



Traceability of Bovine Meat and Bone Meal in Poultry by Stable Isotope Analysis

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ABSTRACT

Bovine meat and bone meal (MBM) was widely used in animal diets until outbreaks of Bovine Spongiform Encefalopathy (BSE) occurred in some countries. It has not been confirmed yet whether or not BSE may be transmitted to man through chicken meat originated from poultry that had been fed diets containing MBM. Therefore, consumers nowadays express preference for meat originated from birds fed exclusively vegetable diets. This study analyzed samples of major breast muscle (*Pectoralis major*) using mass spectrometry of stable isotopes (carbon and nitrogen) as a means to assess the presence of MBM in broiler diets, a technique that might be used in the certification of poultry quality. A total of 150 day-old chicks were reared in five randomized treatments with increasing MBM dietary inclusion levels (0, 1, 2, 4 and 8%). On day 42, breast muscle samples were collected from three birds per treatment and used in the determination of ¹³C/¹²C and ¹⁵N/¹⁴N isotope ratios. The breast muscle isotope values were expressed as delta in parts per thousand (‰). The following carbon isotope values (¹³C) were found: -18.74‰±0.11, -18.51‰±0.19, -18.24‰±0.10, -17.79‰±0.12, and -17.15‰±0.15 for 0, 1, 2, 4 and 8% MBM dietary levels, respectively. Nitrogen isotope values (¹⁵N) were 1.65‰±0.14, 1.65‰±0.28, 1.72‰±0.08, 1.95‰±0.16, and 2.52‰±0.09 for 0, 1, 2, 4 and 8% MBM dietary levels, respectively. This study showed important differences in δ¹³C and δ¹⁵N values in breast meat, evidencing a simultaneous enrichment of this isotopic pair, which allowed tracing MBM in bird diets. Analysis of carbon and nitrogen stable isotopes may be used to ensure feeding with exclusively vegetable diets, and might also be used as a reliable evaluation tool in broiler meat certification. The diet with 1% inclusion level of MBM and the exclusively vegetable diet showed similar results.

INTRODUCTION

Bovine meat and bone meal (MBM) was widely used in animal diets until the occurrence of outbreaks of Bovine Spongiform Encefalopathy (BSE) in Europe, Japan, and, more recently, Canada. Nowadays, the use of MBM is prohibited in ruminant feeding as a means to prevent new cases of BSE, although it is still used in broiler feeding as a substitute for soybean meal, a high-cost protein source of vegetable origin. As a result, sanitary authorities worldwide are increasingly concerned with quality control and certification of origin of fresh meat and animal products. Recently, authentication and objective feed information have been the main objectives of consumers (Monin, 1998).

In the past 30 years, the analysis of naturally occurring variations on the abundance of stable isotopes has been increasingly used in physiological and metabolism research (Gannes & Koch, 1998), and has



also helped to reconstruct the dietary history of animals through the analysis of different tissues (De Niro & Epstein, 1978; Merve van der, 1982). Less scrupulous producers commonly adulterate food products by substituting high-cost ingredients for less expensive ones, or by omitting/including some ingredients (Silva *et al.*, 1999). The $^{13}\text{C}/^{12}\text{C}$ isotope ratio has been used to test the authenticity, quality, and geographical origin of several products such as orange juice (Bricout & Koziat, 1987), honey (Brookes *et al.*, 1991), and vegetable oils (Kelly *et al.*, 1997). Analysis of meat, liver and fat samples using ^{13}C stable isotope technique has also been used to characterize and differentiate the dietary regimen of Iberian swine, and it has enabled classification of animals according to the type of food given during the fattening period (González-Martin *et al.*, 1999; Gonzáles-Martin *et al.*, 2001).

Piasentier *et al.* (2003) demonstrated that it is possible to certify the geographical origin and food regimen of lamb by analyzing carbon and nitrogen isotope levels in muscle and fat using mass spectrometry.

It has not been confirmed whether or not BSE in humans may be caused by the consumption of poultry originated from birds fed diets containing MBM. Therefore, consumers nowadays show preference for chicken meat from birds fed an exclusively vegetable-based diet.

The objective of this study was to trace the presence of MBM in broiler diets. Breast muscle (*Pectoralis major*) was analyzed by mass spectrometry, evaluating stable carbon ($^{13}\text{C}/^{12}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$) in dual isotope ratio analysis in the certification of poultry quality.

MATERIAL AND METHODS

General management of birds

The experiment was performed in the Laboratory of Avian Nutrition – FMVZ/ UNESP, Botucatu-SP, Brazil. One hundred and fifty one-day-old Cobb broiler chicks were housed in cages and raised until 42 days old under similar conditions and standard management procedures. Isoprotein and isocaloric diets were provided. Water and food were supplied *ad libitum* using nipple drinkers and automatic feeders. Birds were given 24 hours of continuous light supplied by incandescent 60W light bulbs.

The birds were distributed into 5 treatments of 30 birds each, according to a completely randomized experimental design. Experimental corn and soybean meal-based diets were formulated according to

Rostagno *et al.* (2000). Tables 1 and 2 show the percentage composition of the diets. Inclusion levels of MBM were 0% in the control treatment (MBM 0), 1% (MBM 1), 2% (MBM 2), 4% (MBM 4), and 8% (MBM 8). Isotopic values of the MBM included in experimental diets were -12.97‰ and 8.06‰ for carbon and nitrogen, respectively.

Isotope ratio measurement by mass spectrometry

At 42 days of age, three birds were randomly taken from each treatment and sacrificed by cervical dislocation. Samples of approximately 20g were collected medially at the proximal third of the left breast muscle. Samples were identified and frozen at -20°C for isotopic analyses.

Breast muscle samples were thawed, washed in distilled water, and dried at 56°C for 48h in a forced ventilation oven (MA 035, Marconi, Piracicaba, Brazil). All samples were ground in a cryogenic grinder with liquid nitrogen (Spex 6700-230 freezer/mill – Spex Industries, Edison, USA) at -196°C for 3 minutes. Individual tubes were used to avoid contamination between samples (Ducatti, 2000).

The samples were analyzed at the Center of Stable Isotopes–IB/UNESP, Botucatu-SP, Brazil. Approximately 0.35mg and 0.45mg of each sample were used for $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ isotopic ratio measurements, respectively. They were weighed inside metal capsules and placed into a Multi-element analyzer (EA 1108 – CHN – Fisons Instruments, Rodano, Italy) by automatic sampler. The samples were quantitatively burned in the presence of oxygen (O_2) and copper oxide (CuO) to obtain CO_2 and NO_x that was then reduced to N_2 in the presence of copper. The gases formed were separated in a gas chromatograph column and analyzed in an isotope ratio mass spectrometer (Delta S – Finnigan MAT, Bremen, Germany).

Isotope ratio values were expressed as delta per thousand ($\delta\text{‰}$) in relation to *Pee Dee Belemnite* (PDB) international standards for $\delta^{13}\text{C}$ and atmospheric air for $\delta^{15}\text{N}$, according to the following equation:

$$\delta\text{‰}_{(\text{sample, standard})} = \left[\frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \right] \times 1000$$

where R represents the ratio between the least and most abundant isotopes, in particular $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$. Each sample was analyzed twice to obtain mean values and analysis was repeated when the standard deviation was higher than 0.2‰ for $\delta^{13}\text{C}$ and 0.3‰ for $\delta^{15}\text{N}$.



Table 1 – Percentage composition of starter diets (1 to 21 days).

Ingredients (%)	Bovine meat and bone meal (%)				
	0	1	2	4	8
Corn, grain	60.87	61.65	62.48	64.11	64.82
Soybean, meal	34.14	33.10	32.02	29.89	26.13
Meat and bone, meal	-	1.00	2.00	4.00	8.00
Soybean oil	1.25	1.00	0.73	0.20	-
Limestone	1.00	0.85	0.70	0.39	-
Bicalcium Phosphate	1.77	1.44	1.10	0.46	-
DL – Methionine	0.16	0.16	0.16	0.16	0.16
L – Lysine	0.18	0.18	0.20	0.22	0.25
Kaolin	-	-	-	-	0.11
Salt (NaCl)	0.43	0.42	0.41	0.37	0.33
Choline chloride	0.05	0.05	0.05	0.05	0.05
Vitamin supplement ¹	0.10	0.10	0.10	0.10	0.10
Mineral supplement ²	0.05	0.05	0.05	0.05	0.05
Calculated values					
ME, kcal/kg	2950	2950	2950	2950	2950
CP, %	21.0	21.0	21.0	21.0	21.0
CF, %	3.21	3.17	3.14	3.07	2.91
Ca, %	0.95	0.95	0.95	0.95	1.25
Available P, %	0.44	0.44	0.44	0.44	0.60
Met, %	0.48	0.48	0.48	0.48	0.48
Met + Cys, %	0.82	0.82	0.82	0.82	0.82
Lys, %	1.24	1.24	1.24	1.24	1.24
δ ¹³ C	-17.47	-17.72	-16.41	-16.37	-15.91
δ ¹⁵ N	0.85	0.99	1.06	1.60	2.10

1- Initial vitamin supplement Vaccinar® (levels per kg of product): Vit. A, 14,000,000 IU; Vit. D₃, 2,500,000 IU; Vit. E, 25,000mg; Vit K₃, 3,000mg; Vit. B₁, 2,000mg; Vit. B₂, 5,000mg; Vit. B₆, 4,000mg; Vit. B₁₂, 25,000mcg; Niacin, 35,000mg; Pantothenic acid, 12,000mg; Biotin, 100mg; Folic acid, 1,000mg; Vit C, 50,000mg; antioxidant (BHT), 125mg. 2- Mineral supplement Vaccinar® (per kg of product): Selenium, 360mg; Iron, 96,000mg; Manganese, 156,000mg; Iodine, 1,400mg; Copper, 20,000mg; Zinc, 110,000mg.

Table 2 - Percentage composition of final diets (22 to 42 days).

Ingredients (%)	Bovine meat and bone meal (%)				
	0	1	2	4	8
Corn, grain	63.75	64.52	65.31	66.91	67.00
Soybean, meal	29.83	28.77	27.71	25.59	21.95
Meat and bone, meal	-	1.00	2.00	4.00	8.00
Soybean oil,	2.96	2.71	2.45	1.95	1.95
Limestone	0.92	0.77	0.60	0.32	-
Bicalcium Phosphate	1.62	1.30	1.00	0.31	-
DL – Methionine	0.15	0.15	0.15	0.15	0.16
L – Lysine	0.21	0.22	0.23	0.25	0.27
Kaolin	-	-	-	-	0.21
Salt (NaCl)	0.37	0.36	0.35	0.32	0.26
Choline chloride	0.05	0.05	0.05	0.05	0.05
Vitamin supplement ¹	0.10	0.10	0.10	0.10	0.10
Mineral supplement ²	0.05	0.05	0.05	0.05	0.05
Calculated values					
ME, kcal/kg	3100	3100	3100	3101	3101
CP, %	19.3	19.3	19.3	19.3	19.3
CF, %	3.01	2.97	2.94	2.87	2.71
Ca, %	0.87	0.87	0.87	0.87	1.24
Available P, %	0.41	0.41	0.41	0.41	0.59
Met, %	0.45	0.45	0.45	0.45	0.45
Met + Cys, %	0.76	0.76	0.77	0.77	0.78
Lys, %	1.15	1.15	1.15	1.15	1.14
δ ¹³ C	-17.03	-16.86	-16.57	-16.49	-16.25
δ ¹⁵ N	1.09	1.37	1.29	1.49	2.24

1- Final vitamin supplement Vaccinar® (levels per kg of product): Vit. A, 10,000,000 IU; Vit. D₃, 2,000,000 IU; Vit. E, 20,000mg; Vit K₃, 2,000mg; Vit. B₁, 2,000mg; Vit. B₂, 4,000mg; Vit. B₆, 4,000mg; Vit. B₁₂, 20,000mcg; Niacin, 30,000mg; Pantothenic acid, 10,000mg; Biotin, 60mg; Folic acid, 1,000mg; Vit C, 50,000mg; antioxidant (BHT), 125mg. 2- Mineral supplement Vaccinar® (per kg of product): selenium, 360mg; iron, 96,000mg; manganese, 156,000mg; iodine, 1,400mg; copper, 20,000mg; zinc, 110,000mg.

Results were statistically analyzed using General Linear Models (SAS, 1996), and multivariate analysis for the isotopic pair. Confidence intervals (95%) were obtained by the difference between each treatment and the control treatment using joint variance matrix and discriminant analysis with further analysis of variance in the discriminating function.

RESULTS AND DISCUSSION

The results of the isotopic analysis for d¹³C and d¹⁵N of initial (1 – 21 days) and final diets (22 – 42 days) of each treatment are shown in Table 3. The isotope signature of the exclusively vegetable diet was -17.47 for ¹³C in the starter diet, and -17.03 in the final diet. These are characteristic values of corn and soybean meal-based diets used by the poultry industry in Brazil. The use of MBM instead of soybean meal changes these values and the diets are expected to become heavier in both ¹³C and ¹⁵N. Isotopic enrichment in both carbon-13 and nitrogen-15 was observed due to the increase in MBM inclusion levels in the diets. Carbon-13 enrichment might be explained by the origin of MBM

produced in Brazil. Tropical pastures consist of C₄-photosynthetic grasses. Nitrogen-15 enrichment in diets is in agreement with Delgado & Garcia (2001), who showed the importance of animal by-products in order to enrich diets with ¹⁵N.

Table 3 - Values of δ¹³C and δ¹⁵N in initial (1–21 days) and final (22-42 days) diets containing different levels of bovine meat and bone meal (MBM).

MBM level*	Initial Diet		Final Diet	
	δ ¹³ C	δ ¹⁵ N	δ ¹³ C	δ ¹⁵ N
0	- 17.47	0.85	- 17.03	1.09
1	- 17.77	0.99	- 16.86	1.37
2	- 16.41	1.06	- 16.57	1.29
4	- 16.37	1.60	- 16.49	1.49
8	- 15.91	2.10	- 16.25	2.24

*Levels in MBM: δ¹³C = -12.97; δ¹⁵N = 8.06

Hobson & Clark (1992) reported that the choice of tissue type for isotopic analysis depends on the speed with which the different tissues reflect the isotopic signature. Therefore, it depends on the metabolism rate of the tissue. Since the turnover rate in breast muscle of growing broiler chickens is very high and



99% of the tissue carbon is replaced in approximately 17 days (Cruz, 2002; Carrijo *et al.*, 2000; Ducatti *et al.*, 2002), the isotopic signature should safely represent the diets given to birds. Therefore, breast muscle was chosen for analysis due to the higher commercial value of this cut, the low fat levels in the tissue, and mainly the higher turnover rate.

The mean $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ isotopic ratios in breast muscle of broiler chickens fed an exclusively vegetable-based diet (MBM 0) were $-18.74\text{‰} \pm 0.11$ and $1.56\text{‰} \pm 0.14$, respectively. The values obtained for $\delta^{13}\text{C}$ corroborate a previous study with birds fed a vegetable diet (Cruz, 2002). This evidenced that the turnover rate in the breast muscle of growing broiler chickens is much higher than the same tissue in adult Japanese quails (Hobson & Clark, 1992) and gerbils (Tieszen *et al.*, 1983).

The mean results for ^{13}C and ^{15}N isotopic pair in the breast muscle of birds fed 1% MBM (MBM 1) were -18.51 ± 0.19 and 1.65 ± 0.28 , respectively. This isotopic pair was not significantly different ($p > 0.05$) from the control diet (MBM 0%). However, 2%, 4%, 8% inclusion levels of MBM resulted in values significantly different from those of birds fed an exclusively vegetable-based diet ($p < 0.05$). Values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for MBM 2, MBM 4, and MBM 8 were respectively -18.24 ± 0.10 and 1.72 ± 0.08 ; -17.79 ± 0.12 and 1.95 ± 0.16 ; -17.15 ± 0.15 and 2.52 ± 0.09 .

Figure 1 shows simultaneous isotopic enrichment for both ^{13}C and ^{15}N with the increasing MBM levels in the diets. This enrichment demonstrated that the turnover rate of breast muscle in growing broilers is very high and actually represents the recent dietary regimen to which the birds have been submitted. The difference between the isotope values in the diets and the muscular tissue is due to isotopic fractionating; this is in accordance with DeNiro & Epstein (1976), who reported that an isotopic signature of the animal represents what was consumed until $\pm 2\text{‰}$ for ^{13}C and 3‰ for ^{15}N .

In addition, considering that the different links in the food chain are normally accompanied by enrichment with heavy isotopes (DeNiro & Epstein, 1978), it is possible that mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in muscular tissue are higher in birds fed MBM enriched diets than in birds fed an exclusively vegetable-based diet. The variability of delta values between diets and muscle is also due to isotopic fractionating during nutrient assimilation.

Isotope enrichment of the muscular tissue in birds fed higher levels of MBM is in agreement with Delgado & Garcia (2001), who emphasized the role of protein

of animal origin as a source of relatively high levels of ^{15}N in animal feeds. These authors showed that carnivores are richer in ^{15}N than omnivores, which in turn have higher levels than herbivores, and finally local plants. This demonstrates that the inclusion of animal by-products in feeds would enrich these with nitrogen-15 and thus change the isotopic signature of animals that have been fed the ^{15}N enriched diet.

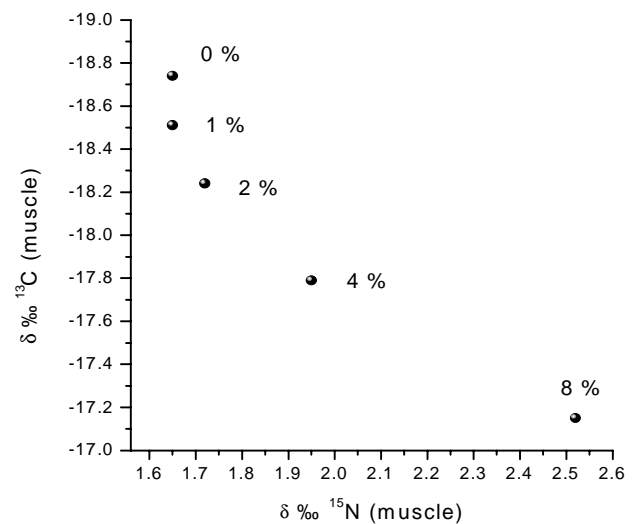


Figure 1 - Mean values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ at 42 days in breast muscle of broiler chickens submitted to diets with different levels of bovine meat and bone meal ($n=3$).

A multivariate analysis was used to simultaneously assess $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ results of the different treatments. Figure 2 shows the confidence interval (95%) for the differences between each MBM level and MBM 0 using a joint covariance matrix. The regions that do not include the intercept (0,0) indicate significant difference between the isotopic pairs.

Piasentier *et al.* (2003) reported that it was possible to certify the geographical origin of lamb and dietary regimen of sheep by means of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis in meat and fat. Similar results have been found for other species (Gonzalez-Martin *et al.*, 1999). Carrijo (2003) found similar results of ^{13}C and ^{15}N isotopic enrichment in the breast muscle of broilers fed diets containing different animal by-products, such as poultry offal meal and/or blood and feather meal.

Our results indicated that the isotope analysis of muscular tissue in broiler breast allowed to trace the presence of bovine meat and bone meal in the diets fed to the birds. Thus, the potential stable isotope ratio as a tool to characterize animal diets was confirmed and the possibility of using this technique to trace animal feeding history was clearly indicated.

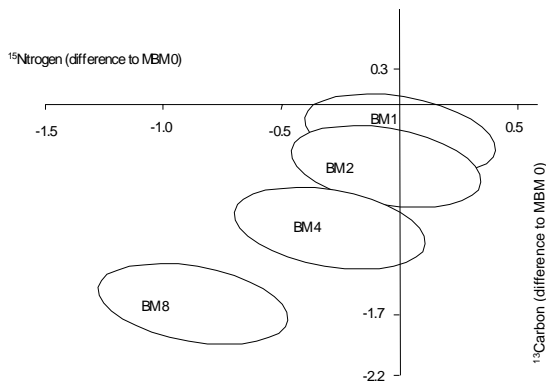


Figure 2 - Confidence intervals for the difference between the control treatment (MBM 0) and treatments with different inclusion levels of bovine meat and bone meal (levels assessed in breast muscle).

CONCLUSIONS

This study showed important differences in the values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in the breast meat of broiler chickens. There was a simultaneous enrichment of the isotopic pair, allowing to trace the addition of bovine meat and bone meal in bird diets. Multivariate analysis followed by establishment of confidence intervals indicated that the analysis of carbon and nitrogen stable isotopes might be used as a reliable evaluation tool for certification of poultry from birds fed exclusively vegetable-based diets. It is important to consider that there were no differences between the groups fed the exclusively vegetable-based diet and 1% MBM in the diet. Studies should be performed to improve the technique and reinforce its applicability, since nitrogen sources might show higher variability than sources with carbon isotopes.

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