and 0.40 and 40.0 µg mL^{-1} for milk) were also evaluated for stability and the results obtained for solutions stored at −20 °C for 24 and 48 h, respectively, were compared to those obtained for freshly-prepared solutions.

The robustness of the analytical method was evaluated by extracting samples of plasma and milk spiked with 5 ng mL^{-1} of thymol and carvacrol in plasma and 20 ng mL^{-1} of thymol and carvacrol in milk, under slightly different conditions than those considered the ideal: extraction time of 38 and 42 min instead of 40 min, extraction temperature of 88 and 92 °C instead of 90 °C and the amount of NaCl of 1.7 and 1.3 g instead of 1.5 g, respectively.

Method application

The developed method was used to analyze milk and plasma samples obtained from six healthy cows (Girolando race with five years old) after administration of an

intra-mammary formulation containing 1% essential oils rich in thymol and carvacrol. The samples (30 mL milk and 10 mL blood) were collected 12 h after administration. Milk samples were stored frozen at –20 °C until analysis. Plasma samples were prepared by centrifugation (1800 g, 40 min) and also stored frozen at –20 °C until analysis.

All experimental procedures involving the use of animals were previously approved by the Ethic Committee for Research on Human Beings of University of Ribeirão Preto (UNAERP), and registered on Comet 03/12.

Results and Discussion

Chromatographic analysis of thymol and carvacrol by GC-MS

The chromatogram in Figure 1 shows the resolution of propofol (internal standard), thymol and carvacrol, respectively, eluted with retention times of 6.39; 6.54 and 7.26 min, obtained by injection of 5 µL (split ratio of 1:10) of standard solutions at the concentration of 2 mg mL^{-1}.

Optimization of the solid-phase microextraction procedure for milk

The results for the influence of variables evaluated in the solid-phase microextraction of thymol and carvacrol from milk were plotted on bar graphs, denoting peak areas obtained for each analyte *versus* variables analyzed. The results obtained are shown in Figures 2 and 3.

As shown in Figure 2A, the PMDS/DVB and PA fibers were almost equivalent for the extraction of thymol. However, the PMDS/DVB fiber resulted in insignificant higher areas for carvacrol and was chosen for the subsequent tests. This fiber was also used by Kohlert *et al.*[15] for determination of thymol and its metabolites in human plasma after administration of a phytomedicine. Data in Figure 2B show that the temperature of 90 °C enhanced the extraction, thus being considered ideal for the present protocol. The optimum temperature determination for HS-SPME extraction is complex because higher temperatures facilitate the transfer of analytes from sample to headspace. However, higher temperatures decrease adsorption/absorption of analytes into the fiber. In addition, the optimum temperature depends on the composition of matrix and type of fiber used.[18]

As described for SPME, extraction increases rapidly at the beginning of the extraction, followed by a slower increase until reaching a plateau, in which the analyte extraction remains constant over time. To obtain maximum sensitivity and reproducibility, the extraction should be conducted when equilibrium is reached. However, with a careful control of the extraction time, this procedure can be carried out in short time pre-equilibrium to reduce the analysis time.[19,20] Figure 2C shows that peak areas for analytes increased up to 40 min, followed by a decrease in the extraction. The equilibrium not reached for SPME might be associated to the samples used. Heating milk samples to high temperatures for long periods may change sample viscosity (protein denaturation), affecting their extraction.[21,22] Moreover, extraction time must be precisely monitored when extraction is carried out without reaching equilibrium conditions.[1]

The 40 min extraction time used for analysis of thymol and carvacrol in milk of dairy cows is similar to that

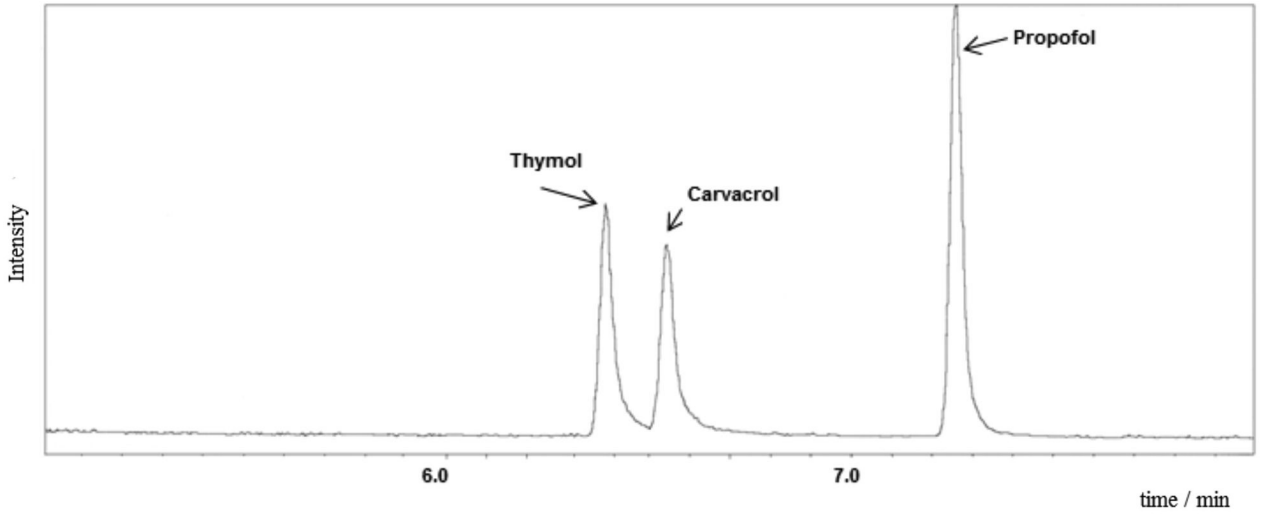

Figure 1. Chromatogram for the analysis of thymol and carvacrol using propofol as internal standard. Chromatographic conditions: RTX-5ms® capillary column, helium as carrier gas at a flow rate of 1 mL min^{-1}, injector and detector interface temperature set at 250 °C and column temperature starting at 100 °C (1 min) and programmed to increase 10 °C min^{-1} to 160 °C (1 min).

used by Kohlert *et al.*,[15] who used 35 min to extract thymol from human plasma. In addition, the 40 min extraction time was appropriate to reach a satisfactory limit of quantification of thymol and carvacrol in milk and plasma.

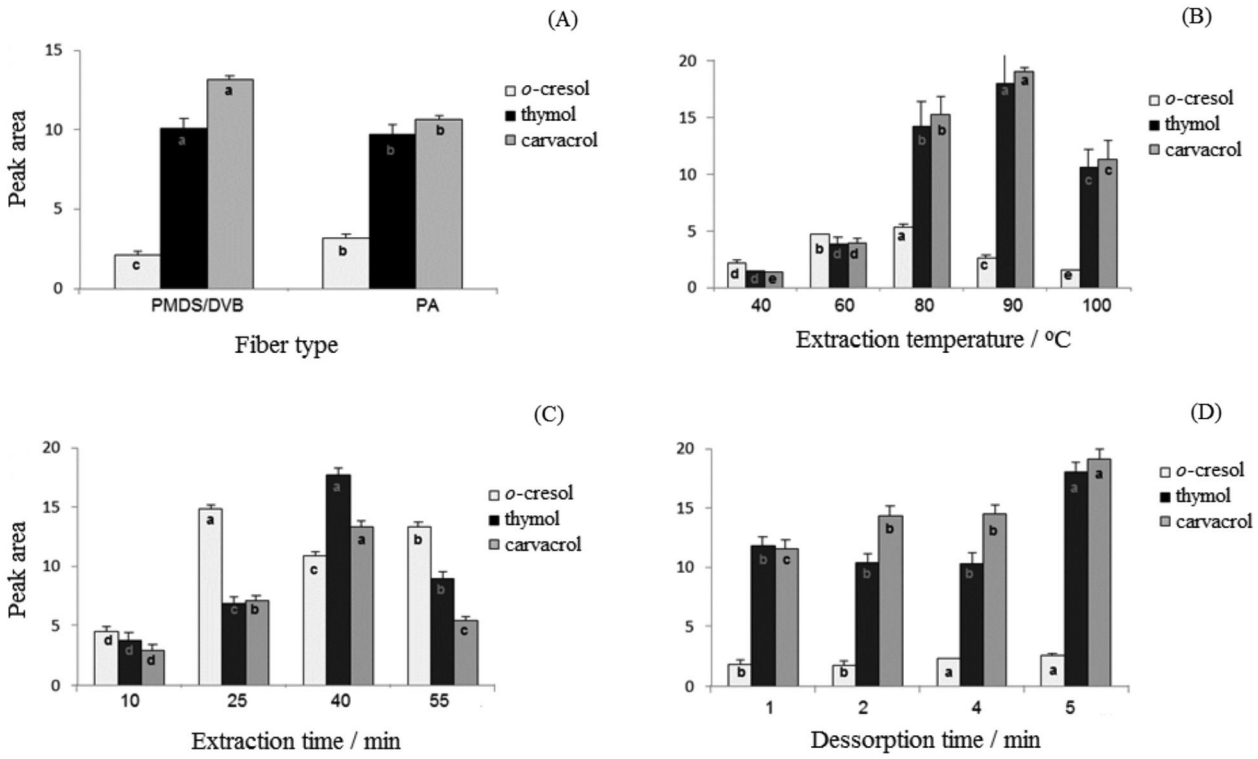

Figure 2. Influence of fiber type (A), extraction temperature (B), extraction time (C) and desorption time (D) in the solid-phase microextraction of thymol and carvacrol form milk. The notation a, b, c, d and e refers to statistical differences in the areas.

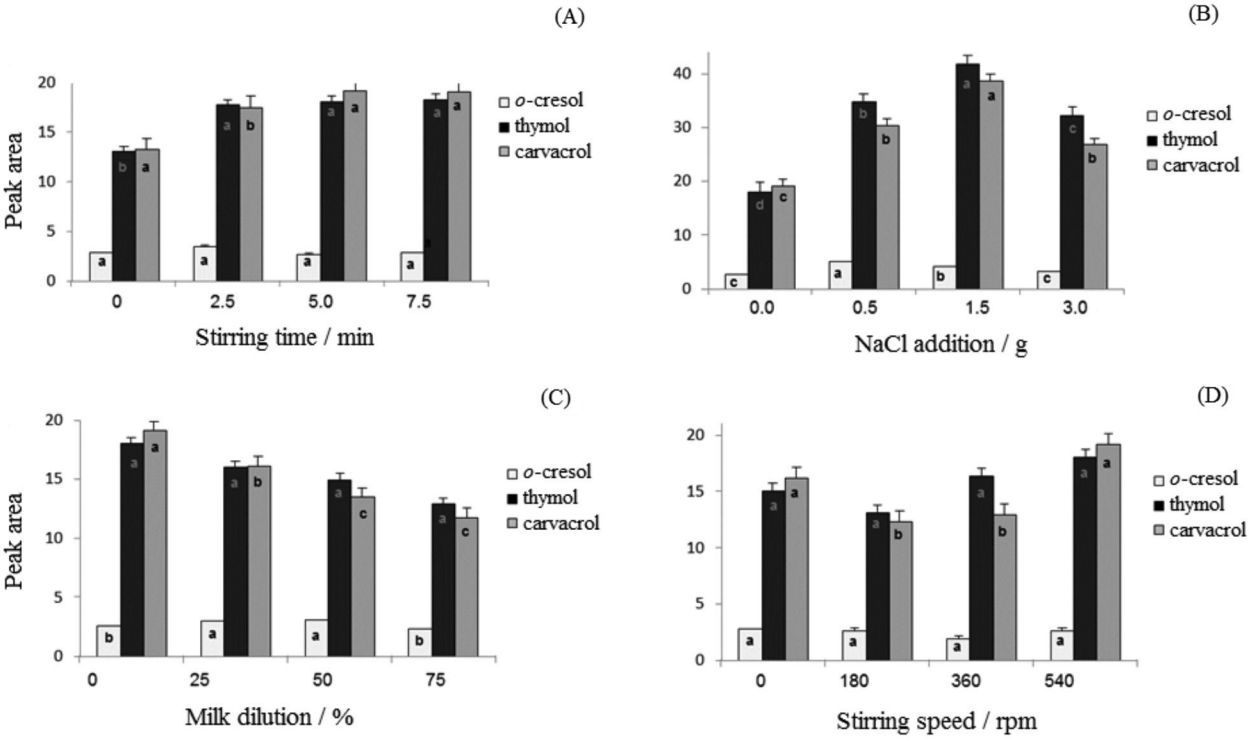

Figure 3. Influence of temperature (90 ºC) and agitation (540 rpm) before extraction (A), addition of NaCl (B), milk dilution (C) and agitation speed (D) in the solid-phase microextraction of thymol and carvacrol form milk. The notation a, b, c and d refers to statistical differences in the areas.

After extraction, the analytes were thermally desorbed into the GC injector at a temperature of 250 °C. Figure 2D shows larger peak areas of analytes with desorption time of 5 min.

The influence of secondary parameters affecting SPME extraction is shown in Figure 3 A-D.

Figure 3A shows the influence of stirring the sample prior to exposure of SPME fiber to headspace. Larger peak areas were observed when the samples were stirred. However, increasing the stirring time does not promote the extraction efficiency. The 5 min stirring period was selected to improve reproducibility. McPhee *et al.*[7] also chose a 5 min stirring period to analyze thymol in an intra-mammary formulation.

Figure 3B shows that extraction efficiency was improved by increasing the amounts of NaCl. Therefore, the amount of 1.5 g of NaCl was chosen for further analysis. Risticevic *et al.*[20] used NaCl to decrease the solubility of phenolic compounds in the aqueous phase and to force them towards the headspace. Moreover, the use of NaCl for determining thymol and carvacrol in plasma has been reported.[15,23]

Figure 3C shows decreased peak areas for carvacrol by using diluted milk samples, resembling a reduction in the extraction efficiency. Thus, it was decided not to dilute the samples.

Figure 3D shows that sample agitation improved extraction, with agitation at 540 rpm enhancing the carvacrol extraction. Our results are consistent with those reported by Risticevic *et al.*[20] and Sitaramaraju *et al.*,[23] who found that higher stirring speed improved mass transfer from the aqueous phase to the headspace.

Finally, the following optimized conditions were established for HS-SPME: milk sample (10 mL), (PMDS/DVB) fiber, NaCl addition (1.5 g), 5 min pre-heating of the samples (90 °C) under agitation (540 rpm), exposure of the SPME fiber to headspace (40 min) at stirring speed of 540 rpm, and desorption in the injector at 250 °C for 5 min.

Solid-phase microextraction of thymol and carvacrol from plasma

Having optimized the analytical method and conditions for extraction of thymol and carvacrol from milk, additional experiments were performed following the same procedures to determine thymol and carvacrol in the plasma of cows.

The influence of plasma constituents, manly proteins, on the SPME efficiency demands attention since drugs are associated with them in different degrees depending on their physicochemical properties.[24] The dilution of plasma samples in water may improve SPME, thus increasing the analyte diffusion coefficient from the plasma samples to the extraction fiber.[25] Thus, plasma samples were diluted in a ratio of 1:10 in water and the other conditions were the same as those used for milk.

Bio-analytical method validation

The analytical method developed for analysis of thymol and carvacrol in milk and plasma of dairy cows was rather selective because no peak of other substance was eluted at the same retention times of the analytes (Figures 4 and 5).

The method was linear for both analytes and biological samples, showing determination coefficients (r^2) higher than 0.99 (Tables 1 and 2).

Moreover, the method had acceptable precision and accuracy, with coefficients of variation and relative errors lower than 15%, except for the limit of quantification as its values were ≤ 20% for both analytes and biological samples.[16,17] The results are shown in Tables 1 and 2.

The limits of quantification for thymol and carvacrol in milk (Table 1) and plasma (Table 2) were 2.0 and 0.5 ng mL^{-1}, respectively. These values were lower than those obtained by McPhee *et al.*,[7] who reported limits of quantification of 75 ng mL^{-1} for thymol in milk and 50 ng mL^{-1} in plasma, with limits of detection of 50 and 10 ng mL^{-1}, respectively. The limits of quantification of thymol were also higher than that reported by Kohlert *et al.*,[15] that is 8.14 ng mL^{-1}.

To evaluate the method robustness, some variables were changed during the procedure (extraction time, extraction temperature and amount of NaCl) and the results obtained (Tables 3 and 4) indicated that the developed method for analysis of thymol and carvacrol is robust, for both matrices.

Data obtained in the evaluation of thymol and carvacrol stability in milk and plasma of dairy cows are shown in Tables 5 and 6, respectively. By investigating the stability of standard solutions, it was observed that solutions of thymol and carvacrol in methanol are stable only for 24 h, i.e., it is necessary to prepare the standard solutions daily.

Studies on short-term stability showed that plasma samples at low concentrations (0.5 ng mL^{-1}) are stable only for 2 h at room temperature, whereas at high concentrations (50 ng mL^{-1}) this stability reaches 4 h. Therefore, it is suggested that plasma samples should be processed as quickly as possible (maximum 2 h after thawing). On the other hand, milk samples at low (2 ng mL^{-1}) or high concentrations (200 ng mL^{-1}) were shown to be stable for at least 4 h. Studies on long-term stability showed that plasma and milk samples can be stabilized for 2 months, i.e., after sample collection, they

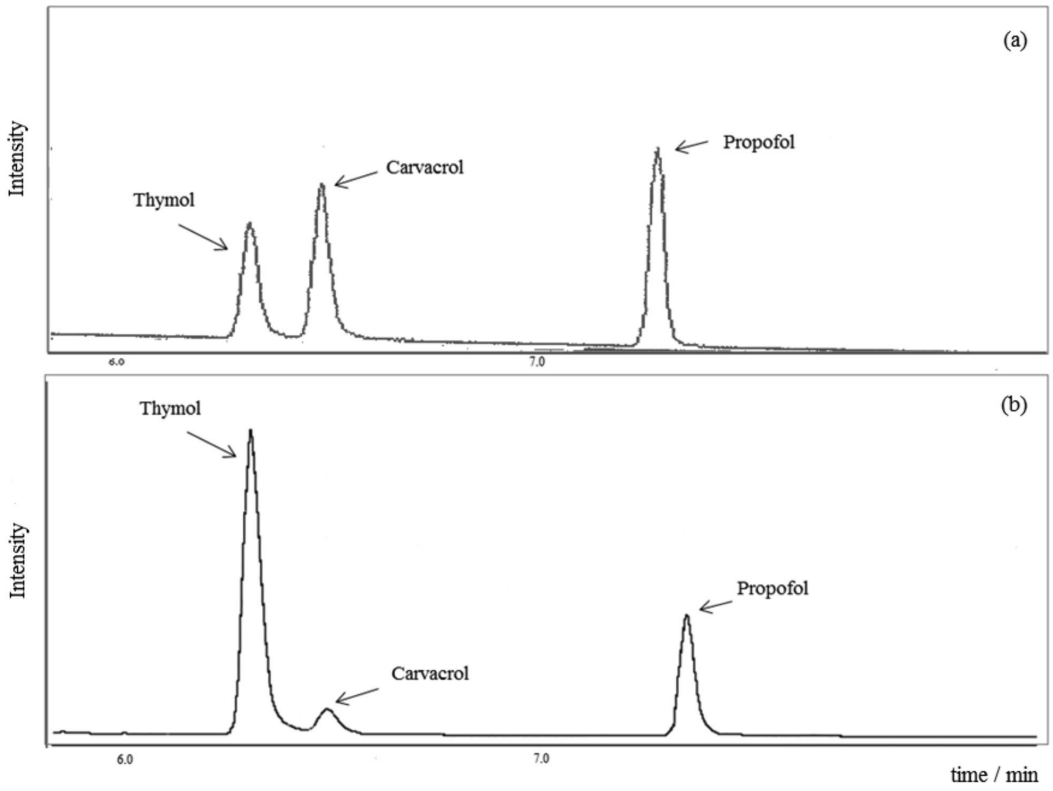

Figure 4. Chromatograms referring to the analysis of thymol and carvacrol in milk of cows. Milk spiked with 20 ng mL^{-1} of thymol and carvacrol and 25 ng mL^{-1} of propofol (internal standard) (a). Milk obtained 12 h after administration of an intra-mammary formulation (b), chromatographic conditions as in Figure 1.

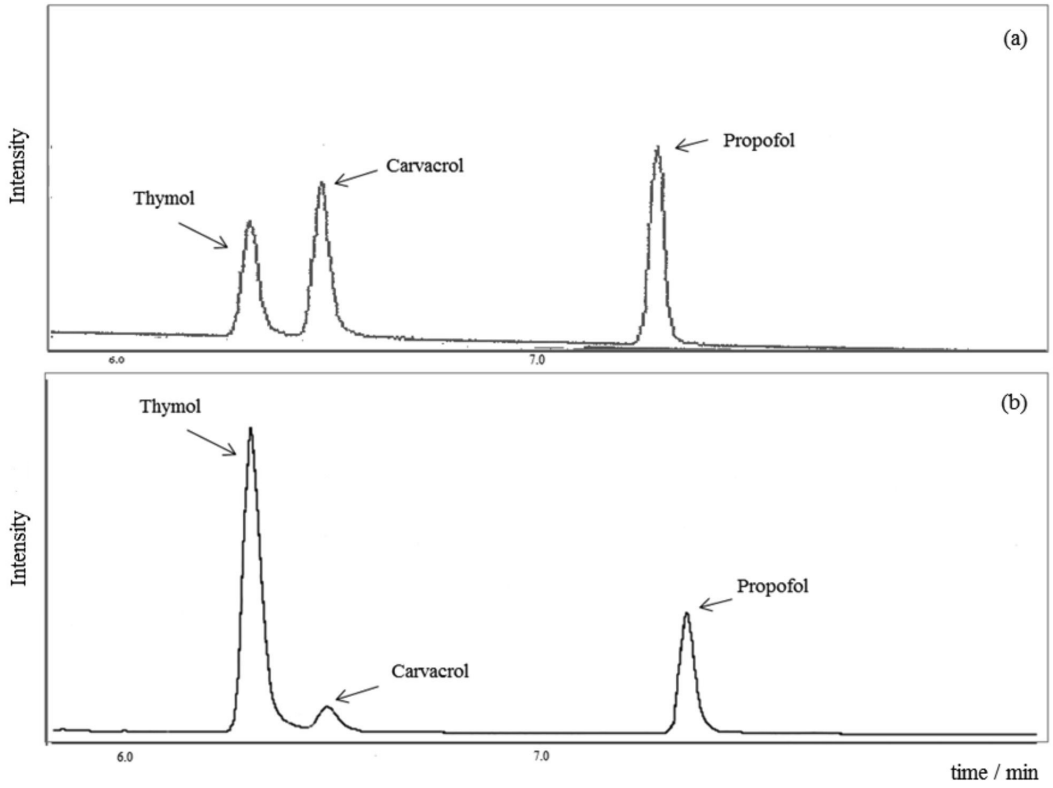

Figure 5. Chromatograms referring to the analysis of thymol and carvacrol in plasma of cows. Plasma spiked with 5 ng mL^{-1} of thymol and carvacrol and 2.5 ng mL^{-1} of propofol (internal standard) (a). Plasma obtained 12 h after administration of an intra-mammary formulation (b), chromatographic conditions as in Figure 1.

Table 1. Confidence limits for the method of analysis of thymol and carvacrol in milk of cows

	Thymol	Carvacrol
Linearity		
Range / (ng mL^{-1})	2.0-400.0	2.0-400.0
Linear equation	$y = 0.054x + 0.0545$	$y = 0.06x + 0.0643$
r^2	0.9994	0.9997
Quantification limit / (ng mL^{-1}) (n = 5)	2.0	2.0
Precision: CV / %	3.5	4.0
Accuracy: deviation / %	−3.9	−6.6
Between day precision: CV / %		
2 ng mL^{-1} (n = 3)	11.3	4.4
20 ng mL^{-1} (n = 3)	5.4	5.1
200 ng mL^{-1} (n = 3)	3.0	2.8
Within day precision: CV / %		
2 ng mL^{-1} (n = 5)	2.2	0.8
20 ng mL^{-1} (n = 5)	3.3	9.8
200 ng mL^{-1} (n = 5)	3.4	2.6
Between day accuracy: relative error / %		
2 ng mL^{-1} (n = 3)	−0.1	−5.1
20 ng mL^{-1} (n = 3)	−2.2	−4.0
200 ng mL^{-1} (n = 3)	−2.4	−0.4
Within day accuracy: relative error / %		
2 ng mL^{-1} (n = 5)	16.3	−16.1
20 ng mL^{-1} (n = 5)	1.5	−5.4
200 ng mL^{-1} (n = 5)	−5.2	−10.1
Limit of detection / (ng mL^{-1})	0.89	0.57

CV: coefficient of variation; r^2: determination coefficient; n: number of determinations (5 replicates for within day and 3 days for between day assays).

Table 2. Confidence limits for the method of analysis of thymol and carvacrol in plasma of cows

	Thymol	Carvacrol
Linearity		
Range / (ng mL^{-1})	0.5-75.0	0.5-75.0
Linear equation	$y = 0.1476x + 0.0012$	$y = 0.1587x + 0.022$
r^2	0.999	0.997
Quantification limit / (ng mL^{-1}) (n = 5)	0.5	0.5
Precision: CV / %	5.2	4.2
Accuracy: relative error / %	17.8	16.6
Between day precision: CV / %		
0.5 ng mL^{-1} (n = 3)	7.6	10.5
5 ng mL^{-1} (n = 3)	5.6	5.2
50 ng mL^{-1} (n = 3)	4.1	3.6
Within day precision: CV / %		
0.5 ng mL^{-1} (n = 5)	8.4	14.9
5 ng mL^{-1} (n = 5)	6.7	6.0
50 ng mL^{-1} (n = 5)	2.3	6.0
Between day accuracy: relative error / %		
0.5 ng mL^{-1} (n = 3)	11.0	6.6
5 ng mL^{-1} (n = 3)	2.6	4.0
50 ng mL^{-1} (n = 3)	0.1	1.6
Within day accuracy: relative error / %		
0.5 ng mL^{-1} (n = 5)	−9.8	8.7
5 ng mL^{-1} (n = 5)	8.4	6.1
50 ng mL^{-1} (n = 5)	3.1	3.1
Limit of detection / (ng mL^{-1})	0.41	0.31

CV: coefficient of variation; r^2: determination coefficient; n: number of determinations (5 replicates for within day and 3 days for between day assays).

Table 3. Robustness of the method for the analysis of thymol and carvacrol in milk of cows

Variables of the extraction procedure	Thymol (relative error)	Carvacrol (relative error)
Extraction time / min		
38	–4.7	–4.0
42	–4.1	-4.2
Extraction temperature / ºC		
88	–3.2	3.3
92	–10.5	–1.8
NaCl amount / g		
1.3	–12.2	–4.9
1.7	–11.9	2.1

Table 4. Robustness of the method for the analysis of thymol and carvacrol in plasma of cows

Variables of the extraction procedure	Thymol (relative error)	Carvacrol (relative error)
Extraction time / min		
38	–4.6	0.8
42	12.4	11.8
Extraction temperature / ºC		
88	9.2	4.7
92	9.7	9.8
NaCl amount / g		
1.3	–7.6	–6.7
1.7	–13.5	–7.0

Table 5. Stability study of thymol and carvacrol in milk of cows

Stability	Thymol concentration (relative error)	Carvacrol concentration (relative error)
Standard solution, 24 h	0.40 μg mL^{-1} (–17.6) 40 μg mL^{-1} (–9.1)	0.40 μg mL^{-1} (–16.2) 200 ng mL^{-1} (–12.4)
Short-term		
4 h	2 ng mL^{-1} (–2.0) 200 ng mL^{-1} (–5.0)	2 ng mL^{-1} (–9.6) 200 ng mL^{-1} (–0.7)
Long-term	2 ng mL^{-1}	2 ng mL^{-1}
1 month	(–12.4)	(–11.5)
2 months	(–13.8) 200 ng mL^{-1}	(–12.0) 200 ng mL^{-1}
1 month	(–9.1)	(–8.6)
2 months	(–10.6)	(–9.1)
Cycles of freezing and thawing	2 ng mL^{-1} (–19.9) 200 ng mL^{-1} (–10.8)	2 ng mL^{-1} (–18.7) 200 ng mL^{-1} (–5.7)

Table 6. Stability study of thymol and carvacrol in plasma of cows

Stability	Thymol concentration (relative error)	Carvacrol concentration (relative error)
Standard solution, 24 h	1 μg mL^{-1} (–7.3)	1 μg mL^{-1} (–4.9)
Short-term		
2 h	0.5 ng mL^{-1} (–13.5)	0.5 ng mL^{-1} (–12.1)
4 h	50 ng mL^{-1} (–11.6)	50 ng mL^{-1} (–10.9)
Long-term	0.5 ng mL^{-1}	0.5 ng mL^{-1}
1 month	(–10.2)	(–7.9)
2 months	(–13.5) 50 ng mL^{-1}	(–11.5) 50 ng mL^{-1}
1 month	(–8.8)	(–3.0)
2 months	(–13.2)	(–9.4)
Cycles of freezing and thawing	0.5 ng mL^{-1} (–15.0) 50 ng mL^{-1} (–3.9)	0.5 ng mL^{-1} (–14.6) 50 ng mL^{-1} (–3.5)

could be stored at –20 ºC for 2 months with no influence of degradation processes or loss of volatile analytes. Stability studies indicated that after the freezing and thawing cycles, the samples are also stable.

Method application

The validated method was used to analyze milk and plasma samples from healthy dairy cows 12 h after administration of an intra-mammary phytoformulation containing essential oils rich in thymol and carvacrol. Chromatograms obtained for analytes are shown in Figures 4 and 5. Samples were analyzed against calibration curves obtained simultaneously and the concentrations in milk samples were 104 ng mL^{-1} for thymol and 8 ng mL^{-1} for carvacrol. In plasma samples, the concentrations were 8 and 4 ng mL^{-1} for thymol and carvacrol, respectively.

Conclusions

The chromatographic method developed and validated here showed the applicability of gas chromatography-mass spectrometry and solid-phase microextraction for quantification of thymol and carvacrol in plasma and milk of dairy cows. This method was more efficient for determining thymol than the one previously reported in the literature because of the lower limits of quantification obtained. Regarding the determination of carvacrol, no methodology has been described before for analysis of this compound in plasma and milk.

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