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Evaluating different bone processing methods used in assessing mineralisation in broilers

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Short title: Processing affects ash content of broiler bones

SUMMARY

The study determined the effect of bone processing method on broiler tibia ash content. First, a total of 288-day-old, Ross 308 male broilers were fed from hatch for 35 days on one of 6 diets differing in P level (8 pens of 6 chicks /treatment). Tibias of 2 birds/pen were used to determine the effect of Soxhlet fat extraction (left tibias) and cartilage caps exclusion (right tibias) on ash content (trial 1). Secondly, to examine the effect of duration of Soxhlet extraction on fat extraction efficiency, 264-day-old Ross 308 male broilers were allocated to 33 pens with 8 birds each (trial 2) and all birds were fed a standard broiler diet. On day 42 of trial 2, one bird per pen was sampled and right tibia bones were collected. In a final trial, 384 one-day Hubbard JA787 male chicks were distributed to three treatments differing in energy and protein density (16 pens/treatment and 8 birds/pen) (trial 3). At D42 of trial 3, 2 birds/pen were sampled, and tibias were used to determine the effect of autoclaving before flesh removal on ash content. The statistical residuals were used to express the accuracy of ash measurement. Autoclaving and cartilage cap inclusion significantly increased ash content. Fat extraction significantly improved the accuracy of ash determination. The fat extracted from tibias was significantly increasing up to 6 hours only. Cartilage inclusion and autoclaving did not significantly alter ash determination accuracy. Autoclaving to ease flesh removal followed by a 5.76-hour fat extraction is recommended before bone ashing for ash determination. Details of bone processing before ash determination should be stated to allow for comparison of the results of unrelated studies.

Key words

Tibia; ashing; fat extraction; cartilage caps; autoclaving

DESCRIPTION OF PROBLEM

Lameness in broiler was reported to be a major issue in poultry industry affecting negatively both welfare and growth performance. Broilers with lameness suffer from severe pain (Danbury et al., 2000; McGeown et al., 1999) which could compromise locomotion leading to a considerable reduction in the ability of birds to reach feed and water. Furthermore, lameness in poultry could increase culling rate. It has been reported that 27% of birds lame and 3% are unable to walk at the age of 40 days (Knowles et al., 2008).

Bone ash content has been widely used to evaluate the skeletal status of poultry (Kim et al., 2008) and is the preferred criterion for estimating phosphorus availability due to its simplicity (Hall et al., 2003). The ash content of various bones that have been used to evaluate bone mineralisation in poultry include toe (Karimi et al., 2013), feet (Garcia and Dale, 2006), tibia (Olukosi and Fru-Nji, 2014), and femur (Dickey et al., 2012). Nonetheless, tibia ash is the most common bone used in evaluating bone mineralisation in poultry research (Hall et al., 2003); as tibia ash shows sensitivity to dietary mineral levels in broilers of all ages (Scholey and Burton, 2017).

A range of different bone processing methods prior to ash determination have been used in poultry studies. Removing all adhering flesh from the bones may be performed manually using enzymatic maceration techniques (Shastak et al., 2012), autoclaving (Boling-Frankenbach et al., 2001; Kim et al., 2008), or by boiling (Ruiz-Feria et al., 2014).

Fat extraction before ash determination of the bone is a well cited process. This includes soaking in diethyl alcohol for 48 hours (Perney et al., 1993), a 2-phase fat extraction process with ethyl alcohol for 36 hours followed by diethyl ether for 36 hours (Sun et al., 2013) or 48 hours (Hamdi et al., 2015), 2-phase extraction process with ethanol for 48h followed by ether for 48 hours (Payne et al., 2005), and Soxhlet method (6 hours (Olukosi and Fru-Nji, 2014); 16 hours (Li et al., 2016)).

Retaining cartilage caps intact might affect ash content of broiler bones as they contain variable amount of ash. Furthermore, some diseases were reported to alter bone ash content of broilers. Bacterial chondronecrosis with osteomyelitis increases femurs and tibia ash content of broilers (Thorp and Waddington, 1997).

Variations in bone processing methods in studies may affect ash content results (Orban et al., 1993) and therefore make it difficult comparing bone ash methods from different studies. Difficulties in comparing across studies are exacerbated by many of the studies which include bone ash determination failing to report sufficient details about bone processing in ash determination method.

Therefore, the main goal of this study is to identify the effect of processing method (fat extraction, inclusion of cartilage caps, autoclaving prior to fat extraction, fat extraction time) on ash content of broiler tibia.

MATERIALS AND METHODS

Ash determination is a destructive analysis. Thus, three trials were used to collect a sufficient number of tibia bones. The three feeding trials were carried out in Nottingham Trent University poultry research unit. All birds were raised in-house in a thermostatically controlled room and provided with an initial room temperature of 32°C which was gradually reduced to 21°C by day 21 and maintained till the end of the experiment. Lighting was provided with 1h darkness from day 1 and was increased by 1h a day to 6h, then maintained until the end of the experiment. Wood shavings were spread on the pen floors (approximately 3cm thick) and was topped up as required during each trial. The experimental diets and water were provided *ad libitum*, and birds were checked twice daily to monitor birds and environmental conditions. Mortalities were recorded along with the date and weight of the bird and reason if culled. Institutional and UK national NC3R ARRIVE guidelines for the

care, use and reporting of animals in research (Kilkenny et al., 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee. Ash content of all bones in the current study was determined for each bone by burning at 650 ^{o}C for 24h in a muffle furnace.

The effect of fat extraction: Trial 1

Right and left tibia bones of this trial was obtained from a previously reported phosphorus availability study (Scholey et al., 2018). Briefly, 288-day-old Ross 308 male broiler chicks were fed 6 diets with different P levels (8 pens/treatment) for 35 days. On day 35, 2 birds/pen were euthanised by cervical dislocation and tibia bones were removed and individually stored at -20 ^{o}C until further processing. Details about the experimental diets and study procedures are presented in (Scholey et al., 2018). Right tibia bones (n = 96) were allowed to thaw at room temperature and the adhering flesh was removed manually while keeping bone cartilage cap intact. Afterwards, the bones were individually dried, fat extracted in petroleum ether using (in labelled thimbles) the Soxhlet extraction method (CAS 64742-49-0, Fisher Scientific, UK; Boiling point 40 - 60 °C (1013 hPa); density 0.645 - 0.665 g/cm^3 (15 °C), viscosity kinematic 0.45 mm^2/s (20 °C); solubility 0.01 g/l practically insoluble) for eight hours and then dried until constant weight was achieved.

These tibias were individually ashed and their ash content was expressed as a percentage of fat extracted bone weight. Fat was not extracted from the corresponding left tibia bones (n = 96) but tibias were dried until constant weight was achieved. The left tibias were individually ashed and ash content of this group of bones was expressed as a percentage of dry fat-unextracted bone weight.

Effect of fat extraction time: Trial 2

A total 264-day-old Ross 308 male broiler chicks were allocated to 33 replicate pens with 8 birds each. The birds were fed *al libitum* on standard broiler diets for 42 days (Table 1). On day 42, one bird per pen was euthanised by cervical dislocation and right and left tibia bones were removed and individually stored at -20 ^{o}C until further processing. All tibia bones were completely thawed at room temperature before all adhering tissues were manually excised using laboratory scalpels, whilst ensuring cartilage caps were kept intact. The right tibia bones were individually dried at 105 ^{o}C for 24h prior to 1h of fat extraction in petroleum ether using the Soxhlet extraction method to determine the amount of fat extracted. All bones were individually fat-extracted in labled thimbles. This process was cumulatively repeated until 8 hours of total extraction time was achieved. The additional hourly fat extracted was determined to evaluate fat extraction efficiency.

Effect of autoclaving: Trial 3

A total of 384 one-day Hubbard JA787 male chicks were distributed equally to three treatments (16 pens/treatment and 8 birds/pen), high density (crude protein (metabolizable energy): 23% (12.5 MJ/kg), 20.4% (13 MJ/kg), 19.1% (13.7 MJ/kg) for starter grower and finisher diets, respectively), medium density (22.3% (12.2 MJ/kg), 19.7% (12.6 MJ/kg), 18.2% (13 MJ/kg) for starter grower and finisher diets, respectively) and low density (21.6% (11.9MJ/kg), 19 (12.1 MJ/kg), 17.3% (12.4 MJ/kg) for starter grower and finisher diets, respectively). At D42, 2 birds/pen were euthanised by cervical dislocation and all tibias were removed and individually stored at -20 ^{o}C until further processing. Detailed description of the experiment is published by (Alkhtib et al., 2023). All bones were thawed at room temperature before flesh removal. The flesh of the right tibias was removed without autoclaving. The left tibia bones were placed in labled trays then autoclaved for 15 minutes ((Boxer Laboratory

Equipment, UK, 121 °C and 15 bars) and the adhering flesh was removed. The bone cartilage was kept intact in all bones. The clean right and left tibia bones were then fat extracted for 8 hours using the Soxhlet extraction method, dried until constant weight was achieved then ash content of each bone was determined. Ash content of these tibias was expressed as a percentage of fat extracted bone weight.

Effect of cartilage removal

The fat extracted right tibias in trial 1 were randomly divided into two equal groups, cartilage caps removed vs. cartilage caps retained. None of the bones were autoclaved before removing the adhering tissues. Ash content of these tibias was determined for each bone and expressed as a percentage of fat extracted bone weight.

Calculations and Statistical Analysis

The bone was considered as an experimental unit in all trials. Each data set in the current study was analysed separately. The effect of fat extraction on tibia ash content was analysed using ANOVA according to the following model:

$$Y_{(ijk)} = Mean + Pen_{(i)} + TRT_{(j)} + ETH_{(k)} + TRT \times ETH + E_{(ijk)}$$

Where $Y_{(ijk)}$ is the dependent variable, Mean is the overall mean, $Pen_{(i)}$ is the effect of the pen, $TRT_{(j)}$ is the effect of the dietary treatment, $ETH_{(k)}$ is the effect of ether extraction, $TRT \times ETH_{(jk)}$ is the interaction between the dietary treatment and ether extraction and $E_{(ijk)}$ is the residual. Ash content means of fat extracted and dry tibia were not compared because no ash is removed from tibias during the process of fat extraction.

The effect of duration of fat extraction on the efficiency of extraction was analysed using one-way ANOVA. The effect of autoclaving prior to fat extraction of tibia and the dietary treatment on ash content was analysed according to the following model:

$$Y_{(ijk)} = Mean + Pen_{(i)} + AUT_{(j)} + TRT_{(k)} + E_{(ijk)}$$

Where $Y_{(ij)}$ is the dependent variable, Mean is the overall mean, $Pen_{(i)}$ is the effect of the pen, $TRT_{(j)}$ is the effect of the dietary treatment, $AUT_{(j)}$ is the effect of autoclaving, $TRT_{(k)}$ is the effect of the dietary treatment and $E_{(ijk)}$ is the residual.

The effect of cartilage cap removal on ash content of tibias was analysed according to the following model:

$$Y_{(ijk)} = Mean + Pen_{(i)} + TRT_{(j)} + CART_{(k)} + TRT \times CART_{(jk)} + E_{(ijk)}$$

Where $Y_{(ijk)}$ is the dependent variable, Mean is the overall mean, $Pen_{(i)}$ is the effect of the pen, $TRT_{(j)}$ is the effect of the dietary treatment, $CART_{(k)}$ is the effect of cartilage cap removal, $TRT \times CART_{(jk)}$ is the interaction between the dietary treatment and cartilage cap removal and $E_{(ijk)}$ is the residual.

Wherever analysis of variance was applied, means were separated using Fisher's Least Significant Difference. Analysis of variance models were used to calculate the predicted ash content of tibia and the residuals of each pen in each data according to the following equation:

$$Residual\ (\%) = 100 \times \frac{(Oi - Pi)}{Oi}$$

Where RE is the residual, Oi is the observed (measured) ash content and Pi is the ash content predicted using ANOVA models. The RE of pens in each data set was analysed according to the corresponding ANOVA model. All data analyses were done using R (R core Team, 2017).

RESULTS AND DISCUSSION

To properly evaluate the effect of optimised dietary phosphorus content on skeletal integrity, it is important to quantify bone mineralisation and accurately compare data which may be derived from unrelated studies. However, the lack of a standardised procedure for evaluating bone mineralisation, which leads to differences in results, as previously highlighted by Orban et al. (1993), remains a pertinent issue in current poultry research. This hampers meaningful comparison of bone ash data particularly from unrelated studies where different processing methods are employed and prevents industry practitioners from using published data as a reference standard for commercially reared birds.

The need for standardising the methodology of determining ash content of bones has been recognised for many decades. Bethke and Record (1934) observed different variations in the bone processing methodology in poultry e.g. use of different fat extraction solvents, different drying temperature, inclusion or exclusion of cartilage caps and determining bone ash % either on air dry or moisture free basis. These variations affect the result obtained (Orban et al., 1993). It is therefore important to examine which bone processing method best reflect sensitivity to changes in bone mineralisation to improve accuracy when comparing bone mineralisation data, especially from unrelated studies.

Fat Extraction

The fat extraction procedure is a well-cited methodology routinely employed prior to bone ash determination (Driver et al., 2006; Waldroup et al., 2000). However, this process requires substantial use of organic solvents and laboratory processing time, which raises the question of whether fat extraction is an essential step in the methodology of bone ash determination. The results of the current study showed that ether extraction of tibia before ashing significantly decreased RE of ash content by 38% (from 7.95% to 5.1%) (Table 2). This means the accuracy of ash determination of broiler bone was substantially improved by ether extraction. Our results are in disagreement with Garcia and Dale (2006) who analysed bones from 2-week old broilers and reported that sensitivity of ash content in detecting dietary differences was not improved by fat extraction. This could be due to low fat content of the bones in 2-week old broilers used in Garcia and Dale (2006). Yan et al. (2005) reported that the accuracy of determination of broiler tibia ash was not affected by ether extraction at 21 days of age. In both Garcia and Dale (2006) and Yan et al. (2005), the accuracy of tibia ash determination of fat extracted and unextracted bones was based on the ability of ash content to separate the dietary treatments which leads to high level of speculation. The accuracy of an ANOVA model is expressed by determining the deviation between the observed data and the model-based predicted data which was used in our study (statistical residuals). This will clearly reflect the individual variability among the birds in bone ash content. Mineralisation data obtained from unextracted fat bone might be misleading where lipid metabolism is compromised by factors like aflatoxicosis (Huff, 1980). In many laboratories, ashing large numbers of high-fat samples produces large amounts of CO2 which raises safety issues. Accordingly, fat extraction would increase the efficiency laboratories in bone mineralisation and decrease hazards related to the high release of CO2. Thus, determination tibia ash in

broilers should include fat extraction to increase the accuracy and the efficiency of bone mineralisation assessment.

The Effect of Fat Extraction Time

A number of different methods of fat extraction from bones before ash determination have been previously reported. These methods include soaking in diethyl alcohol for 48 hours (Perney et al., 1993), a 2-phase fat extraction process with ethyl alcohol for 36 hours followed by diethyl ether for 36 hours (Sun et al., 2013) or 48 hours (Hamdi et al., 2015), 2-phase extraction process with ethanol for 48h followed by ether for 48 hours (Payne et al., 2005), and Soxhlet method (6 hours (Olukosi and Fru-Nji, 2014); 16 hours (Li et al., 2016)). Although it is long established that the type of organic solvent does not affect bone ash proportion (Bethke and Record, 1934), no studies to date have reported on the minimum time required to extract fat from bones.

The cumulative fat extracted from tibia bones was significantly different up to 6 hours, beyond which no significant differences were found (Table 3). Cubic model had the highest R^2 compared to both linear and quadratic models. Therefore, cubic model is the best model to explain the relationship between extraction time and the cumulative fat extracted. The maxima of the cubic model in the current study was found to be 5.76 hours (which is, in other words, 5 h and 46 minutes). This suggests 5 hours 46 minutes is adequate to extract fat from bones using petroleum ether. This reduction would reduce processing time, cost and negative environmental impact of bone ash determination in broilers.

Autoclaving Prior to Fat Extraction

The manual removal of flesh from the bones is laborious and time-consuming. Autoclaving tibia bones prior to ash determination facilitates flesh removal by softening the adhering tissues

(Boling-Frankenbach et al., 2001; Hall et al., 2003; Kim et al., 2008). However, autoclaving might compromise bone content of ash. Autoclaving bones prior to fat extraction has significant effect on ash proportion in 42-day old broilers (P= 0.002) (Table 4). Autoclaving increased tibia ash content by 3% compared to the non-autoclaved tibia. Therefore, autoclaving broiler bones before ashing would compromise the comparison of bone ash content of unrelated studies. Although, not autoclaving the bones provides an opportunity to capture other bone measurements, e.g. bone histology and strength which will add value to understanding bone mineralisation data. Autoclaving the tibia did not have significant effect on RE. Accordingly, autoclaving did not compromise the accuracy of tibia ash determination.

Retaining Cartilage Caps

Retaining tibia cartilage caps did not significantly affect RE of bone ash content (Table 5). Therefore, retaining cartilage caps would not alter the accuracy of tibia ash determination of broilers. However, retaining the cartilage cap of tibia decreased ash content of tibia by ~22%. Therefore, the results of unrelated studies where cartilage caps condition (intact or removed) is not clearly documented make a limited contribution to the discipline beyond use in the controlled study where the data are reported. Although the accuracy of tibia ash determination dose not decline by retaining or removing cartilage caps, it is important to state details on cartilage caps status in bone mineralisation studies to allow for results comparison among studies.

CONCLUSIONS AND APPLICATIONS

1- Adopting a standard bone processing method would enable accurate comparisons of results from unrelated studies.

2- A reliable, fast and cheap method includes autoclaving to facilitate soft tissue removal followed by 5.76 hours fat extraction as a step before ash determination in broiler tibias.

3- Bone cartilage inclusion or removal and autoclaving prior to flesh removal in ash determination did not change the accuracy. However, they affected ash bone content.

4- It is essential to report bone processing method and whether cartilage caps are intact or not to enable a robust contrast of results from unrelated studies.

DISCLOSURES

The authors declare no conflicts of interest.

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Table 1. Feed composition of experimental diets (g/kg as fed basis), Trial 2

Ingredient	Starter (D1 - D14)	Grower (D15 - D28)	Finisher (D29 - D42)
Wheat	585	612	695
Soybean meal 48	352	318	240
Soy oil	15.9	31.7	28.7
Salt	4.3	4.0	4.2
DL-Methionine	3.3	2.0	1.3
Lysine HCl	2.5	0.5	0.3
Threonine	0.9		
Limestone	13.3	11.2	10.3
Dicalcium phosphate	18.0	15.3	15.3
Vitamin and trace mineral premix[1]	4	4	4
Nutritional composition			
Dry matter (g/kg)	881	881	881
Ash (g/kg)	51.1	44.3	4.1
Protein (g/kg)	250	232	204
Fat (g/kg)	61.4	73.4	60.9
Ca (g/kg)	12.85	11.69	8.75
P (g/kg)	6.7	5.59	4.32
Gross energy (MJ/kg)1	18.61	19.02	20.3

1

Vitamin premix : Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg.

Table 2. The effect of fat extraction on tibia ash content and the statistical residuals of broilers (trial1).

	Ash (%)	Residuals (%)
Ether no extracted[1]	28.7	5.2
Ether extracted[2]	44.4	3.23
SEM[3]	0.266	0.513
P value		
Treatment	<0.001	0.216
Method	<0.001	<0.001
Treatment*method	0.077	0.731

1 % of dry tibia weight.

2 % of fat extracted tibia weight.

3 Standard error mean.

Table 3. The effect of increasing fat extraction time on fat extraction efficiency (trial 2).

Extraction time (hour)	Cumulative fat extracted (%)
1	5.72^{f}
2	9.39^{e}
3	11.6^{d}
4	12.9^{c}
5	13.5^{ab}
6	13.9^{ab}
7	14.2^{a}
8	14.5^{a}
SEM[1]	0.243
P value	
Anova	<0.001
Linear ($y = 1.1073x + 6.9811$; $R^2 = 0.803$)	<0.001
Quadratic ($y = -0.2582x^2 + 3.4307x + 3.1087$; $R^2 = 0.977$)	<0.001
Cubic ($y = 0.0493x^3 - 0.9233x^2 + 5.9679x + 0.67$; $R^2 = 0.999$	<0.001

a-f

Means within the same column with different subscripts differ significantly ($P \leq 0.05$).

1

Standard error mean.

Table 4. The effect of autoclaving tibia bone before fat extraction on ash content and the statistical residuals of broiler tibia (trial 3).

	No autoclaving	Autoclaving	SEM[2]	*P* value
Tibia ash (%)[1]	37.8	40.8	0.655	0.002
Residuals (%)	6.21	4.47	1.88	0.514

1

Ash content of these tibias was expressed based on fat extracted bone weight.

2

Standard error mean.

Table 5. The effect of cartilage removal on tibia ash content and the statistical residual of broilers (trial 1)[1].

	Cartilage cap excluded	Cartilage cap included	SEM[2]
Tibia ash (%)	36.3	44.4	0.283
P value			
Method	<0.001		
Treatment	<0.001		
Method*treatment	0.918		
Residual (%)	1.89	1.53	0.148
P value			
Method	0.09		
Treatment	0.236		
Method*treatment	0.86		

1

Ash content of these tibias was expressed based on fat extracted bone weight.

2

Standard error mean.