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Multivariate analysis of stable isotope data in the traceability process for birds

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ABSTRACT. Isotope analysis has proved to be an extremely important tool in the traceability process; however, statistical analyses of the results show discrepancies, as the data depend on and originate from several chemical elements such as carbon, hydrogen, oxygen, nitrogen and sulfur (CHONS). In order to establish the proper analysis of traceability data for birds using the stable isotope technique and evaluate the need for a combined analysis of the variables, data for carbon-13 and nitrogen-15 were used from eggs (albumen + yolk) of laying hens and the pectoral muscle of broilers, which were subjected to univariate statistical analysis (ANOVA and complemented with Tukey's test) and multivariate statistical analysis (MANOVA and Discriminant Analysis). The data were analyzed using Minitab 16 software, and the results, corroborated in the theory, confirm the need for multivariate analysis, showing also that discriminant analysis clarifies questions from the results of the other analysis methods compared in this study.

Keywords: ANOVA, discriminant analysis, carbon-13, MANOVA, nitrogen-15.

Análise multivariada em dados de isótopos estáveis no processo de rastreabilidade em aves

RESUMO. A análise isotópica tem se mostrado uma ferramenta de suma importância ao processo de rastreabilidade, no entanto, existem divergências nas análises estatísticas dos resultados, uma vez que os dados são dependentes e advindos de vários elementos químicos tais como Carbono, Hidrogênio, Oxigênio, Nitrogênio e Enxofre (CHON'S). Com o intuito de estabelecer a análise propícia para os dados de rastreabilidade em aves pela técnica de isótopos estáveis e avaliar a necessidade da análise conjunta das variáveis, foram usados dados de carbono-13 e de nitrogênio-15 de ovos (albúmen + gema) de poedeiras e músculo peitoral de frangos de corte, os quais foram submetidos à análise estatística univariada (Anova e complementada pelo teste de Tukey) e multivariada (Manova e Discriminante). Os dados foram analisados no software Minitab 16, e os resultados, consolidados na teoria, confirmam a necessidade de análise multivariada, mostrando também que a análise discriminante esclarece as dúvidas apresentadas nos resultados de outros métodos de análise comparados nesta pesquisa.

Palavras-chave: Anova, análise discriminante, carbono-13, Manova, nitrogênio-15.

Introduction

In animal traceability, the isotopic composition of carbon in tissues serves as a natural tracer of the different diets, each with distinct isotopic signatures. Conversely, the difference between the isotopic composition of the ingested material and that of animal tissue can be sensitive to nutritional status, turnover rate and biosynthetic route (KOCK et al., 1994). Some authors have evaluated the stable isotopes of carbon and nitrogen independently (univariate analysis).

Rogers (2009) analyzed 18 different chicken egg brands under three rearing types (in poultry houses, confined in cages and free range) using the stable isotope technique to evaluate the effect of diet (conventional and organic) on egg characteristics. Statistical analysis of the results for carbon and nitrogen was performed independently, that is, for the whole egg and its components (yolk and albumen) and for each variable (carbon and nitrogen). The author observed that the results for the variables carbon and nitrogen were different within the same sample; it was therefore concluded that the analysis of stable nitrogen isotopes in egg components is a potentially useful technique to determine the diet to which the birds were submitted, serving as an important authentication tool in the egg industry.

However, Denadai et al. (2009), while evaluating eggs from two producers in the area of Bastos, São

Paulo State – one using only plant-based products, the other using animal byproducts – detected a 1.5% inclusion of bovine meat and bone meal in the albumen of eggs from birds fed with that ingredient. They concluded that the combined analysis of $\delta^{15}N$ and $\delta^{15}C$ – that is, multivariate analysis of data from stable carbon and nitrogen isotopes – makes it possible to track the inclusion of animal-based meals in the diets of laying hens, by detecting it in the eggs.

Carrijo et al. (2006) observed a need for data analysis on the relative enrichment of carbon-13 ($\delta^{13}C$) and nitrogen-15 ($\delta^{15}N$) (multivariate analysis), which made it possible to detect the inclusion of bovine bone meal in broiler diets, found in the pectoral muscle. Also working with chickens, Gottmann et al. (2008) identified in the pectoral muscle the inclusion of poultry viscera meal in diets, even when other alternative ingredients (wheat bran and yeast) were added. Oliveira et al. (2010) analyzed the pectoral muscle, keel and tibia of broilers to identify which of these tissues would best track poultry viscera meal in the diets of broilers at a given rearing stage.

Móri et al. (2007) detected animal byproducts used in the diets of meat quails, in the pectoral muscle, keel and tibia.

Researchers worldwide have analyzed data on traceability using multivariate analysis known as discriminant analysis (GUO et al., 2010; HEATON et al., 2008). The literature shows that works on carbon and nitrogen isotopes have been analyzed for traceability using both univariate and multivariate analysis.

Multivariate (MANOVA) and univariate (ANOVA) analysis of variance

Both univariate (ANOVA) and MANOVA techniques are used to evaluate the statistical significance of intergroup differences. In ANOVA, the null hypothesis tested is the equality of the means of the dependent variable throughout the groups. In MANOVA, the null hypothesis tested is the equality of mean vectors on multiple dependent variables throughout the groups. The unique aspect of MANOVA is that the statistical variable optimally combines the multiple dependent measurements into a single value that maximizes the differences throughout the different groups.

The use of separate univariate ANOVAs can create a problem when attempting to control the general or experimental error rate. For instance, consider that that a series of five dependent variables were analyzed using separate ANOVAs, always using 0.05 significance. Given that there are real differences in the dependent variables, a significant effect is to be expected 5% of the time for any given dependent variable. However, Hair et al. (2009), observed that in five separate tests, the probability of a Type I error is about 5% if all dependent variables are perfectly correlated, and 23% (1- 0.95^5) if the dependent variables are non-correlated. Thus, a series of separate statistical tests leaves the effective general or experimental Type I error rate without any control. Should the researcher wish to keep control over the experimental error rate and there is any degree of intercorrelation among the dependent variables, then MANOVA is appropriate.

To complement MANOVA, two-dimensional graphs can be created evaluating the differences between one response vector and the others. This type of evaluation is mostly used when a single comparison is sought – with the standard, for example – given that if there are 10 treatments, 10 graphs would be created to evaluate all possible differences, which greatly hinders the analysis process.

Discriminant analysis

Discriminant analysis is an appropriate statistical technique for types of problems in which the dependent variable consists of two or more groups (classifications) – for instance, different treatments.

This analysis involves determining a statistical variable, the linear combination between two or more independent variables that will best discriminate between groups defined *a priori*. Discrimination is achieved by establishing the weights of the statistical variable for each variable, in order to maximize intergroup variance relative to intragroup variance. The linear combination for a discriminant analysis, also known as the discriminant function, is determined by the following equation:

$$Z_{jk} = \alpha + W_1 X_{1k} + W_2 X_{2k} + ... + W_i X_{ik}$$

where:

Z_{jk} = discriminant Z-score of discriminant function *j* for object *k;* α = intercept;

W_i = discriminant weight for independent variable *i*;

X_{ik} = independent variable *i* for object *k*.

Discriminant analysis is the appropriate statistical technique for testing the hypothesis that the means of a set of independent variables for two or more groups are equal. To that end, discriminant analysis multiplies each independent variable by its corresponding weight and adds both products. The

result is a discriminant Z-score for each individual in the analysis. Calculating the mean between the discriminant scores for all individuals in a group, the group mean is obtained. This group mean is known as the centroid.

The statistical significance test for a discriminant function is a generalized measurement of the distance between group centroids. It is computed by comparing the distributions of the discriminant scores for the groups. If the overlap in the distributions is small, the discriminant function separates the groups well. If there is large overlap, the function is a poor discriminator between the groups (HAIR et al., 2009).

The objective of this article was to shed light on the differences between the existing analysis techniques and establish a statistical analysis featuring greater detail on the results of stable carbon-13 and nitrogen-15 isotopes for traceability in birds. To that end, the study analyzed carbon-13 and nitrogen-15 data from the eggs, pectoral muscle and keel of birds using univariate (ANOVA and Tukey's test) and multivariate statistical methods (MANOVA and discriminant analysis).

Material and methods

Data on eggs

The data on eggs were obtained in an experiment performed at the School of Veterinary Medicine/FMVZ, Botucatu, São Paulo State, Brazil in 2008, in which 240 laying hens were used, in a completely randomized design, with treatments and six replications. Treatments consisted of five inclusion levels (0, 1.5, 3.0, 4.5 and 6.0%) of bovine meat and bone meal in the corn- and soybean meal-based diet. On the 35th day, 12 eggs were collected at random per treatment, two per replication, to measure the isotopic enrichment of $\delta^{13}C$ and $\delta^{15}N$. Isotope analysis was carried out at the Stable Isotopes Center (CIE) at IB/UNESP/Botucatu.

Data on the pectoral muscle of broiler chickens

The experiment on the pectoral muscle of broilers was carried out at the School of Veterinary Medicine and Animal Science/UFMS, Campo Grande, Mato Grosso do Sul State, Brazil in 2007, measuring the relative isotopic enrichment of $\delta^{13}C$ and $\delta^{15}N$ at the CIE from 10 samples, per treatment and per collection date, of pectoral muscle from three treatments with inclusion of 0, 6 and 12% poultry viscera meal in the corn- and soybean meal-based diet of broilers, collected at 21 and 42 days of age.

Statistical analyses of traceability data

The first phase consisted of separately analyzing the data on carbon-13 and nitrogen-15 stable isotopes, using univariate analysis of variance (ANOVA) complemented with Tukey's test. This analysis was carried out to assess the influence of the rate of Type I errors described by Hair et al. (2009), thus attesting whether the variables were perfectly correlated.

In the second phase, the data were explored by MANOVA multivariate analyses, evaluating the comparison between the standard (0% inclusion) and the other treatments, represented by the two-dimensional graph, as well as discriminant analysis.

Lastly, the results obtained by the different methods were evaluated, and the most adequate method was determined for use in traceability data for birds through the stable isotopes of carbon-13 and nitrogen-15.

Results and discussion

Statistical analysis results for eggs

A significant difference was evident between treatments, through univariate analysis of variance (ANOVA) (Table 1) for variable carbon-13 in data on eggs. Only the treatments with 4.5 and 3.0% inclusion of bovine meat and bone meal did not show significant differences between one another; the others differed by Tukey's test at 5% significance (Table 1).

For variable nitrogen-15, a significant difference was observed through ANOVA (Table 1); also, the treatment with 6.0% inclusion of bovine meat and bone meal did not differ from the treatment with 4.5% inclusion, the treatment with 4.5% inclusion did not differ from the treatment with 3.0% inclusion, and the treatment with 1.5% inclusion did not differ from the standard by Tukey's test at 5% significance (Table 1). These results differed from that observed for the variable carbon-13.

Table 1. Results of statistical analyses of variables carbon-13 and nitrogen-15 for eggs.

Treatments	Average values	
	Carbon-13	Nitrogen-15
6.0	-16.8758^{a}	5.1700^{a}
4.5	-17.3150^{b}	5.0175^{ab}
3.0	-17.4283^{b}	4.8100^{b}
1.5	-17.7717^{c}	4.5783^{c}
0.0	-18.3050^{d}	4.4308^{c}
p*	< 0,01	< 0,01

*Result of variance analyses. Same letters in the same column do not differ by Tukey test.

MANOVA shows that there is a significant difference between treatments (Table 2) for all criteria used. Figure 1 shows the difference

between the standard (0% inclusion of bovine meat and bone meal) and the other treatments, where it is seen that only the treatment with 1.5% inclusion did not differ from the standard.

Table 2. MANOVA for the variables carbon-13 and nitrogen-15 in eggs.

Criterion	Test estatístico	F	P
Wilks'	0.05383	44.688	0.000
Hotelling	15.61062	103.420	0.000
Pillai's	1.05207	15.261	0.000
Roy's	15.48356		

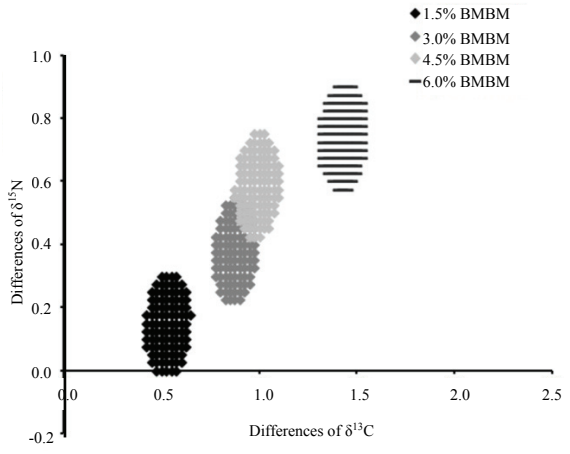

Figure 1. Two-dimensional representation of MANOVA, comparison between the different treatments with inclusion of bovine meat and bone meal and the treatment without inclusion, for eggs.

However, in the discriminant analysis (Table 3) it was observed that the treatments with inclusion of 0 and 1.5% of bovine meat and bone meal did not differ from one another; the same happened between treatments with 1.5 and 3.0%, and treatments with 3.0 and 4.5% inclusion. The treatment with 6% meal inclusion differed from all other treatments.

When the statistical analysis was performed for each ANOVA variable, it was observed that the treatments differed, as there was a difference in one variable between the treatments. Thus, the treatment with 6% inclusion of meat meal would not show any statistical difference from the treatment with 4.5% inclusion of meal (Table 2), for this analysis.

However, when the variables were analyzed collectively (Multivariate Discriminant Analysis, Table 3) it was observed that the treatment with 6% inclusion differed from the other treatments, showing a different result than the univariate analysis. This difference can be attributed to the general or experimental error rate (HAIR et al., 2009). The differences obtained for the other treatments were common to both types of statistical analysis, univariate and multivariate.

Table 3. Groups formed by discriminant analysis for the variables carbon-13 and nitrogen-15, in eggs original.

Groups Formed	Groups original				
	0	1.5	3.0	4.5	6.0
0.0	11	1	0	0	0
1.5	1	11	1	0	0
3.0	0	0	9	2	0
4.5	0	0	2	10	0
6.0	0	0	0	0	12
Total N	12	12	12	12	12
N correct	11	11	9	10	12
Percentage	0.92	0.92	0.75	0.83	1.00

Statistical analysis results for the pectoral muscle

For the data on the pectoral muscle of 21-day-old birds, there was a difference between the treatments using univariate analysis of variance (ANOVA) for variable carbon-13 (Table 4). The treatments that did not differ from one another were those with 6 and 12% inclusion of poultry viscera meal (Table 4).

ANOVA also showed a significant difference (Table 4) for variable nitrogen-15 in the pectoral muscle of 21-day-old birds. The treatments with 0 and 6% inclusion did not differ from one another; the same was observed between treatments with 6 and 12% inclusion. However, the treatments with 0 and 12% inclusion were statistically different by Tukey's test at 5% significance (Table 4).

Table 4. Results of the statistical analyses of variables carbon-13 and nitrogen-15 for the pectoral muscle, at 21 days of age.

Treatments	Average values	
	Carbon-13	Nitrogen-15
12	-17.6820^{a}	2.8760^{a}
6	-17.7120^{a}	2.7820^{ab}
0	-18.8340^{b}	2.5940^{b}
p	< 0.01	< 0.05

*Result of variance analyses. Same letters in the same column do not differ by Tukey test.

Using multivariate analysis of variance (MANOVA) it can be verified that there was a significant difference between the treatments for the variables (Table 5), confirming the same result obtained through ANOVA. In the graph (Figure 2), it was observed that the treatment with 6% inclusion of viscera meal did not differ from the 0% treatment; the 12% treatment, however, showed statistical difference from the treatment with 0% inclusion. This graph does not provide any conclusion on differences between the treatments with 6 and 12% inclusion.

Table 5. MANOVA for variables carbon-13 and nitrogen-15 of the pectoral muscle, at 21 days of age.

Criterion	Test estatísticos	F	P
Wilks'	0.32095	9.947	0.000
Lawley-Hotelling	2.06890	12.931	0.000
Pillai's	0.69411	7.175	0.000
Roy's	2.04597		

Discriminant analysis showed that only the treatment with 12% inclusion of viscera meal showed statistical difference from the treatment with 0% inclusion; the treatments with 0 and 6% meal inclusion did not show statistical difference by that method in only one sample (Table 6). That fact, confirmed by MANOVA, could not be detected for the pectoral muscle by carbon-13 analysis, only for variable nitrogen-15. It can be observed that the treatments with 6 and 12% inclusion did not differ from one another. Therefore, the variable nitrogen-15 caused the influence in the non-differentiation between the treatments.

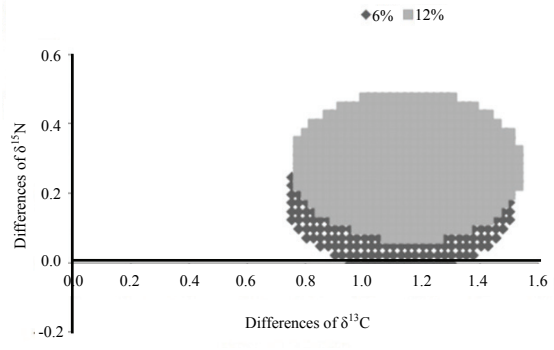

Figure 2. Two-dimensional representation of MANOVA, comparison between the treatments with inclusion of 6% and 12% and the treatment without inclusion of viscera meal, for the pectoral muscle, at 21 days of age.

Table 6. Groups formed by discriminant analysis for variables carbon-13 and nitrogen-15 in the pectoral muscle, at 21 days of age.

Groups Formed	Groups original		
	0	6	12
0	9	1	0
6	1	5	4
12	0	4	6
Total N	10	10	10
N correct	9	5	6
Percentage	0.90	0.50	0.60

The variable carbon-13, for 42-day-old birds, showed statistical difference between all three treatments (Table 7). However, for the nitrogen variable the treatments with 0 and 6% inclusion of viscera meal did not differ from one another, and the same could be observed between the treatments with 6 and 12% inclusion (Table 7).

Table 7. Results of the statistical analyses of the variables carbon-13 and nitrogen-15 for the pectoral muscle, at 42 days of age.

Treatments	Average values	
	Carbon-13	Nitrogen-15
12	-17.8540[a]	2.9770[a]
6	-18.4460[b]	2.7620[ab]
0	-19.2170[c]	2.6310[b]
p	0.000	0.012

*Result of variance analyses. Same letters in the same column do not differ by Tukey test.

Using MANOVA it was observed that the combined variables showed differences between the treatments (Table 8). That difference was found only for the treatments with 0 and 12% inclusion; the same was not observed between the treatments with 0 and 6% inclusion of viscera meal (Figure 3).

Discriminant analysis demonstrated that only one sample of the treatment with 0% inclusion was related to the treatment with 6% inclusion; the same was observed for the treatment with 6% inclusion in relation to the 12% treatment – that is, even though they were statistically different, only two of the 30 total samples were part of groups other than the original ones (Table 9).

Thus, it was concluded that those two samples likely influenced the result obtained through MANOVA (Figure 3), and that nitrogen-15 was probably the variable that led to this result, as the same result was repeated for nitrogen-15 data (Table 7).

Table 8. MANOVA for the variables carbon-13 and nitrogen-15 of the pectoral muscle, at 42 days of age.

Criterion	Tests estatísticos	F	P
Wilks'	0.20315	15.843	0.000
Hotelling	3.85594	24.100	0.000
Pillai's	0.81038	9.196	0.000
Roy's	3.83859		

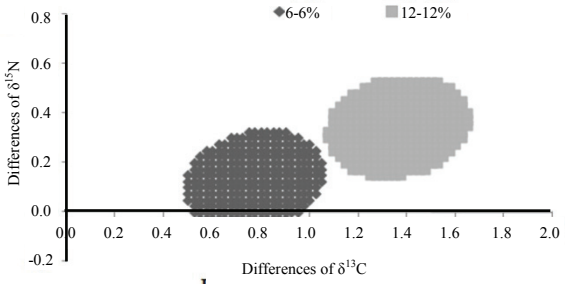

Figure 3. Two-dimensional representation of MANOVA, comparison between the treatments with inclusion of 6% and 12% and the treatment without inclusion of viscera meal, for the pectoral muscle, at 42 days of age.

Hair et al. (2009) highlight that a large sample (more than 20 elements) is necessary; however, the number of sample elements in traceability analyses is not always that large. In those cases, it is extremely important to investigate the obtained results in greater detail.

Table 9. Groups formed by discriminant analysis for variables carbon-13 and nitrogen-15 in the pectoral muscle, at 42 days of age.

Groups	Groups original		
Formed	0	6	12
0	9	0	0
6	1	9	0
12	0	1	10
Total N	10	10	10
N correct	9	9	10
Percentage	0.90	0.90	1.00

The need for carbon-13 and nitrogen-15 analysis for bird traceability is thus emphasized. Furthermore, the combined data analysis showed a more adequate result for problems with more than one dependent variable, which can be confirmed by Carrijo et al. (2006), Gottmann et al. (2008), Denadai et al. (2009) and Oliveira et al. (2010); discriminant analysis gave a more detailed result showing the relationship between each sample (replication) and the treatments in question, which was also observed by Heaton et al. (2008) and Guo et al. (2010).

Conclusion

Traceability using stable isotopes should be evaluated by more than one variable, such as carbon-13 and nitrogen-15.

The proper statistical analysis for problems with more than one dependent variable – in this case carbon-13 and nitrogen-15 – could be multivariate analysis (MANOVA) complemented with discriminant analysis.

Among the methods analyzed in this research, discriminant analysis also gives the relationship between each sampled value and the others.

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