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Standardization of A Sample-Processing Methodology for Stable Isotope Studies in Poultry

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ABSTRACT

The objective of this study was to determine if lipid extraction processes alter the isotopic value of $\delta^{13}C$ and $\delta^{15}N$ of tissues (pectoral muscle, thigh and liver) and eggs and if the use of anticoagulants interferes with blood and plasma $\delta^{13}C$ and $\delta^{15}N$ isotopic values. Samples were acquired from the same flock of birds. The 32 egg samples were randomly divided into four treatments (liquid, dehydrated, and fat-extracted with ether or chloroform + methanol) with eight replicates each. The 24 samples of pectoral muscle, thigh muscle and liver of broilers were randomly divided into three treatments (dehydrated, fat-extracted with ether and chloroform + methanol) with eight replicates each. Blood samples were divided into a 3x3 factorial arrangement with three physical forms (liquid, oven-dried or freeze-dried) and three collection methods (with no anticoagulant, with EDTA or heparin). Plasma samples were distributed in a 3x2 factorial arrangement, with three physical forms (liquid, oven-dried, or freeze-dried) and two anticoagulants (EDTA or heparin). The obtained isotopic results were submitted to the multivariate analysis of variance (MANOVA) and univariate (ANOVA, complemented by Tukey' test), using the GLM procedure of the statistical program SAS (1996) or Minitab 16. The results show that it is possible to use the evaluated methods of fat extraction, drying and anticoagulants in the isotopic analyses of carbon-13 and nitrogen-15 in chicken tissues.

INTRODUCTION

The future application of methodologies involving the natural variation of stable isotope abundance in animal nutrition and physiology studies should be stimulated for health, environmental and economic reasons, as well as due to the possibility of developing new models that include details of acquisition rates, synthesis, degradation and destination of nutrients in the various animal tissues.

According to Ducatti (2007), for the progress of this line of research, studies that contribute to the knowledge on carbon isotopic assimilation in different poultry tissues during production phases are needed, in addition of the development of methods that aid sample processing in order to allow faster results.

Considering that the lipid content of the muscular tissues of the animal species, in general, is highly variable, and it is influenced by factors such as size, sex, sexual maturity condition and sampling time, lipid extraction is commonly used to remove depleted carbons from lipids. In addition, it and allows standardization when evaluating and comparing lipid muscle content among different species (Hussey *et al.*, 2008).

The yolk is composed of more than 50% lipids in most species of birds (Carey *et al.*, 1980; Sotherland & Rahn, 1987; Burley & Vadehra, 1989), and lipids are depleted in 13-carbon isotope compared with proteins and carbohydrates (DeNiro & Epstein, 1978). Therefore, differences in the lipid content of samples may confound the interpretation of stable carbon isotopes (Post *et al.*, 2007), and many ecological studies chemically remove lipids from tissues or apply an arithmetic correction to explain the greater abundance of isotopes (Larsen *et al.*, 2006).

Anticoagulants are typically used to prevent the coagulation cascade, allowing subsequent centrifugation. Heparin interacts with antithrombin, forming a ternary complex that inactivates several clotting enzymes, such as clotting factors (II, IX and X) and significantly more thrombin (Shuman & Majerus, 1976). EDTA acts as a strong calcium-chelating agent (Rand *et al.*, 1996), and when complexed with magnesium, prevents the coagulation cascade.

In a study evaluating different storage and sample collection methods of elasmobranchs (Kim & Koch, 2012), no statistical differences were found in the isotopic ratio of blood components collected in tubes coated with heparin or with no additives. In that study, muscle lipids and urea were also removed with petroleum ether and deionized water, respectively. Although untreated and treated muscles had similar amino acid compositions, the treated muscle lost ^{14}N and had a higher C: N ratio, indicating that urea affects isotope ratios and that water treatment removes urea without altering muscle composition.

Blood tissue is often used in stable isotope studies, as it is a relatively non-invasive sampling procedure, and serves as a benchmark for muscle tissue (Wallace *et al.*, 2006; Caut *et al.*, 2008; Dodge e*t al.*, 2011).

In order to advance the application of stable carbon and nitrogen isotopes in the field of animal science, a deeper understanding of complicating factors, such as tissue lipid content, the effects of the lipid extraction process on the species in question, and the use of anticoagulants for the collection of blood and plasma samples and their effects on the isotopic signal of the samples is required.

Therefore, in order to standardize sample preparation methods in isotopic studies, the objectives of this study were to determine if the lipid extraction processes alter the isotopic ratio ($\delta^{13}C$ and $\delta^{15}N$) of selected tissues (breast muscle, thigh muscle, and liver) and eggs and determine if the use of anticoagulants interferes with the isotopic ratio ($\delta^{13}C$ and $\delta^{15}N$) of blood and plasma.

MATERIAL AND METHODS

The experiment was conducted at the Department of Breeding and Animal Nutrition, School of Veterinary Medicine and Animal Science, University of the State of São Paulo (UNESP), Botucatu campus, Brazil.

Egg samples were obtained from a layer flock fed the same diet, with $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios of -17.28 ± 0.06 ‰ and 2.24 ± 0.08 ‰, respectively. Thirty-two eggs were randomly divided into four treatments with eight replicates of one egg each. The treatments consisted of liquid eggs, dehydrated eggs (oven-dried, or freeze-dried), and eggs submitted to lipid extraction by ether or and chloroform + methanol.

Samples of the pectoral muscle, thigh and liver were obtained from a flock of broilers fed a diet with isotopic ratios of -17.78 ± 0.38 ‰ $\delta^{13}C$ and 1.03 ± 0.40 ‰ $\delta^{15}N$. Twenty-four samples of each tissue were randomly divided into three treatments with eight replicates each. The treatments consisted of dehydrated tissues and tissues submitted to lipid extraction by ether or chloroform + methanol.

Blood samples were collected from layerflock fed the same diet, with $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios of -17.28 ± 0.06 ‰ and 2.23 ± 0.07 ‰, respectively. Blood samples were distributed according to a randomized experimental design in a 3x3 factorial arrangement, consisting of three blood physical forms (liquid, oven-dried, or freeze-dried), and blood collection with no anticoagulant, with EDTA or with heparin. Plasma results were analyzed according to a 3x2 in a factorial arrangement, consisting of three blood physical forms (liquid, oven-dried, or freeze-dried), and from blood collected with EDTA or heparin.

All samples were stored in a freezer at -20 ° C for up to 2 months until analyses. The dehydrated samples were obtained by drying the samples in a forced-ventilation oven (Marconi, model MA 035, SP, Brazil) at 56ºC for 48 hours or freeze-drying in a lyophilizer (model L108, Liobras®, SP, Brazil). The duration of the lyophilization process was 48 hours and the samples were dried under vacuum at -55°C and pressure around 50 μHg, and then cryogenically ground (Spex-6750 freezer-mill, Metuchen, USA) at -196ºC (Carrijo *et al.*, 2000; Denadai *et al.*, 2008).

Fat was extracted from the dehydrated and cryogenically-ground samples in a Soxhlet apparatus in the ether treatment (Carrijo *et al.*, 2000; Denadai *et al.*, 2008) and according to the method of Folch *et al.* (1957) in the chloroform + methanol treatment.

Isotopic analysis

The isotopic analyses were carried out at the Center for Stable Isotopes of the Institute of Biosciences of UNESP, Botucatu, SP, Brazil.

Liquid samples were thawed and immediately pipetted into tin capsules (0.2 µL for carbon-13 and 2 µL for nitrogen-15 analyses).

Samples weighing approximately 50-120 µg and 500-600 µg were used to measure $^{13}C/^{12}C$, $^{15}N/^{14}N$, respectively, whose CO_2 and N_2 gases were obtained by combustion and measured in a mass spectrometer, which determines the obtained$\delta^{13}C$ and $\delta^{15}N$ enrichment values relative to their respective international standards.

The isotopic composition values were obtained relative to the international standard V-PDB (Vienna Pee Dee Belemnite) for $\delta^{13}C$ and atmospheric air N_2 for $\delta^{15}N$, with analysis error of 0.2 ‰ and calculated by equation 1:

$$\delta X_{(sample,\ standard)} = [(R_{sample}/R_{standard}) - 1] \times 10^3 \quad (1)$$

where:

δX represents the isotope enrichment of the chemical element X (^{13}C or ^{15}N) of the sample relative to the respective international standard, and R represents the ratio between the least abundant and the most abundant isotope.

Isotopic fractionation was calculated according to Hobson & Clark (1992) as the difference between the δ in the tissue and in the diet (equation 2), both for $\delta^{13}C$ and for $\delta^{15}N$:

$$\Delta = \delta_{tissue} - \delta_{diet} \quad (2)$$

Negative uppercase delta (Δ) values indicate that the tissue is depleted in carbon-13 relative to diet (Hobson & Clark, 1992).

Statistical analysis

The obtained isotopic results were submitted to the multivariate analysis of variance (MANOVA) and univariate (ANOVA, complemented by Tukey's test) using the GLM procedure of the statistical program SAS (1996) or Minitab 16.

RESULTS AND DISCUSSION

Egg samples

The mean $\delta^{13}C$ and $\delta^{15}N$ values of eggs were statistically analyzed (MANOVA) and generated regions with 95% confidence (Figure 1). MANOVA showed a statistical difference among the evaluated treatments studied ($p<0.01$). However, the ANOVA of the $\delta^{13}C$ and $\delta^{15}N$ values, separately, complemented by Tukey's test, detected statistical differences only for carbon-13 (Table 1). The results in Table 1 show no statistical differences ($p>0.05$)carbon-13 and nitrogen-15 between dehydrated and liquid eggs or between eggs submitted to fat extraction either by ether or by chloroform + methanol; however, fat-extracted eggs presented higher carbon-13 isotopic values than liquid and dehydrated eggs ($p<0.01$).

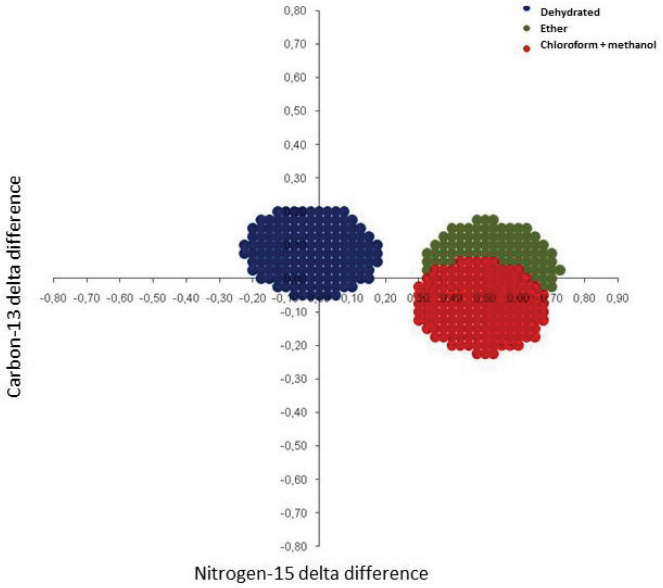

Figure 1 - Confidence region determined by the difference between isotopic δ13C and δ15N values of eggs in each treatment compared to the liquid treatment (n = 8).

Lipids are depleted in 13-carbon compared with proteins and carbohydrates (DeNiro & Epstein, 1978). However, the lipid-extracted eggs were enriched in approximately 0.52 ‰ of carbon-13 and depleted in 0.06 ‰, on average, ofnitrogen-15 (Table 2). These results conflict with those of Oppel *et al.* (2010), who examined the effects of the chemical extraction of avian egg yolk lipids on $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ and found that lipid extraction by chemicals led to an increase of 3.3 ‰ in $\delta^{13}C$, 1.1 ‰ in $\delta^{15}N$, and 2.3 ‰ in $\delta^{34}S$.

The isotopic fractionation determined dietary $\delta^{13}C$ and $\delta^{15}N$ isotopic values of -17.28 ± 0.06 ‰ and 2.24 ± 0.08 ‰, respectively. The calculation of isotopic fractionation (Table 2) showed a decrease of 0.51 ‰ in carbon-13 and of 0.07 ‰ in nitrogen-15 as a result of lipid extraction. These values are much lower than those found by Hobson (1995), who studied the effects of lipid extraction in wild duck, quail, and falcon eggs.

Table 1 – Isotopic δ13C and δ15N values (mean ± SD) of liquid, dehydrated and lipid-extracted (ether or chloroform and methanol) eggs.

Treatments	$\delta^{13}C$	$\delta^{15}N$
Liquid	-20.01 ± 0.25b	4.45 ± 0.19
Dehydrated	-20.03 ± 0.24b	4.53 ± 0.19
Ether lipid extraction	-19.48 ± 0.26a	4.49 ± 0.11
Chloroform + methanol lipid extraction	-19.52 ± 0.19a	4.37 ± 0.16
p-value	$p<0.01$	$p=0.11$

*Different letters in the same column differ statistically by the Tukey's test at $p<0.05$

Table 2 – Isotopic fractionation factor (Δtissue - diet) of liquid, dehydrated and lipid-extracted (ether or chloroform and methanol) eggs.

Treatments	^{13}C	^{15}N
Liquid	-2.72	2,22
Dehydrated	-2.75	2.30
Ether lipid extraction	-2.20	2.25
Chloroform + methanol lipid extraction	-2.24	2.13

According to Denadai *et al.* (2008), the determination of the isotopic fractionation (Δ tissue - diet) aims at reconstructing the diet, that is, finding the isotopic signature of the feed to which the animal submitted, and thus trying to predict the probable components of this diet. Considering the results of isotopic fractionation obtained in the present study (low variation among treatments), it is suggested that the feed and egg samples should be collected from the same batch in order to provide greater certainty of the birds' feeding history (Kennedy & Krouse, 1990).

Muscle and liver samples

The carbon-13 and nitrogen-15 isotopic compositions of the individual tissues analyzed were influenced by lipid extraction. When tissues were not submitted to lipid extraction (dehydrated tissues), carbon-13 and nitrogen-15 values ranged from -19.87 ‰ to -19.79 ‰ and 1.76 ‰ to 3.19 ‰, respectively between tissues (Table 3). After fat extraction by ether, ^{13}C and ^{15}N components ranged between -19.52 ‰ and -19.37 ‰ and between 1.75 ‰ to 2.97 ‰, respectively (Table 3), and after fat extraction by chloroform + methanol, ^{13}C and ^{15}N varied between -20.06 and -19.66 ‰ and 1.92 ‰ and 3.33 ‰, respectively (Table 3).

The multivariate statistical analysis (Figure 2) showed that the isotopic ratios of the isotopic torque of the dehydrated pectoral muscle were statistically different ($p<0.05$) from that defatted by ether. Isotopic ratios of defatted thigh muscles were statistically different ($p<0.05$) from the dehydrated ones. The isotopic liver particle defatted with ether was statistically different ($p<0.05$) from that of defatted with chloroform + methanol.

Table 3 – Isotopic δ13C and δ15N values (mean ± SD) of broiler tissues submitted to dehydration and lipid extraction by ether or chloroform and methanol.

Tissues	Isotopes	Dehydrated	Ether	Chloroform + methanol
Pectoral muscle *	$\delta^{13}C$, (‰)	-19.87 ± 0.23b	-19.52 ± 0.06a	-19.70 ± 0.12b
	$\delta^{15}N$, (‰)	1.76 ± 0.27	1.75 ±0.21	1.92 ± 0.18
Thigh muscle *	$\delta^{13}C$, (‰)	-19.79 ±0.32	-19.51 ± 0.16	-19.66 ± 0.16
	$\delta^{15}N$, (‰)	2.00 ± 0.11b	2.04 ± 0.08b	2.17 ± 0.07a
Liver *	$\delta^{13}C$, (‰)	-19.85 ± 0.32ab	-19.37 ± 0.18a	-20.06 ± 0.63b
	$\delta^{15}N$, (‰)	3.19 ±0.14ab	2.97 ± 0.20b	3.33 ± 0.21[a]

Different letters in the same row differ statistically by the Tukey's test at $p<0.05$, *isotopic pair every two lines differ statistically by MANOVA $p<0.01$

Hussey *et al.* (2008) did not find any significant difference in $\delta^{13}C$ between fat and fat-free muscle tissues of two shark species, as observed in the thigh muscle in the present study. Despite the statistical $\delta^{13}C$ difference in the pectoral muscle and liver between fat removal methods, the numerical difference was small ($\Delta_{\text{dehydrated-ether (pectoral m.)}}$ = 0.35 ‰ and $\Delta_{\text{dehydrated-ether (liver)}}$ = 0.48 ‰) between dehydrated and ether treatments, and there was no significant difference between dehydrated and chloroform + methanol samples. These results differ from those reported in several ecological studies, where the carbon-13 isotopic ratio differences of up to3 ‰ due to lipid extraction were found (Kiljunen *et al.*, 2006, Sweeting *et al.*, 2006, Post *et al.*, 2007; Mintenbeck *et al.*, 2008).

Relative to $\delta^{15}N$ (Table 3), no significant differences ($p> 0.05$) the pectoral muscle among treatments were detected; however, in the thigh muscle, significantly higher $\delta^{15}N$ ($p<0.05$) was detected in samples defatted with chloroform+methanol compared with ether-defatted and dehydrated samples, which were not different from each other ($p> 0.05$) and contained higher fat concentrations. Lower arithmetic difference in nitrogen-15 isotopic ratio between defatted and dehydrated samples ($\Delta_{\text{dehydrated-defatted (average)}}$ = 0.12 ‰) was determined compared with carbon-13 ratio (f $\Delta_{\text{dehydrated-defatted (average)}}$ = 0.27 ‰). In ecological studies, lipid extraction generally enriched the samples in +0.25 to +1.60 ‰ nitrogen-15 values, which are higher than those found in the present study (Pinnegar & Polunin,

1999; Murry *et al.*, 2006; Post *et al.*, 2007; Logan *et al.*, 2008).

The calculation of the isotopic fractionation of broiler tissues aimed at determining the dietary isotopes incorporated into the tissue and whether the lipid extraction method would cause greater differences between the tissues and the diets. The isotopic fractionation data (Table 4) show that, in general, the lipid extraction methods changed the diet-tissue fractionation ($\Delta_{TISSUE - DIET}$). In tissues that have a high fat content (thigh muscle and liver), the process of fat extraction by ether caused a decrease in the isotopic fractionation of carbon-13. However, the isotopic fractionation of nitrogen-15 in the thigh muscle increased and did not change in the liver.

Table 4 – Isotopic fractionation factor (Δtissue - diet) of broiler tissues submitted to dehydration and lipid extraction by ether or chloroform and methanol.

Tissues	Isotopes	Dehydrated	Ether	Chloroform + methanol
Pectoral muscle *	$\delta^{13}C$	-2.09	-1.74	-1.92
	$\delta^{15}N$	0.73	0.72	0.89
Thigh muscle *	$\delta^{13}C$	-2.00	-1.73	-1.87
	$\delta^{15}N$	0.72	1.00	1.14
Liver *	$\delta^{13}C$	-2.07	-1.58	-2.27
	$\delta^{15}N$	2.16	1.94	2.30

Ecological studies typically evaluatecarbon-13 and nitrogen-15 isotopic ratios in bird tissues and fluids in order to predict their diet composition (Kennedy & Krouse, 1990). The advantage of working with poultry for animal science purposes is that it is possible to determine the actual isotopic fractionation of the tissues in relation to the diet, and not only to estimate this fractionation, with advantages of applying these data in several studies hereafter.

A number of authors have reported that lipid extraction is performed to normalize the ratio of stable carbon isotopes, which, however, has also been shown

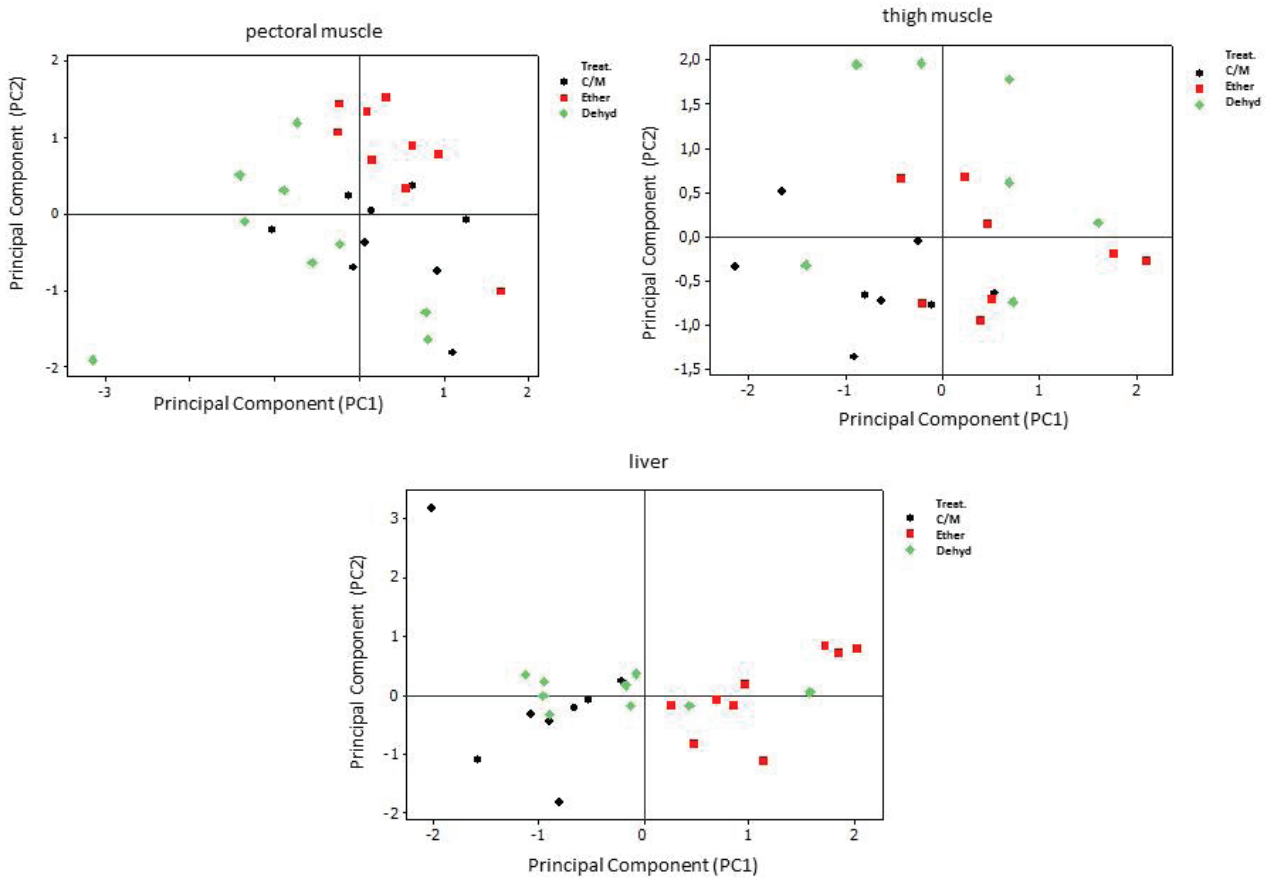

Figure 2 – Principal component analysis carbon-13 and nitrogen-15 in broiler tissues submitted to dehydration and lipid extraction using ether or chloroform + methanol, as determined by multivariate analysis of variance (MANOVA).

to affect the stable nitrogen isotope ratio in fish and invertebrate tissues (Pinnegar & Polunin, 1999; Murry *et al.*, 2006; Post *et al.*, 2007; Logan *et al.*, 2008). Considering the results of the present study, the possibility of not performing lipid extraction needs to be considered, since lower isotopic variation was found in eggs and broiler tissues in the present study when compared with that reported in other species. This isotopic difference from ecological studies may be caused by a number of factors, such as diet diversity, diet heterogeinity during the day, and lipid deposition, which in wild birds may have different purposes compared with poultry, such as temperature control, energy storage for migratory flight, etc. (Kennedy & Krouse, 1990; Hobson & Clark, 1992), leading led to greater isotopic fractionation for the production of the body lipids of migratory birds compared to laying hens and broilers.

Blood samples

The mean $\delta^{13}C$ and $\delta^{15}N$ values of blood samples submitted to different drying methods and anticoagulants are shown in Table 5, and the $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios of the plasma are compiled in Table 6. No interaction between drying methods and anticoagulants were detected in either fraction (blood and plasma) for both elements ($\delta^{13}C$ and $\delta^{15}N$), with P values of 0.473 and 0.690 for $\delta^{13}C$ and $\delta^{15}N$ in the blood, respectively, and of 0.983 for $\delta^{13}C$ and 0.802 for $\delta^{15}N$ in the plasma.

Table 5 – Isotopic values of δ13C and δ15N (‰) (mean ± SD) of blood samples submitted to different drying methods and collected or not with anticoagulants*.

Treatments	No anticoagulant	Heparin	EDTA	Mean
		$\delta^{13}C$, ‰		
Liquid	-18.7 ± 0.30	-18.5 ± 0.47	-19.3 ± 0.75	-18.8
Oven-dried	-18.9 ± 0.32	-19.0 ± 0.32	-19.1 ± 0.73	-19.0
Freeze-dried	-19.0 ± 0.39	-19.0 ± 0.39	-19.2 ±0.65	-19.1
Mean	-18.9	-18.8	-19.2	
		$\delta^{15}N$, ‰		
Liquid	4.7 ± 0.26	4.7 ± 0.11	4.6 ± 0.34	4.7[a]
Oven-dried	4.4 ± 0.15	4.5 ± 0.19	4.5 ± 0.63	4.5[ab]
Freeze-dried	4.2 ± 0.13	4.4 ± 0.25	4.5 ±0.43	4.4[b]
Mean	4.4	4.5	4.5	

*Different letters in the same column differ statistically by the Tukey's test at $p<0.05$.

Blood $\delta^{13}C$ isotopic values (Table 5) were not affected either by the drying methods or by the use of anticoagulants ($p>0.05$); however, the drying process influenced $\delta^{15}N$ values ($p<0.05$), which were lower in lyophilized than in liquid blood.

Plasma $\delta^{15}N$ isotopic ratio was not influenced ($p>0.05$) by drying processes or anticoagulants (Table 6). EDTA-containing plasma presented 0.2 ‰ higher $\delta^{13}C$ ratio ($p<0.05$) compared with heparin-containing plasma.

Table 6 – Isotopic values of δ13C and δ15N (‰) (mean ± SD) of plasma samples submitted to different drying methods and collected or not with anticoagulants*.

Treatments	Heparin	EDTA	Mean
		$\delta^{13}C$, ‰	
Liquid	-17.5 ± 0.34	-17.8 ± 0.36	-17.6
Oven-dried	-17.6 ± 0.40	-17.9 ± 0.27	-17.7
Freeze-dried	-17.6 ± 0.38	-17.8 ±0.25	-17.7
Mean	-17.6[a]	-17.8[b]	
		$\delta^{15}N$, ‰	
Liquid	5.2 ± 0.28	5.3 ± 0.19	5.3
Oven-dried	5.1 ± 0.19	5.2 ± 0.21	5.2
Freeze-dried	5.1 ± 0.30	5.2 ±0.18	5.2
Mean	5.1	5.2	

*Different letters in the same column differ statistically by the Tukey's test at $p<0.05$.

The objective of calculating the isotopic fractionation (Δ tissue - diet) (Tables 7 and 8) of the blood fractions submitted to the experimental treatments was to determine how the dietary isotopes were incorporated into the tissues and if the drying and the fat removal methods would cause greater differences between the tissues and diets. Both lyophilization and EDTA resulted in higher carbon-13 tissue (blood and plasma) to diet ratios. Among blood collection methods, the blood and plasma of laying hens containing the anticoagulant EDTA promoted the highest nitrogen-15 tissue to diet ratio whereas lyophilization resulted in the lowest ratio among blood processing methods.

Lemons *et al.* (2012) studied the effect of the use of anticoagulants for collection of blood samples and its fractions in turtles on $\delta^{13}C$ $\delta^{15}N$ isotopic values, and observed ^{15}N depletion in plasma containing EDTA and ^{15}N enrichment in heparin-collected blood, which was not found in the present study.

Table 7 – Isotopic fractionation factor (Δtissue - diet) of blood samples submitted to different drying methods and collected or not with anticoagulants.

Treatments	No anticoagulant	Heparin	EDTA	Mean
		$\Delta_{tissue-diet}$ ^{13}C, ‰		
Liquid	-1.42	-1.24	-2.04	-1.57
Oven-dried	-1.61	-1.76	-1.82	-1.73
Freeze-dried	-1.77	-1.72	-1.97	-1.82
Mean	-1.60	-1.57	-1.94	
		$\Delta_{tissue-diet}$ ^{15}N, ‰		
Liquid	2.50	2.47	2.41	2.46
Oven-dried	2.20	2.23	2.30	2.24
Freeze-dried	2.00	2.16	2.30	2.15
Mean	2.23	2.29	2.33	

The comparison of the fractionation factors among eggs, broiler tissues and layer blood and plasma suggests that, when it is not possible to frequently sacrifice animals for isotopic studies, blood plasma and blood can be used to determine tissue to diet carbon-13 and nitrogen-15 isotopic ratios, respectively, due to their low isotopic fractionation value. According to Hobson & Clark (1992), blood can be used as a substitute for tissues without the need of sacrificing animals in dietary studies.

Table 8 – Isotopic fractionation factor (Δtissue - diet) of plasma samples submitted to different drying methods and collected or not with anticoagulants.

Treatments	Heparin	EDTA	Mean
	$\Delta_{tissue-diet}$ ^{13}C, ‰		
Liquid	-0.32	-0.60	-0.46
Oven-dried	-0.32	-0.42	-0.46
Freeze-dried	-0.26	-0.49	-0.37
Mean	-0.30	-0.56	
	$\Delta_{tissue-diet}$ ^{15}N, ‰		
Liquid	2.91	2.99	2.95
Oven-dried	2.91	2.99	2.95
Freeze-dried	2.92	3.11	3.01
Mean	2.91	3.03	

CONCLUSIONS

The fat extraction methods applied to layer eggs and broiler tissues did not affect the $\delta^{13}C$ or the $\delta^{15}N$ isotopic values of the studied samples.

The drying processes of the egg and blood samples from laying hens and broilers did not result in $\delta^{13}C$ $\delta^{15}N$ isotopic differences in any of the processes studied.

The tested anticoagulants did not interfere with the $\delta^{13}C$ or $\delta^{15}N$ isotopic ratios of the blood and plasma of the laying hens.

Therefore, it is possible to use the evaluated methods of fat extraction, drying and anticoagulants for the isotopic analyses of carbon-13 and nitrogen-15.

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