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Determination of cinnamaldehyde, carvacrol and thymol in feedstuff additives by pressurized liquid extraction followed by gas chromatography–mass spectrometry☆

Marta Jiménez-Salcedo, María Teresa Tena*

Department of Chemistry, Faculty of Science and Technology, University of La Rioja, C/Madre de Dios 53, E-26006 Logroño (La Rioja), Spain

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ABSTRACT

Specific blends of essential oils (BEOs) are promising substitutes for antibiotics to promote livestock performance and to reduce the incidence of intestinal disorders. Microencapsulation of BEOs has shown to improve their stability, bioavailability and to control their release rate once they are added to the feedstuff. The development and validation of a method for determining essential oil components such as carvacrol, thymol and cinnamaldehyde in a microencapsulated material used as feed additive is presented. Analytes were extracted from feed additives and feedstuff by pressurized liquid extraction (PLE) with methanol at 50 °C for 5 min. Methanol provided good recovery values and cleaner extracts than other polar organic solvents tested. However, for certain kind of composite additives ethyl acetate showed to be a better option because *trans*-cinnamaldehyde undergoes chemical reaction in methanol. Then PLE extracts were analysed by gas chromatography coupled to ion trap mass spectrometry in selected ion storage (SIS) mode. The analyte stability and the absence of analyte losses during the PLE process was checked by a recovery study. Also, the matrix effect was studied to assess accuracy. Recovery values were between 85 and 115% in most cases. Intra- and inter-day relative standard deviation values were less than 4 and 14%, respectively. Finally, the developed method was applied to the analysis of a microencapsulated feed additive, several composite feed additive samples containing microencapsulated BEOs and a spiked feedstuff, for quality control and in stability studies.

1. Introduction

One of the reasons of the appearance of resistant bacteria can be attributed to the excessive use of antibiotics in animal production, not only to control pathogens but also to improve and accelerate livestock growth. The concern about antibiotic resistance and the European Union ban on the use of antibiotics [1] has prompted feed industry to search for alternatives to antibiotics to promote livestock performance and to reduce the incidence of intestinal disorders due to the proliferation of gut pathogens.

Essential oils have been shown as promising substitutes for antibiotics in calves [2]. Specific essential oil blends and probiotics used as dietary supplementation have shown to promote broiler growth [3,4], and in combination with benzoic acid have improved gut integrity and intestinal microbiota in turkey poults [5]. In addition, a microencapsulated, plant-based essential oil blends (containing α-pinene, linalyl acetate, p-cymene, and thymol octanoate) showed promising results as a daily supplement to reduce helminth infections in both pigs and human beings [6]. Moreover, the essential oil treatments tended to diminished diarrhoea incidence in pigs that were subjected to [7]. To sum up, the results of these studies indicate that supplementation of the diet with blended essential oils could replace treatment with antibiotics to improve growth performance and faecal characteristics. However, there is studies that set some controversy about the positive effect of thyme on animal performance [8] inasmuch as no improvement in weight gain was observed and also because with high dietary levels of thyme herb, thymol concentrations increased in gut contents and plasma but were very low in edible tissues such as liver and flesh.

Microencapsulation of natural products, for instance polyphenols from grape and carvacrol, has been proposed to improve the stability, bioavailability and in order to control their release rate once they are added to the functional and enriched foods [9,10].

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* Corresponding author.
E-mail address: maria-teresa.tena@unirioja.es (M.T. Tena)

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With this aim, some essential oil components were microencapsulated to prepare a feed additive candidate to promote livestock growth. In order to control the quality, stability and manufacture of this new product, it was necessary to develop a method to assess the concentration levels of its constituents: carvacrol, thymol and trans-cinnamaldehyde.

In addition, additives in animal feed must be controlled because their potential impact on food safety. Isotope ratio mass spectrometry has been proposed to assess the authenticity of several spice essential oils used as feed additives [11]. To our knowledge, there are no methods reported for the analysis of food or feedstuff containing microencapsulated essential oil constituents. Up to date, the reported methods deals with plants [12–19], food [20–25] and pharmaceuticals [25,26]. These methods usually involve the separation of the essential oil components from the sample and a further chromatographic analysis of the extract obtained.

The extraction of essential oil components has been carried out by steam distillation [12,15–17], solvent extraction [20,23], supercritical fluid extraction [27], ultrasound assisted extraction [14,19,26] pressurized liquid extraction [28] and by microextraction techniques such as single-drop [29], stir bar sorptive extraction [24] and solid-phase microextraction [18,22,25]. The subsequent chromatographic analysis is mainly performed by gas chromatography [12,13,15–18,20,22–24] and liquid chromatography [14,19,21,25] coupled to different detectors. The most popular detector is MS but UV photometers [25], fluorimetric [21] and electrochemical detectors [14] have been also reported for liquid chromatography.

The solvents used for removing essential oil components from sample matrix used to be ethyl acetate [13,20,23] and methanol [28] or methanol-water mixtures [14,19]. In the case of trans-cinnamaldehyde, methanol and ethanol are reported to be not available for PLE because this compound reacts with these solvents through an aldol reaction [13].

The aim of this work was to develop and validate of a method for the quality control in the production of feed additives containing microencapsulated essential oil components such as carvacrol, thymol and cinnamaldehyde. The method proposed is based on pressurized liquid extraction followed by gas chromatography coupled to mass spectrometry analysis and has been applied to the analysis of different feed additives and feedstuff. To our knowledge, this is the first time that a method is reported for the determination of essential oil components in these feed-related samples.

2. Experimental

2.1. Materials and samples

Carvacrol (5-isopropyl-2-methylphenol CAR, ≥98%), thymol (2-isopropyl-5-methylphenol, THY, ≥99%), eugenol (2-methoxy-4-(2-propenyl)phenol, EUG, ≥99%) and *trans*-cinnamaldehyde ((2*E*)-3-phenylprop-2-enal, CIN, ≥99%) and (+)-fenchol (1,3,3-trimetil-2-norbornanol, FEN) were obtained from Sigma-Aldrich (Madrid, Spain). Ethyl benzoate (EBZ, ≥99%) and methyl trans-cinnamate (MtC, ≥99%), also from Sigma-Aldrich, were used as internal standards. Individual analyte stock standard solutions were prepared in methanol and stored at 4 °C. Working standard solutions containing 100 μg ml^{-1} of each analyte were used to prepare calibration solutions. Internal standard (IS) concentration in calibration solutions was 1.00 or 25.0 μg ml^{-1} depending on the experiment.

LCMS-grade methanol and HPLC-grade ethyl acetate, ethanol and acetone, all of them provided by Scharlab (Barcelona, Spain), were tested as extraction solvents. 20-mm diameter cellulose filters for PLE cells were purchased from Restek (Bellefonte, PA, USA) and washed sea sand from Scharlab was used to fill up PLE cell void volume.

Three different types of samples were analysed: 1) Three batches of a microencapsulated feed additive (MFA) containing cinnamaldehyde, carvacrol and thymol in a fatty matrix, 2) three different composite feed additive samples (CFA) prepared from MFA by dilution with other additive ingredients (such as vitamins and mineral salts), 3) A feedstuff sample containing MFA. All samples were provided by Igusol Advance S. A. (La Rioja, Spain).

2.2. Pressurized liquid extraction

A Dionex ASE200 pressurized liquid extractor furnished with 11-mL stainless-steel extraction cells was used. Samples (0.1 g of MFA, 0.5 g of CFA or 1.4 g of feedstuff) were extracted in one cycle with methanol at 50 °C and 1500 psi for 5 min. The heating time was 5 min and the flush volume 90%. The void volume of the extraction cell was filled with sand. Extracts were made up to 25 ml with methanol. MFA extracts were diluted 1:100 in methanol before GC analysis. Different dilution ratios were used for CFA extracts, for instance 1:10, 1:10 and 1:20 for P11, CV8 and CV4 extrats, respectively. Feedstuff extracts were not diluted. Different dilution ratios were used because analyte concentrations were different in the samples analysed. Extract dilution was aimed to get a concentration level within the linear range of calibration. Final extract solutions were filtered through a 0.45 μm nylon filter before GC analysis.

2.3. Gas chromatography–mass spectrometry analysis

Essential oil components were determined using a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2200 ion trap mass detector (Walnut Creek, California, USA) and equipped with a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland). Analyte separation was performed in a HP-5 MS 30 m × 0.25 mm i.d. fused-silica column with a 0.25 μm 5% polydiphenylsiloxane/95% polydimethylsiloxane stationary phase. Helium (99.996%) at a flow-rate of 1 ml min^{-1} was used as a carrier gas. Injection was performed at 240 °C with a 1:50 split ratio and the injection volume was 1 μl. The oven temperature was programed as follows: an initial temperature of 90 °C for 3 min, then increased at a rate of 3 °C min^{-1} to 115 °C, increased again at a rate of 6 °C min^{-1} to 140 and finally increased at 40 °C min^{-1} to 200 °C.

Mass spectrometry detection was performed by electron-impact ionisation (70 eV) and the electron multiplier set at 1600 V. Trap, manifold and transfer line temperatures were set at 200, 60 and 280 °C, respectively. Chromatograms were recorded in scan and SIS MS detection modes depending on the experiment. A 5-min solvent delay was used. The *m*/*z* range for scan mode chromatograms was 35–350. Extracted ions from scan chromatograms were *m*/*z* 80, 81, 103, 122, 131, 135, 150, 162 and 164. The SIS program was as follows: 79–82 *m*/*z* range from 5.00 to 7.90 min, 121–123 *m*/*z* range from 7.90 to 10.00 min, 102–132 *m*/*z* range from 10.00 to 12.50 min, 134–151 *m*/*z* range from 12.50 to 14.00 min, 163–165 *m*/*z* range from 14.00 to 15.30 min and 130–163 *m*/*z* range from 15.30 to 16.50 min. The quantification ions were *m*/*z* 122 for EBZ, *m*/*z* 103 + 131 for CIN, *m*/*z* 135 + 150 for THY and CAR, *m*/*z* 164 for EUG and *m*/*z* 131 + 162 for MtC. Other ions for identification of the compounds are: *m*/*z* 132 and 77 for CIN and *m*/*z* 107 and 91 for THY and CAR.

Both scan and SIS modes were used during the method development and in preliminary experiments involving MFA sample. Scan mode was also used in a preliminary study on standard solution stability. CIN degradation was observed after several months at room temperature: CIN signal decreased while a peak at 15.8 min (MS base peak at *m*/*z* 147) appeared. Therefore, CIN degradation can be detected in the 130–163 *m*/*z* acquisition segment of SIS program,