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Traceability of Animal Byproducts in Quail (*Coturnix coturnix japonica*) Tissues using Carbon ($^{13}C/^{12}C$) and Nitrogen ($^{15}N/^{14}N$) Stable Isotopes

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ABSTRACT

Consistent information on meat products consumed by the public is essential. The technique of stable isotopes is a powerful tool to recover consumers' confidence, as it allows the detection of animal byproduct residues in poultry meat, particularly in quail meat. This study aimed at checking the presence of poultry byproduct mixtures in quail diets by applying the technique of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) stable isotopes in quail breast muscle, keel, and tibia. Sixty four one-day-old male quails were obtained from a commercial farm. Birds were housed in an experimental house from one to 42 days of age, and were randomly distributed into 8 experimental treatments, and fed diets containing poultry offal meal (POM), bovine meat and bone meal (MBM) or poultry feather meal (PFM), or their mixtures. Four birds per treatment were slaughtered at 42 days of age, and breast (*Pectoralis major*), keel, and tibia were collected for analyses. The inclusion of animal byproducts in quail diets was detected by ^{13}C e ^{15}N analyses in the tissues of the birds; however, it was not possible to specify which byproducts were used. It was concluded that quail meat can be certified by the technique of stable isotopes.

INTRODUCTION

The landmark for changes in the knowledge of feeding livestock used for human consumption was the emergence of Bovine Spongiform Encephalopathy or "mad cow disease" in the Europe, USA, Japan and Canada. Consumers in these countries took a new attitude, demanding high quality animal proteins with proven health safety.

According to Peupert (2003), quality and safety are influenced throughout the production chain. The improvement of the traceability process of agricultural products is an essential element of quality management systems in the food industry. This trend is observed in several agribusiness industries, but it is more evident in the meat sector, where incidents related to zoonoses decreased the confidence of consumers on the quality and safety of food products.

The survival and success of companies in a global competitive and demanding market depend on the voluntary option to use mechanisms and techniques that allow the success of traceability programs, thereby preventing sanctions from importers, particularly those from Europe.

Regulation CE n. 1774/2002 of the European Parliament and European Union Council, Consolidated Text (Consleg, 2004), Chapter 1, Article 22, determines that it is forbidden to feed an animal species with transformed animal proteins derived from bodies or body parts of animals of the same species.

Under this scenario, researchers have improved technologies to detect foods derived from animal tissues. Some of these technologies

may infer definitive inferences as to the history of these foods, while other may be used to confirm the presence of specific components (Schwägele, 2005). One of these techniques has shown to be effective to detect frauds in the food chain is the use of carbon-13 and nitrogen-15 stable isotopes.

The stable isotope technique was initially used in geological and archeological studies. However, it has been lately increasingly and continuously applied in agricultural, ecological, and physiological research as an alternative technique in studies on nutrient digestion, absorption, and metabolism and humans and animals, as well as to identify and to determine the origin of plant and animal products (Gannes *et al.*, 1998).

The isotopic ratio of the chemical element carbon has been successfully used to test the authenticity, quality, and geographical origin of several products, such as fruit juice (Bricout & Koziet, 1987; Koziet *et al.*, 1993), wine (Martin *et al.*, 1988), honey (Brookes *et al.*, 1991; Reniero *et al.*, 1997; White *et al.*, 1998), dairy products (Rossmann *et al.*, 1998; Rossmann *et al.*, 2000; Manca *et al.*, 2001), and vegetable oils (Kelly *et al.*, 1997), as well as to characterize and to differentiate Iberian pork products, allowing to classify animals according to the type of feeding offered during the finishing period (Gonzáles-Martin *et al.*, 1999).

The isotopic ratio of the chemical element nitrogen ($^{15}N/^{14}N$) allowed the certification of the geographical origin and feeds of sheep (Piasentier *et al.*, 2003), as well as to determine the feeding ecology of sharks (Domi *et a*l., 2005), quality of oysters produced in impacted areas (Piola *et al.*, 2005), and feeding studies of seals raised in captivity (Zhao *et al.*, 2006).

According to Oliveira (2005), the technique of carbon and nitrogen stable isotopes can be used as a tool to trace the inclusion of animal byproducts in broiler feed diets by analyzing the isotopic ration in the breast muscle, keel, and tibia.

Aiming at improving this technique and to produce more information on other species, the objective of the present study was to detect the inclusion of poultry visceral meal (POM), bovine bone and meat meal (MBM) and feather meal (PFM) and/or its possible mixtures in the breast muscle, keel, and tibia of 42-day-old broilers using the technique of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) stable isotopes.

MATERIAL AND METHODS

The experiment was carried out in the facilities of the Poultry Sector located at Edgárdia Experimental Farm of the School of Veterinary Medicine and Animal Science of UNESP, Botucatu campus. Sixty four one-day-old male quails derived from a commercial farm were used. Birds were housed in a rearing house measuring 15 x 4 m, covered with asbestos tiles, and provided with plastic side curtains. Eight birds were housed per cage, which measured 100 cm x 80 cm x 35 cm and were originally used for layer chick brooding. The floor of the cages was covered with newspaper sheets, on which a 1-cm black plastic screen was placed to prevent birds from fleeing and from injuring their legs. Below the cages, the floor was covered with 5-cm deep wood shavings to absorb excreta. Each cage was equipped with a 250-watts infrared lamp, suited to brood quails up to 16 days of age. There was a 0.5-L capacity chick cup drinker per cage, which water was changed twice daily. When birds were 14 days of age, the cup drinkers were replaced by trough drinkers placed in the rear of the cage. During the first seven days of age, quail chicks were fed in a tray feeder, over which a 1-cm mesh plastic screen was placed to prevent feed wastage. After 1 days of age, feed was offered in a trough feeder placed in front of the cage. Birds were offered water and feed *ad libitum* during the entire experimental period. Birds received 24 hours of light until three weeks of age by incandescent 100-watts lamps. After this period, birds were submitted to natural light.

The following experimental treatments were applied: T1, diet based on corn and soybean meal (control); T2 POM inclusion; T3 MBM inclusion; T4 POM+PFM inclusion; T5, POM+PFM+MBM inclusion; T6 POM+MBM inclusion; T7 MBM+PFM inclusion; and T8 PFM inclusion. Birds were fed the same experimental diets during the entire rearing period (one to 42 days of age). Feeds were formulated to supply quails' nutritional requirements. The same feeding program used by a commercial farm was used, i.e., starter feed from one to 21 days of age, and grower feed from 22 to 42 days of age. All experiment diets contained equal energy, protein, phosphorus, and methionine levels. The inclusion of animal byproducts in the experimental diets were based on maximal inclusion of meat and bone meal (MBM), as this ingredient presents high phosphorus content, which limits its utilization. The maximal MBM level provided 2.61% crude protein, which was then fixed in the formulation of the other diets containing one single animal byproduct and their possible mixtures. Table 2 shows percentage values of dry matter (DM%), crude protein (CP%), ether extract (EE%), mineral matter (MM%), and mean isotopic

Table 2 - Chemical analysis and mean isotopic values of corn, soybean meal (SBM), poultry offal meal (POM), poultry feather meal (PFM), and bovine meat and bone meal (MBM).

Ingredients	DM%	CP%	EE%	MM%	Mean isotopic values	
					$\delta^{13}C$	$\delta^{15}N$
Corn	87.11	8.26	3.61	1.27	-13.07	3.57
SBM	92.10	41.00	1.00	4.00	-26.58	0.43
POM	96.14	65.54	12.47	14.49	-16.28	4.30
PFM	93.26	88.18	7.92	2.25	-16.98	4.44
MBM	93.75	45.75	8.43	48.17	-12.82	7.43

values of corn, soybean meal, poultry offal meal, feather meal, and bovine meat and boné meal. Tables 3 and 4 present percentage composition, calculated nutritional levels, and mean isotopic values of the experimental feeds. Each feed ingredient belonged to the same production batch.

On day 42 of the experiment, four birds per treatment (n = 4) were chosen at random, and sacrificed by neck dislocation to collect samples of the breast muscle (*Pectoralis major*), keel, and tibia for isotopic analyses. Breast muscle samples were collected by removing a transversally cut 20-g section of the longitudinal intermediate third of the right *Pectoralis major*. Keel samples were collected by dissecting the cartilaginous extension of the sternum, and transversally cutting its insertion to the bone in a right angle relative to its dorsal surface. The longitudinal intermediate third of the right tibia was collected, and its bone marrow was removed by washing with distilled water. All tissue samples were duly identified and frozen at –20°C. for analyses, samples were thawed, rinsed in distilled water, placed in Petri dishes, and dried in a forced-ventilation oven (Marconi – model MA 035) at 55°C for 48 hours. After drying, samples were ground in a cryogenic mill (Spex – model 6700 *freezer/mill*) at -196°C at maximum frequency for three minutes, with the objective of obtaining homogenous material with very fine particle size, with talcum powder appearance (Licatti, 1997; Ducatti, 2004). Feeds were ground according the procedure mentioned above, but for approximately ten minutes.

Isotopic analyses of ingredients, feeds, and tissues

Table 3 - Percentage composition, calculated nutritional levels, and mean isotopic values of the starter experimental diets (one to 21 days of age).

	Experimental diets							
Ingredients (%)	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
Ground corn	44.81	48.42	48.10	49.87	50.58	48.28	48.60	49.14
Soybean meal	48.60	43.05	43.08	40.58	39.48	43.07	42.51	41.73
Poultry offal meal	-	3.83	-	2.51	1.60	1.91	-	-
Poultry feather meal	-	-	-	2.10	2.40	-	1.58	3.16
Bovine meat and bone meal	-	-	5.50	-	1.38	2.75	2.75	-
Soybean oil	2.93	1.69	2.01	1.69	1.58	1.84	2.10	2.24
Calcitic limestone	1.03	0.98	0.45	1.02	0.90	0.70	0.74	1.09
Dicalcium phosphate	1.79	1.24	0.05	1.42	1.10	0.65	0.91	1.72
DL-Methionine	0.05	0.04	0.06	0.05	0.06	0.05	0.06	0.07
L-Lysine	-	-	-	-	0.16	-	-	0.10
Kaolin	-	-	-	-	-	-	-	-
Salt	0.39	0.35	0.35	0.36	0.36	0.35	0.35	0.35
Vitamin and mineral suppl.[1]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Total	100	100	100	100	100	100	100	100
Calculated nutritional levels								
Metabolizabel energy (kcal/kg)	2900	2900	2900	2900	2900	2900	2900	2900
Crude protein (%)	26.00	26.00	26.00	26.00	26.00	26.00	26.00	26.00
Crude fiber (%)	3.94	3.70	3.72	3.57	3.56	3.71	3.67	3.60
Calcium(%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Avail. phosphorus (%)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Methionine (%)	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44
Methionine + cystine (%)	0.86	0.86	0.85	0.90	0.90	0.85	0.88	0.92
Lysine (%)	1.50	1.47	1.44	1.40	1.49	1.46	1.41	1.44
Mean isotopic values[2]								
$\delta^{13}C$	-21.11	-20.07	-19.53	-19.54	-19.56	-19.59	-19.76	-19.72
$\delta^{15}N$	0.73	1.23	1.46	1.41	1.58	1.40	1.38	1.26

1 - Composition of vitamin and mineral supplement from Nutron®/kg feed: folic acid 200 mg; pantothenic acid 3,120 mg; choline 75,500 mg; biotin 10,000 mcg; niacin 8,400 mg; Vit. A 1,680 UI; Vit. B1 436.50 mg; Vit. B12 2,400 mcg; Vit B2 1.200 mg; Vit. B6 624 mg; Vit. D3 400,000 UI; Vit. E 3,500 mg; Vit. K3 360 mg; Cu 2.000 ppm; Fe.12,500 ppm; I. 187.50 ppm; Mn.18,.750 ppm; Zn. 17,500 ppm; Se 17,500 ppm. [2] Mean isotopic values expressed as δ ^{13}C relative to Peedee Belemnite (PDB) standard, and δ ^{15}N relative to atmospheric N_2 standard.

Table 4 - Percentage composition, calculated nutritional levels, and mean isotopic values of the grower experimental diets (22 to 42 days of age).

	Experimental diets							
Ingredients (%)	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
Ground corn	44.81	48.42	48.10	49.87	50.58	48.28	48.60	49.14
Soybean meal	40.91	35.30	35.23	34.74	34.05	35.50	34.67	34.06
Poultry offal meal	-	3.83	-	2.51	1.60	1.91	-	-
Poultry feather meal	-	-	-	2.10	2.40	-	1.58	3.16
Bovine meat and bone meal	-	-	5.50	-	1.38	2.75	2.75	-
Soybean oil	4.99	3.61	3.90	4.06	4.04	4.13	4.14	4.41
Calcitic limestone	0.90	0.84	0.27	0.88	0.62	0.58	0.62	0.98
Dicalcium phosphate	1.67	1.14	-	1.40	0.59	0.56	0.80	1.60
DL-Methionine	0.10	0.09	0.11	0.10	0.11	0.10	0.11	0.12
L-Lysine	-	-	0.03	0.05	0.05	-	0.07	0.12
Kaolin	0.26	-	-	0.30	0.47	0.47	0.21	0.50
Salt	0.35	0.34	0.33	0.35	0.35	0.35	0.35	0.35
Vitamin and mineral suppl.[1]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Total	100	100	100	100	100	100	100	100
Calculated nutritional levels								
Metabolizable energy (kcal/kg)	3100	3100	3100	3100	3100	3100	3100	3100
Crude protein (%)	23.00	23.00	23.00	23.00	23.00	23.00	23.00	23.00
Crude fiber (%)	3.54	3.30	3.32	3.25	3.24	3.31	3.26	3.20
Calcium (%)	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Avail. phosphorus (%)	0.42	0.42	0.43	0.42	0.42	0.42	0.42	0.42
Methionine (%)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Methionine + cystine (%)	0.82	0.83	0.81	0.85	0.84	0.82	0.85	0.89
Lysine (%)	1.29	1.26	1.26	1.25	1.25	1.25	1.25	1.25
Mean isotopic values[2]								
$\delta^{13}C$	-20.25	-19.03	-19.04	-18.82	-18.79	-19.27	-18.96	-19.23
$\delta^{15}N$	0.96	1.42	1.80	1.86	1.95	1.53	1.63	1.59

1 - Composition of vitamin and mineral supplement from Nutron®/kg feed: folic acid 200 mg; pantothenic acid 3,120 mg; choline 75,500 mg; biotin 10,000 mcg; niacin 8,400 mg; Vit. A 1,680 UI; Vit. B1 436.50 mg; Vit. B12 2,400 mcg; Vit B2 1.200 mg; Vit. B6 624 mg; Vit. D3 400,000 UI; Vit. E 3,500 mg; Vit. K3 360 mg; Cu 2.000 ppm; Fe.12,500 ppm; I. 187.50 ppm; Mn.18,.750 ppm; Zn. 17,500 ppm; Se 75,00 ppm. [2] Mean isotopic values expressed as $\delta\ ^{13}C$ relative to Peedee Belemnite (PDB) standard, and $\delta\ ^{15}N$ relative to atmospheric N_2 standard.

were carried out at the Environmental Stable Isotope Center of the Biosciences Institute (CIE/IB), UNESP, Botucatu campus. Carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios were determined by isotopic ratio mass spectrometer (IRMS) model DELTA – S (Finnigan Mat) coupled to an Elemental Analyzer (EA 1108 CHN), according to the method described by Ducatti (2004). Carbon and nitrogen analyses were carried out separately for each element, and in duplicate.

Analyses results were expressed as *delta per thousand* of the sample isotopic ratio relative to the international standards Peedee Belemnite (PDB) and atmospheric nitrogen (N_2), for the elements carbon and nitrogen, respectively, according to the equation:

$$\delta‰_{(sample,\ standard)} = [(R_{sample} - R_{standard}) / R_{standard}] \times 10^3$$

where R represents the ratio between the heaviest and the lightest isotope, in particular $^{13}C/^{12}C$ and $^{15}N/^{14}N$, in the sample and in the standard.

Isotopic results were submitted to multivariate analysis of variance (MANOVA) with the aid of the GLM (General Linear Model) procedure of SAS (1999) statistical software. Data were generated by error matrices for each tissue, and were subsequently graphically distributed in regions (ellipses) with 95% confidence of observing possible differences between experimental treatment means and control treatment means. This method allows to verify if the values of the isotopic pair ($\delta^{13}C$ and $\delta^{15}N$) of the control treatment (vegetable feed), are statistically different from the values of the isotopic pair of the treatment with the inclusion of animal proteins.

RESULTS AND DISCUSSION

The mean $\delta^{13}C$ $\delta^{15}N$ isotopic values obtained for the different tissues of 42-day-old meat quails fed diets containing different protein sources are shown in Table 5.

It is observed that all tissues of birds fed the diet based only on corn and soybean meal (treatment T1) presented negative values, and higher ^{13}C as compared to the other treatments. This finding is similar to that obtained by Móri (unpublished data), who observed relative $\delta^{13}C$ and $\delta^{15}N$ enrichment in adult meat quails fed diets containing poultry offal meal.

Table 5 - Mean $\delta^{13}C$ and $\delta^{15}N$ isotopic values and their respective standard deviations of the breast muscle, keel, and tibia of 42-day-old meat quails for the different protein sources.

Treatments	Sampled tissue		Breast muscle		Keel Tibia	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{3}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
T1	-21.70 ± 0.25	2.53 ± 0.07	-19.35 ± 0.21	3.57 ± 0.22	-18.61 ± 0.06	2.71 ± 0.07
T2	-20.74 ± 0.20	2.97 ± 0.10	-18.36 ± 0.21	3.91 ± 0.01	-17.42 ± 0.29	3.30 ± 0.06
T3	-20.78 ± 0.29	3.01 ± 0.12	-18.65 ± 0.20	3.99 ± 0.00	-17.04 ± 0.15	3.40 ± 0.15
T4	-20.58 ± 0.10	3.00 ± 0.17	-18.50 ± 0.10	4.05 ± 0.14	-17.73 ± 0.15	4.06 ±0.13
T5	-20.65 ± 0.18	2.92 ± 0.15	-18.14 ± 0.20	3.88 ± 0.05	-17.58 ± 0.19	4.03 ± 0.15
T6	-20.62 ± 0.18	2.92 ± 0.06	-18.11 ± 0.23	3.95 ± 0.14	-17.86 ± 0.18	3.93 ± 0.10
T7	-20.84 ± 0.33	2.99 ± 0.15	-18.04 ± 0.19	4.10 ± 0.15	-17.78 ± 0.13	4.05 ± 0.07
T8	-20.54 ± 0.09	2.94 ± 0.15	-17.98 ± 0.13	3.91 ± 0.15	-17.38 ± 0.25	3.78 ± 0.05

Variations in the percentage participation of the ingredients in the composition of feeds are directly responsible for isotopic carbon-13 and nitrogen-15 enrichment. Therefore, the analysis of the ingredients and of the produced feeds is important for the certification process of meat product as, according to DeNiro & Epstein (1978), the isotopic composition of animal tissues reflects the composition of the diet they consumed. Figures 1, 2, and 3 show the confidence regions (ellipses) to verify differences among the means of the isotopic pairs $\delta^{13}C$ and $\delta^{15}N$ of treatments T2, T3, and T8 in the breast muscle, keel, and tibia of 42-day-old meat quails as compared to the control treatment (corn- and soybean meal-based diet). In all tissues, there was ^{13}C and ^{15}N enrichment, as the confidence ellipses are far from all graph axes, which represent the control treatment.

The Figures 1 and 2 also show that differences between T2, T3, and T8 and control (T1) treatment mean for ^{15}N were lower than for ^{13}C, making confidence means to shift closer to the carbon axis than to the nitrogen axis. This space distribution demonstrates that ^{15}N is the essential chemical element to detect animal byproduct presence in quail meat, as these ingredients have higher nitrogen content in their composition.

The differences found in the analyzed tissues are probably due to differences in tissue synthesis and their essential and non-essential amino acid composition. According to Moran Jr. (1999), breast muscle amino acid composition consists mainly of essential amino acids, which, when incorporated to tissues, produce little change in their isotopic ratio (Pinnegar & Polunin, 1999). On the other hand, the enrichment differences observed between breast muscle and bone are probably due to type-I collagen, which comprises approximately 95% of the organic bone matrix (Pizauro Jr., 2002), and it is therefore the main source of bone nitrogen, and to the fact that keel cartilage proteins consist mainly of non-essential amino acids.

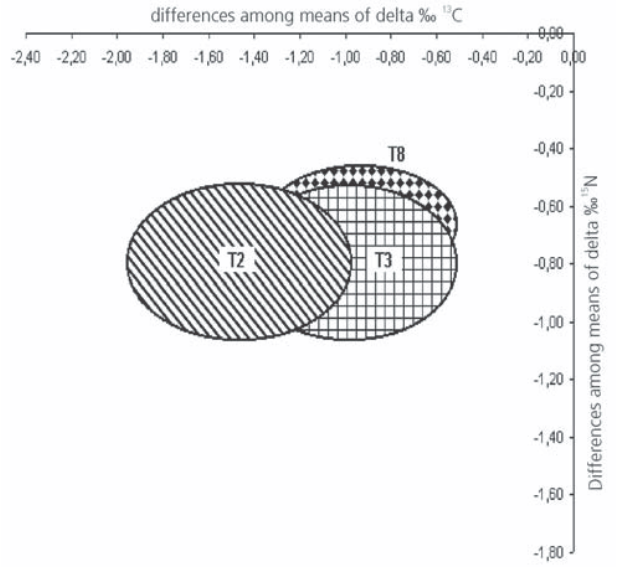

Figure 1 – Confidence regions for differences among means of delta ‰ ^{13}C and ‰^{15}N of the breast muscle of 42-day-old meat quails in treatments T2, T3, and T8.

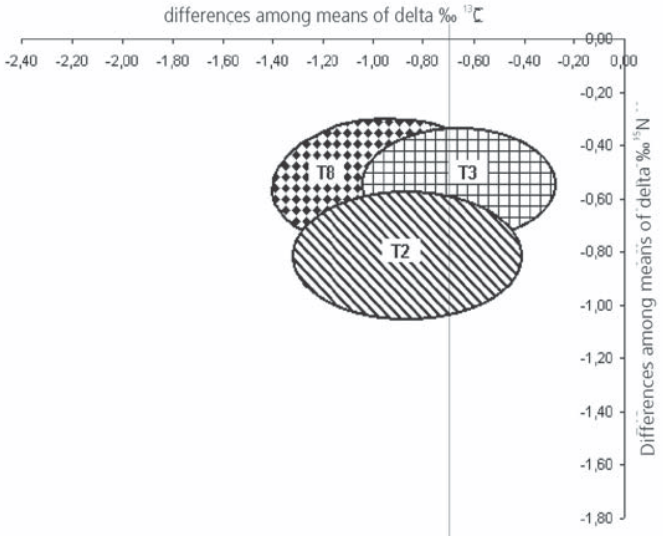

Figure 2 – Confidence regions for differences among means of delta ‰ ^{13}C and ‰^{15}N of the keel of 42-day-old meat quails in treatments T2, T3, and T8.

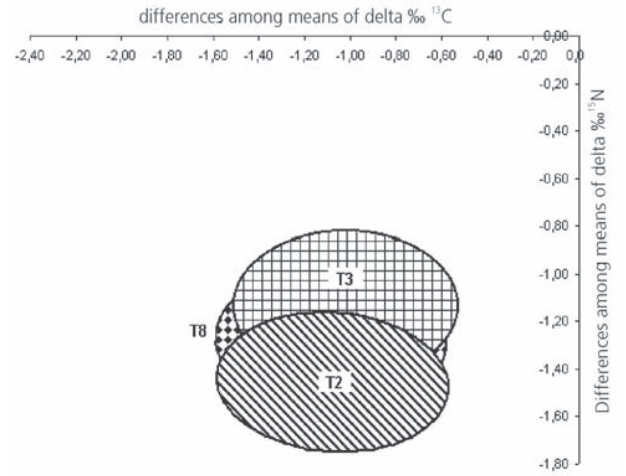

Figure 3 – Confidence regions for differences among means of delta ‰ ^{13}C and ‰^{15}N of the tibia of 42-day-old meat quails in treatments T2, T3, and T8.

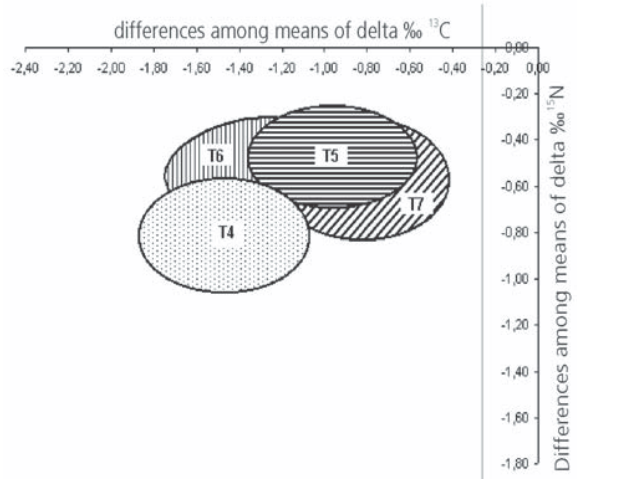

Figure 4 – Confidence regions for differences among means of delta ‰ ^{13}C and ‰^{15}N of the breast muscle of 42-day-old meat quails in treatments T4, T5, T6, and T7.

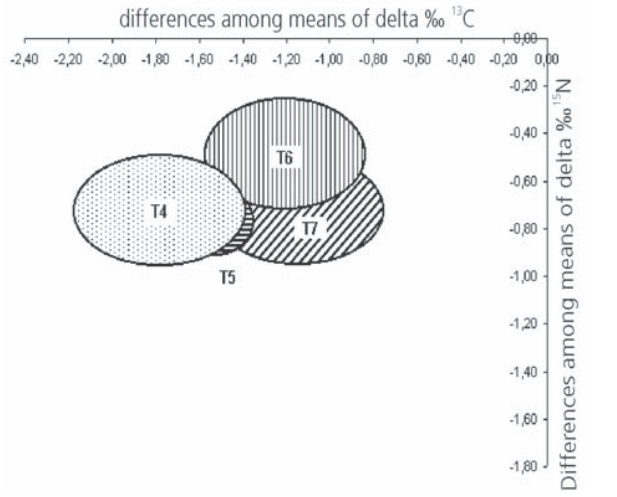

Figure 5 – Confidence regions for differences among means of delta ‰ ^{13}C and ‰^{15}N of keel of 42-day-old meat quails in treatments T4, T5, T6, and T7.

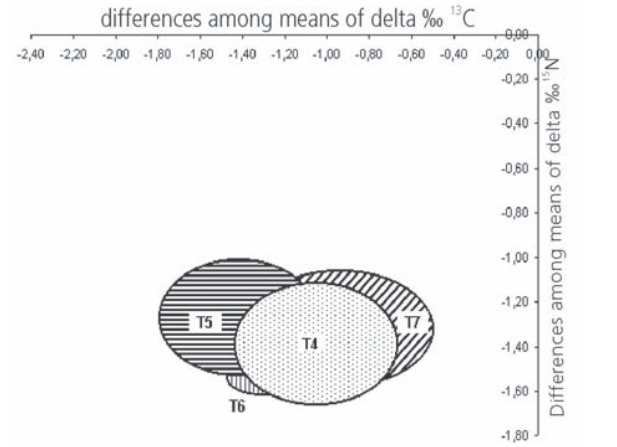

Figure 6 – Confidence regions for differences among means of delta ‰ ^{13}C and ‰^{15}N of the tibia of 42-day-old meat quails in treatments T4, T5, T6, and T7.

The variations among tissues are not fully elucidated yet. According to Tiezen *et al*. (1983), the main biochemical fractions of the body are isotopically different, which may reflect differences in their biochemical composition. Tissues containing higher lipid content would probably have lower d^{13}C values as compared to those with lower lipid content, which are relatively poorer in carbon-13 (Tiezen *et al*., 1983; Piasentier *et a*l., 2003). Similar behavior was observed in treatments T4, T5, T6, and T7, which feeds contained mixtures of different animal proteins, as compared to the control treatment (Figures 4, 5, and 6) in the three analyzed tissues. The overlapping of the confidence regions indicates that there are no differences among treatments (Figures 1, 2, 3, 4, 5, and 6), i.e., it was not possible to establish differences among the byproducts. Tibia confidence ellipses were the most distant from the graph axes, indicating that this tissue is the most adequate for control purposes (Figures 3 and 6). This behavior may be explained by the fact that this tissue presents higher ^{13}C isotopic enrichment as compared to the breast muscle and to the keel (Table 5). Hobson & Clark (1992a,b) state that every tissue in a same animal has specific isotopic signature, fractioning factor, and isotopic turnover.

CONCLUSIONS

Based on the data obtained in the present study, it is concluded that the technique of stable isotopes is efficient for the detection of the presence of animal byproducts in the breast muscle, keel, and tibia of adult quails. Therefore, this technique can be used as an

auxiliary tool in poultry product traceability; however, its limitation as to the detection of specific animal byproducts must be considered.

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