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Thymol detection and quantitation by solid-phase microextraction in faeces and egg yolk of Japanese quail

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ABSTRACT

To measure bioavailability of the active ingredients of phytogenic feed additives in poultry products and subproducts is a key element for developing a rational understanding of its mode of action and biological effects. Hence, we validated a headspace solid phase microextraction (HS-SPME) technique followed by gas chromatography-mass spectrometry as an analytical extraction procedure and as method for detection and quantitation of 2-Isopropyl-5-methylphenol (thymol) in faeces and egg yolk of quail. The suitability of this method for thymol analysis in both matrices was first proved via linearity, limit of detection, limit of quantification, and recovery using m-cresol as internal standard. The optimal HS-SPME extraction conditions were obtained at 40 °C for 5 min in faeces and 60 °C for 30 min in egg yolk. This procedure was found to be precise, sensitive and linear in the range of 2.5–100 ng/gr for faeces and 20–800 ng/gr for the egg yolk. Limits of detection were 0.5 ng/g and 5 ng/g for faeces and yolk, respectively, and the limits of quantitation were 1 ng/g and 10 ng/g for faeces and yolk, respectively. The method was successfully used for measuring thymol in fecal and egg yolk samples, from quails supplemented with thymol in their diets. Thus, in fresh faeces and egg yolk samples obtained from a supplemented group (80 mg thymol per bird per day) were determined as 31.51 ng/g for faeces and 11.83 ng/g for the egg yolk.

1. Introduction

To measure bioavailability of the active ingredients of phytogenic feed additives in poultry products and subproducts is a key element for developing a rational understanding of its mode of action and biological effects. 2-Isopropyl-5-methylphenol (thymol, Fig. 1), is a natural phenol and the main component of "oregano" (*Origanum vulgare*) and "thyme" (*Thymus vulgaris*) essential oils (EO) [1,2]. Thymol is considered a compound of interest in the food industry and is included in the GRAS ("Generally Recognized As Safe") (2016) [3] list of US (United State) government-approved food additives. Moreover, in wide range of animals has been calculated thymol safety levels (European Food Safety Authority) [4], clearance and half life [5–8] on subacute and chronic administration [9–12]. Thymol has intrinsic bioactivities (antioxidant, modulator at GABAa receptors (anti-stress), insecticidal and antifungal, among others) [13–15] which impact beneficially on lipid metabolism, performance, health and welfare issues in animal production [16,17] and thus on products derived for human consumption. Particularly, hens diet supplementation with herbs containing thymol, has improved egg mass, egg shape index, egg production rate and feed conversion ratio [16,18,19]. Additionally, thymol supplementation in the mentioned species has improved the oxidative stability of eggs during storage [20]. Krause and Ternes [21] stated that the chain reaction of oxidation of the consumed lipids could be inhibited by the transfer of the antioxidant constituents of natural supplements, such as thymol, into the hen by feeding, and consequently decreasing the oxidation of constituents transferred into the egg yolk [22]. Also, preliminary results in quail indicated that feed supplementation with thymol increases polyunsaturated fatty acids and reduces saturated fatty acids in total and triglyceride fatty acids of fresh egg yolks [23]. Accordingly, an increment of polyunsaturated fatty acids in egg yolk could potentially improve embryonic development [24], and be related to

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the increment in hatching success reported in quail using thymol as feed supplement [25]. Moreover, from a human consumption perspective, thymol effects on fatty acids and oxidative stability would derive in the production of healthier table eggs responding to current market demands [26,27]. Although, thymol presence in the egg could also produce unexpected odors or flavors for consumers.

There is evidence indicating that thymol can cause effects at various levels [8,28–31], and its magnitude may be related to the supplementation dose [28] and to the amount of the component that is actually incorporated, redistributed into the body (including allocation to the egg yolk) and excreted [8]. Haselmeyer et al. [8] demonstrated that thymol is efficiently absorbed, although much variability between individuals was found, and apparently also eliminated since only traces were found in tissues when the diet of domestic chickens was supplemented with Thyme EO. However, to our knowledge, the excretion mechanism of thymol supplemented into birds diet has still not been elucidated nor a study has evaluated methods for determining the presence of thymol in faeces. Quantitation of unmetabolized thymol may allow inferring the amount of it that is available in the body to perform its functions at their localized sites in relation to the amount ingested, without manipulating the animal. The latter would reduce the adverse effects that stress by manipulation causes on animals or even avoid unnecessary animal slaughtering [32].

Either scenario (human consumption and bioactivity on animals) highlights the importance of evaluating transference and bioavailability of thymol to faeces and eggs in a fast, efficient and accurate manner. Head-space solid-phase microextraction (HS-SPME) is an attractive alternative for the extraction, detection and quantitation of thymol in complex biological matrices, such as egg yolk and faeces, since it reduces the drag of sample preparation, and therefore, the time involved in analysis [33]. This extraction technique was successfully used by Kohlert et al. [34] 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. [35] also used the SPME technique to detect and quantify thymol in milk of goats treated with an intra-mammary formulation containing essential oils. This technique is not only fast but also selective, easily automated and free of organic solvents [36–38] these characteristics simplify the analysis of volatile and non-volatile compounds in solid and liquid complex biological matrices. Additionally, gas chromatography coupled to mass spectrometry (GC–MS) is a powerful tool to separate, identify and quantify volatile organic compounds in most types of complex matrices. Thus, the purpose of this study was to develop a method, based on HS-SPME coupled to GC–MS, to detect and quantitate thymol in faeces and egg yolk of Japanese quail (*Coturnix coturnix*), to better understand the transfer of this monoterpene from the maternal diet to the egg yolk and its excretion; we expect that the obtained information will be useful to address dose-response relationship in future works. First, we optimized the method for quail faeces and egg yolk and, once validated, we applied it to faeces and egg yolk from thymol-supplemented quail.

2. Experimental

2.1. Chemicals and reagents

Ethanol analytical grade was obtained from Porta (Córdoba, Argentina). Thymol standard was purchased from Sigma Aldrich. A stock solution of thymol was prepared in ethanol at 2 mg/mL and stored at 4 °C; then it was used for preparing a matrix spike, in order to optimize the extraction conditions, and for the validation study at different concentration levels. m-Cresol standard was purchased from Sigma Aldrich. A stock solution of m-cresol was prepared in ethanol at 2 mg/mL and stored at 4 °C, and was then used as internal standard in all assays at a concentration of 50 ng/g. Deionized water was employed in all experiments.

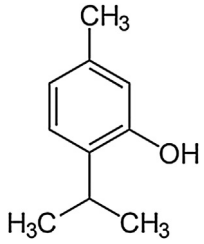

Fig. 1. Chemical structure of thymol.

2.2. Animals and husbandry

We used Japanese quail because they are considered an important species not only because of its relevance in meat and egg production but also because of its usefulness for the extrapolation of data to chickens and other commercially more important poultry species because of their physiological similarities [39] offering the advantages of a short life cycle and a considerably less costly maintenance [40].

All experimental procedures involving the use of animals were carried out in accordance with international standards of care and use of laboratory animals and previously approved by the Institutional Committee on Care and Use of Animals of Facultad de Ciencias Exactas, Físicas y Naturales-Universidad Nacional de Córdoba (approved on September 9, 2015, according to current regulations: Resol. 571-HCD-2014/, act number 4/2015) and the Instituto de Investigaciones Biológicas y Tecnológicas – Consejo Nacional de Investigaciones Científicas y Técnicas (approved on October 08, 2015, according to current regulations, act number 27 of IIByT Board Directors).

The study involved twelve females of Japanese quail, randomly and individually housed in cages within one 6-tier cage battery unit; one battery comprised twenty-four cages. Quail were maintained individually to identify the origin of each egg and avoid any influence of agonistic interactions and hierarchy on egg quality. In addition, quail housed in the individual cages had tactile, visual, and vocal contact among them [41]. Light conditions were a 14 h light cycle (0600–2000 h; approximately 180 Lux) and a 10 h dark cycle. Maintenance and feeding chores were performed at the same time each day (0900 h).

At 100 d of age, females within each cage were randomly assigned to 1 of 2 feed treatments (6 individuals each one): Control (CON; basal diet) or THY (80 mg of THY/kg of basal diet) [42]. Both THY supplemented and CON layers diets had corn, soybean disabled, wheat bran, soybean pellets, sunflower pellets, calcium, salt, vitamins, minerals and phosphate in identical compositions (Min. raw crude protein 20% – Min. fat matter 5.50% – Max. crude fiber 5.15% – Max. total minerals 7.5% – Max. calcium 2.90% – Min. calcium 2.50% – Max. phosphorous 0.85% – Min. phosphorous 0.75%). This feed ration had 20 g/kg of Crude Protein and 2.9 Mega Joules of Metabolizable Energy/kilogram.

Thymol was commercially obtained from Sigma Aldrich, SAFC®, ≥99%, FCC, USA). A 0.5% ethanol/water solution of thymol was uniformly sprayed on fresh commercial feed [25,42]. Following the protocol described by Krause and Ternes [22], quail diet was supplemented for 12 days before collection of egg and fecal samples in order to address the plateau or stationary state of thymol transfer indicated by these authors.

The faeces and egg yolk from control group were used in optimization and validation of the method as blank (Fig. 3) and spiked