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A preliminary study of the poultry body weight effect of carvacrol in litter and of carvacrol residue in organ tissue of exposed chickens

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Abstract

Introduction: Carvacrol is an essential oil extracted from oregano which can be used as a natural additive in poultry litter and could have a positive impact not only on production rates but also on the quality of poultry meat. The aim of this study was to evaluate the effect of the addition of carvacrol to litter on weight gain and the occurrence of residues in chicken tissues. **Material and Methods:** One-day-old Ross 308 chicks were used for the study and were randomly divided into two experimental groups. For 42 days, one group was kept in a room with litter enriched with carvacrol and the second group was kept in a room with litter without carvacrol. After 42 days, the birds were sacrificed and necropsied. Carvacrol content was determined in homogenised organ tissue samples by liquid chromatography–mass spectrometry. **Results:** Weekly weighing results showed that exposure to carvacrol in litter had no impact on chicken body weight. The analysis of plasma, muscle, liver and lung tissue after 42 days' exposure clearly indicated that there were residues of carvacrol in the analysed matrices. **Conclusion:** Exposure of chickens to carvacrol left residues but did not affect body weight.

Keywords: carvacrol, essential oils, litter, liquid chromatography–mass spectrometry, poultry.

Introduction

The usefulness of aromatic and flavour supplements, including herbs, is increasing in animal nutrition and treatment (3, 21). The significant increase of interest in these supplements is due to the trend for healthy nutrition and a return to natural raw materials in animal nutrition. The tendency to reduce or ban the use of antibiotics in animal nutrition has undoubtedly contributed to interest in those supplements in this category which can reproduce the growth-enhancing and other effects gained previously from antibiotics (4, 7, 8, 14). The addition of herbs to feeds has a beneficial effect not only on production indices, but also on the quality of meat products, including their dietetic and taste properties (5, 11, 17).

Multicomponent herbal supplements are alternatives to antibiotics in practical poultry nutrition. Many studies have demonstrated the antibacterial activity of oregano (*Origanum vulgare*), black pepper (*Piper nigrum*), clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*) and essential oil components (thymol, carvacrol, piperine, eugenol, and curcumin) against many strains of pathogenic bacteria, including *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Yersinia enterocolitica* (6, 17, 22, 30). These observations were made in *in vitro* and *in vivo* experiments. Carvacrol and thymol also have insecticidal activity against the brown-winged cicada (*Pochazia shantungensis*), which damages fruit trees (19). Due to carvacrol's very strong antifungal, anti-insect, antiviral and antimicrobial effects and limiting action on the population of parasites responsible for gastrointestinal diseases, it has been selected as a poultry litter additive (1, 5, 6, 11, 17, 19, 24).

Alternative feed additives such as essential oils can be added to the litter. An example of such a litter additive would be eucalyptus leaves added to litter as a potential natural herbicide (22). In this case, a litter additive that contained carvacrol was prepared using plasticisers and

potato starch. Potato starch was used because it is a substance free of smell and taste, which is particularly important when studying essential oils (13, 18). Among the most frequently used plasticisers are glycerine, sorbitol, water, ethylene glycol, urea, formamide and acetamide (2, 10, 19, 23, 33). According to our earlier experience, the best results were achieved with propane-1,2,3-triol (2). Additionally, water was used as a starch modifier because it limits the upper processing temperature (27). Our earlier study also proved that 5% carvacrol is enough to protect chickens from insects such as the lesser mealworm, *Alphitobius diaperinus* Panzer (24).

A litter additive prepared in this way should also improve productivity and meat quality just as a herb additive in feed does (11, 17, 21). When in litter, essential oils enter the animal's body through the respiratory system. To assess whether the addition of carvacrol could improve the efficiency of nutrition and the health status and condition of animals, an experiment was designed in which weight gain was compared in groups reared on litter with carvacrol and without carvacrol. The aim of this study was to evaluate the effect of the addition of carvacrol to litter on weight gain and evaluate the occurrence of residues in chicken tissues. A method for determining carvacrol in plasma, lung, muscles, and liver tissues was used to detect possible residues (25).

Material and Methods

Reagents. Ultrapure water was filtered through a Millipore Milli-Q system (Burlington, MA, USA). Methanol, isopropanol, 1-butanol, anhydrous magnesium sulphate ($MgSO_4$), carvacrol (≥99%), and fipronil-$13C_4$ (≥98%) as an internal standard were obtained from Sigma Aldrich (St. Louis, MO, USA). Pre-heated magnesium sulphate ($MgSO_4$) was prepared in the laboratory for heating at 400°C overnight. Primary secondary amine and octadecylsilane sorbent (C18) were purchased from Supelco (Bellefonte, PA, USA). Acetic acid was procured from Avantor Performance Materials Poland (Gliwice, Poland). Nanosep MF 0.22 µm filters were supplied by Pall (DeLand, FL, USA). All reagents were of analytical grade or higher. DKA Broiler 1 and Broiler Finisher feed were obtained from Agropol Motycz (Motycz, Poland).

Individual stock standard solutions of carvacrol and fipronil-$13C_4$ as internal standard solutions at a concentration of 1 mg mL^{-1} were prepared in methanol and stored in the dark at <−18°C for no longer than six months. The working standard and internal standard solutions at a concentration of 0.01 mg mL^{-1} were prepared in methanol and stored in the dark at <6°C for no longer than three months.

Experimental procedure. The Local Ethics Committee for Animal Experimentation in Lublin gave permission to perform the tests under Resolution No. 84/2018 of 2018.07.02. The experiment was carried out in the National Veterinary Research Institute in Puławy, Poland (NVRI), in two identical rooms of approximately 9 m^2 that met the relevant criteria for experimental poultry housing.

One-day-old Ross 308 chickens were randomly selected for the study and divided into two experimental groups. The broiler chickens had an average body weight of 45 ± 3 g and were not sexed. Group 1 (n = 8) was kept in a room with litter enriched with a carvacrol-containing preparation (1 kg of starch granules enriched with carvacrol preparation and 1.5 kg of litter per m^2 of the room area). Group 2 (n = 6) was kept in a room with litter without the addition of carvacrol (2.5 kg of litter per m^2 of the room area). Fresh litter was added to both rooms once a week (up to 10% of the initial value in both groups).

The chickens had access to water, and antibiotic-free feed *ad libitum*. The rooms were air-conditioned and their temperature was gradually decreased from 33°C on the first day to 23°C on the 42nd day of the experiment. Humidity was regulated and a light/dark cycle was maintained for all experiments. Initially, the chickens were fed with DKA Broiler 1 feed (for broiler chickens from 1 day to 5 weeks of age) and this continued until the final week, when the feed was changed to Broiler Finisher. During the course of the experiment, the chickens were weighed every seven days at the same time of the day. After 42 days of the experiment, the birds were euthanised by decapitation after sedation with isoflurane and subjected to necropsy, during which the appearance and consistency of the internal organs and changes in them were assessed. Tissue samples were collected after 42 days of experiments. Samples of liver, lungs, muscles, and plasma were collected in plastic containers and stored at −80°C until further analysis.

Sample preparation. A 5 ± 0.05 g portion of plasma and homogenised lung, muscle and liver was mixed with 100 µL of internal standard (fipronil-$13C_4$) at the level of 5.0 µg g^{-1} for 15 min on a Stuart STR4 General Rotator from Cole-Parmer (Vernon Hills, IL, USA) at minimum rotation speed (6 rpm) (0.05 × rcf) with 5 mL of 1-butanol and 20 mL of water, then centrifuged at 2,930 × rcf for 10 min at approximately 0°C. A 0.5 mL volume from the top layer was transferred, then 60 mg of octadecylsilane sorbent, 30 mg of primary and secondary amine, and 200 mg of $MgSO_4$ were added. The extract was mixed and centrifuged at 2,930 × rcf for 10 min at approximately 0°C. A 0.3 mL volume of the top layer was filtered and then transferred to an autosampler vial for analysis by high-performance liquid chromatography–tandem mass spectrometry (25). The injection volume was 10 µL.

High-performance liquid chromatography–tandem mass spectrometry. An ExionLC ultra-high performance liquid chromatography system was connected to an API 5500 Qtrap mass spectrometer (both products of AB Sciex, Concord, ON, Canada). The mass spectrometer was operated in the negative electrospray ionisation (ESI) mode with a capillary voltage of −4.5 kV. The flow rate of the mobile phase was 600 µL min^{-1} and the injection volume was 10 µL.

Chromatography was performed in a Kinetex XB-C18 column (50 mm × 2.1 mm × 2.6 μm) connected to a C18 precolumn of 4 mm × 2 mm × 4 μm (Phenomenex, Torrance, CA, USA). The mobile phase for liquid chromatography analysis consisted of two solutions: A was 0.5% isopropanol in 0.1% acetic acid in water and B was methanol. The mobile phase gradient programme started at 20% B, progressed to 95% B from 3 min to 6 min, then was decreased to 20% B at 6.2 min and was held for 2.8 min. The column was equilibrated for 2 min. The column operated at 35°C and the ions were monitored in the multiple reaction monitoring (MRM) mode (25).

Litter preparation. Native potato starch (Wielkopolskie Przedsiębiorstwo Przemysłu Ziemniaczanego, Luboń, Poland) was used in the study. The plasticiser was propane-1,2,3-triol (Chempur, Piekary Śląskie, Poland) with a density of 1.26 g ml^{-1}. Calcium carbonate was added as an extender (Piotrowice II, Tarnobrzeg, Poland). The carvacrol had a density of 0.976 g ml^{-1}.

Preparation of starch granules enriched with carvacrol. Potato starch powder, propane-1,2,3-triol (28%), 2% calcium carbonate and 5% carvacrol were introduced into a planetary mixer (HBI Rheocord 90 system; Haake Buchler Instruments, Dallas, TX, USA). All ingredients were mixed with a stirrer speed of 60–100 rpm for 9 ± 2 min. The pellets were extruded using a single-screw extruder PLV 151 Plasti-Corder (Brabender, Duisburg, Germany). The screw parameters were 25D working length and 3:1 compression ratio. The temperature profile along the cylinder was 70, 80, 90 and 95°C. The experimental stand was equipped with a device for measuring the temperature of the heating zone of the plasticising system and the head. The screw rotation speed was 100 rpm.

Validation. The method was developed according to the International Council for Harmonisation of Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) methodology and following previously described methods (9, 12, 28, 29). The following validation parameters were estimated: selectivity, limit of detection (LOD), limit of quantification (LOQ), working range, repeatability, reproducibility, matrix effect and uncertainty of the method. The matrix effect was calculated for each matrix at the second concentration level (1.0 μg g^{-1}) using the same method as previously described (26). In the selectivity study, possible interferences encountered in the method were assessed by analysing 20 blank samples for each matrix from different sources, and no interferences were found. The analyte standard solutions at 0.2, 1.0, 5.0, 20.0 and 50.0 μg g^{-1} concentrations were added to the blank sample containing an internal standard (5.0 μg g^{-1}) and then subjected to the QuEChERS extraction and the HPLC-MS^2 procedure. The LOD and LOQ were estimated by calculations based on the signal-to-noise ratio, which was determined by comparing the measured signals from samples with known low concentrations of analyte with those from blank samples and establishing the minimum concentration at which the analyte could be reliably detected or quantified. A typical signal-to-noise ratio is 3:1 for LOD and 10:1 for LOQ (9, 12, 28, 29). Spiked blank samples were prepared as follows: standard solutions concentrations of 0.2, 1.0, 5.0, 20.0 and 50.0 μg g^{-1} and internal standard (fipronil–$13C_4$) solutions at 5.0 μg g^{-1} were added to 5.0 g of sample. These spiked blank samples were analysed according to the previously described procedure. Repeatability and reproducibility were determined at the same five concentration levels of 0.2, 1.0, 5.0, 20.0 and 50.0 μg g^{-1} for six samples at each level. The samples were analysed by the same operators on the same day with the same instrument, and this made for a valid repeatability parameter calculated as the relative standard deviation (RSD, %). For within-laboratory reproducibility, another two sets of blank samples were fortified and analysed by different operators on two different days with the same instrument, and reproducibility was also calculated as the RSD (%). Recovery was calculated by comparing the mean measured concentration with the fortified concentration of the samples. Any matrix effect was assessed by analysing five different samples at the second concentration level (1.0 μg g^{-1}) applying an equation proposed previously (15). The expanded uncertainty was calculated at the second concentration level (1.0 μg g^{-1}) by applying a coverage factor of 2, which gave a level of confidence of approximately 95% (32).

Statistical analysis. Prior to analysis, the Grubbs's test was used to find a single outlier in a normally distributed data set, and the Shapiro–Wilk normality test available as an online calculator (https://www.statskingdom.com/shapiro-wilk-test-calculator.html) was used to verify the data distribution. Because the data were normally distributed, the Student's *t*-test was used to verify differences in average weight between the control group and the group with carvacrol. All calculations were performed using Microsoft Excel 2016.

Results

The presented procedure is selective and able to detect carvacrol in various matrices such as muscle, lung, plasma and liver tissue. All required validation parameters, namely repeatability, reproducibility, LOD, LOQ, working range, recovery matrix effect and uncertainty of the method were evaluated. The limit of detection was calculated at 0.06 μg g^{-1} and the limit of quantification was 0.2 μg g^{-1} for all matrices, with relative standard deviation repeatability and reproducibility below <10%. The determination coefficient was above 0.98 for all matrices and the working range was 0.2–50.0 μg g^{-1}. The recoveries calculated based on matrix-matched calibration curves were in the range of 98.9–107.7%. The expanded uncertainty did not exceed 34% (25).

During the course of the experiment, the chickens were weighed every seven days and the weighing results are summarised in Table 1. Weekly weighing results showed that the control group and chickens exposed to carvacrol-containing litter were similar.

Table 1. Chicken body weights

Day of experiment	Average control group weight (g)	SD (g)	Average carvacrol group weight (g)	SD (g)	P-value*
1	44	±1	47	±1	<0.621
7	167	±3	154	±2	<0.756
14	388	±29	366	±35	<0.546
21	710	±70	692	±64	<0.865
28	1,103	±132	1,125	±137	<0.921
35	1,487	±196	1,561	±203	<0.9385
42	1,953	±260	2,053	±232	<0.759

SD – standard deviation; * – number of degrees of freedom = 6 ($P < 0.005$)

During necropsy examination, samples of lung, muscle and liver tissue and blood (from which plasma was separated) were collected. Carvacrol content determinations were performed on these materials. Carvacrol was not found in the control group's samples at concentrations above the LOD of the method used (0.06 μg g^{-1}). The mean carvacrol content determined in the samples taken from birds of the experimental group is presented in Table 2. The lowest concentration of carvacrol was obtained for plasma (0.57 μg g^{-1}), while the highest was for lung tissue (1.78 μg g^{-1}).

Table 2. Mean concentrations of carvacrol residues in chicken tissues (μg g^{-1})

Matrix	Concentration (μg g^{-1})	SD (μg g^{-1})
Plasma	0.57	±0.26
Muscle	0.64	±0.24
Liver	1.39	±0.21
Lung	1.78	±0.29

SD – standard deviation

Discussion

Due to restrictions on the use of antibiotics in animal husbandry (4, 8, 14), there has been considerable interest in the use of herbal supplements or substances contained in these supplements such as essential oils. Therefore, it is necessary to analyse the residues of these substances and to estimate their effects on the animal organism. Carvacrol is an essential oil which has very strong antifungal, antiviral and antimicrobial effects and can reduce the population of parasites responsible for gastrointestinal diseases (1, 5, 6, 11, 17, 19, 22, 30). Our previous studies also showed that carvacrol significantly impaired the growth and development of *Alphitobius diaperinus* larvae and is toxic to this pest (24). Because of carvacrol's properties, it was selected as a litter additive to assess whether the addition of it may have a beneficial impact on the efficiency of nutrition and on the health status and condition of animals. Investigation of the presence of carvacrol residues in chicken tissues is also relevant in regard to food safety. For this reason, an experiment was conducted to test the effect of carvacrol on animal weight gain and to analyse the occurrence of carvacrol residues in plasma, muscle, liver, and lung tissue. Based on previous experiments (24), natural conditions similar to those prevailing on the farm were prepared in this case. The enclosure size was increased to 9 m^2 and the number of animals was reduced. Furthermore, fresh litter was added to the enclosures once a week (up to 10% of the initial value in both groups). We used a 5% addition of carvacrol, which is sufficient to protect chickens from insects like the lesser mealworm, *Alphitobius diaperinus* Panzer (24). Weekly weighing results showed that chickens exposed to carvacrol-containing litter had slightly higher body weight (Table 1), but the Student's *t*-test statistical analysis did not indicate that these differences were significant, which was also confirmed in our previous study (24). During this study, liver fragility was observed in the group of birds exposed to carvacrol. It cannot be ruled out that this change was caused by carvacrol, because a similar fragility was not observed in the control group.

For the determination of carvacrol residues in tissues, a method was developed according to the recommendations of ICH Q2 (R1) (12). All required validation parameters, namely repeatability, reproducibility, LOD, LOQ, working range, recovery matrix effect and uncertainty of the method, were evaluated. The analysis of residues in plasma, muscle, liver, and lung tissue after 42 days of exposure to carvacrol in litter clearly indicated that the animals were in contact with the test substance. When analysing the concentrations in individual tissues, it could be stated that the contact occurred through the lungs, because the highest concentrations of carvacrol residue was in this tissue (Table 2). To the best of our knowledge, this is the first attempt to determine carvacrol residues in poultry tissue after exposure to the substance contained in litter, and these are the first data to confirm such residues after such exposure. Unfortunately, whereas other substances have defined limits for their residues in muscle, liver, plasma or lung tissue, there is no such limit yet established for carvacrol in these matrices. The only information on experiments conducted with carvacrol in chickens is that feeding oregano oil to broilers increases accumulation of this substance in muscles (*i.e.* in breast meat) (1).

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Animal Rights Statement: The experimental protocols involving animals were approved by the Local Ethics Committee for Animal Experimentation in Lublin (Resolution No. 84/2018 of 2018.07.02).

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