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Quantification of soy protein using the isotope method ($\delta^{13}C$ and $\delta^{15}N$) for commercial brands of beef hamburger

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ABSTRACT

Hamburgers (beef patties) may be adulterated through the overuse of protein extenders. Among vegetables, soy protein is the best substitute for animal protein. These ingredients help to reduce the cost of producing a final product, and they maximize profits for fraudulent industries. Moreover, the ingestion of soy or other non-meat proteins by allergic individuals may present a health risk. In addition, monitoring by supervisory bodies is hampered by a lack of appropriate analytical methodologies. Within this context, the aim of this study was to determine and quantify the levels of added soy protein by determination of ^{15}N and ^{13}C stable isotopes. A total of 100 beef hamburger samples from 10 commercial brands were analyzed. Only three samples of the G brand were within the standards set the Brazilian legislation. The remaining 97 samples from 10 commercial brands contained >4% soy protein; therefore, they are adulterated and not in compliance with the current legislation.

1. Introduction

The burger is susceptible to fraud by excessive use of protein extenders. Among the protein extenders, the textured soy protein is the one that has been most used. The soy is considered among the vegetables, the best replacement of animal products.

In the meat industry, binders, fillers, emulsifiers and stabilizers are used to increase the emulsion stability, cut and flavor characteristics and primarily reduce formulation costs (ITAL, 1981). In meat products, soy protein improves the texture and enhances the appearance, firmness, juiciness, sliceability and cooking yield; in addition, soy protein replaces animal fat (Castro-Rubio, Garcia, Rodriguez, & Marina, 2005; Xiong, 2005). Extenders increase manufacturing yield due to increase the weight of the final product compared with the original (Olivo, 2006).

The ground beef used in hamburger may therefore be supplemented with hydrated soy protein and according to the compositional requirements (Brasil, 2000), only the "maximum addition of 4.0% of non-meat protein in aggregate form" is permitted.

The ingestion of soy or other non-meat proteins by allergic individuals may present a health risk; therefore, it should be controlled by providing accurate information to the costumers (Mellenthin & Galensa, 1999).

According to the FDA (2015) there are >160 foods that can cause allergy and 90% of cases of allergic reactions are connected to eight foods (milk, eggs, fish, shellfish, nuts, peanuts, wheat and soy). These foods and other ingredients that contains protein derived from one or more of them, are called allergens and must be indicated on the label.

The Brazilian legislation has specified the maximum amount of meat extenders in meat products; however, it is difficult to detect and quantify these ingredients during inspections because there is a lack of appropriate analytical methods that can identify these ingredients (Belloque, Garcia, Torre, & Marina, 2002).

Flint and Meech (1978) were among the first to use quantitative or stereological microscopy to quantify the textured soy protein in model systems of meat and soy (Rodrigues, 1986). This technique, however, has presented problems due to low accuracy in determination of certain types of soy products, being very time consuming, resulting in a high cost per determination (Griffiths, Billington, & Griffiths, 1981).

The same technique was collaboratively tested in 1980/81, and was considered not to be exact, therefore, the immunoenzymatic method ELISA (enzyme linked immunosorbent assay) has been investigated as an alternative procedure for determination of soy proteins in food (Crimes, Hitchcock, & Wood, 1984).

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The Association of Official Analytical Chemists (AOAC) proposes the use of enzyme linked immunosorbent assay (ELISA) for soy control in meat products (Method 988.10; AOAC, 2005). The technique is a semi-quantitative enzymatic immunoassay (ELISA). However, in order to render quantitative instead of qualitative results, the soy cultivar must be known.

The determination of soy protein in meat products can also be performed by analysis of peptides after enzymatic hydrolysis and the product can be characterized by the amino acid analyzer. It is a time consuming technique (5 to 6 days), which uses a specialized equipment and is not available for laboratories of routine analysis, which require rapidity to a large number of samples (Agater et al., 1986; Bailey, 1976).

Several references propose the use of techniques (High Performance Liquid Chromatography – HPLC, Polymerase Chain Reaction – PCR, Western blotting and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis - SDS-PAGE) as an alternative to ELISA, which is considered of limited accuracy, time consuming, costly and complex. For these disadvantages, the Enzyme Linked Immuno Sorbent Assay have been heavily criticized (Leitner, Castro-Rubio, Marina & Lindner, 2006).

Brazilian law specified limits for the addition of non-meat ingredients in order to promote extension, but its detection and quantification is still a problem for the supervision, due to the shortage of appropriate analytical methodologies for determining these ingredients (Belloque et al., 2002).

Among the techniques that can be used to evaluate the quality of hamburger, analyses of the natural variability of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes are noteworthy. Especially if it is considered that it requires only 25 g of each sample and the official report may be presented within 3 days.

The cost of an isotopic analysis is relatively low, especially if it is considered that requires a much smaller number of samples, compared to other methods (Misi et al., 2004) as the quantitative microscopy, ELISA (enzyme linked immunosorbent assay) or by analysis of peptides after enzymatic hydrolysis.

Mass spectrometry, which is used in $^{13}C/^{12}C$ isotope ratio analysis, has been successfully employed for testing the authenticity and quality of products such as milk (Brescia, Monfreda, Buccolieri, & Carrino, 2005; Renou et al., 2004; Ritz et al., 2005), fish (Focken, 2004; Gaye-Siessegger, Focken, Muetzel, Abel, & Becker, 2004; Trueman, Mcgill, & Guyard, 2005), cheese (Camin et al., 2004; Manca et al., 2001), meat (Bahar et al., 2005; Boner & Forstel, 2004), honey, syrup, vinegar, ethanol, distillates, beer, brandy, oil, aromatic compounds, additives, etc. (Winkler & Schmidt, 1980).

The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratio values have allowed the use of mass spectrometry to assess the presence of soy protein in beef hamburgers sold to consumers. However, there are no references in the literature on the use of stable isotope techniques to investigate these products, especially in investigations of adulteration related to excess soy protein in Brazilian commercial brands. The technique of stable isotopes can be used as a tool aimed at the authenticity of the food, particularly where conventional analytical methods may not provide fully reliable results (Bahar et al., 2005).

Within this context, the present study aimed to determine the amount of soy protein in the commercial beef hamburgers and compared our findings with the information given on the label.

2. Materials and methods

In preliminary tests, the ingredients emulsifier, stabilizer, mixture of dextrin with antioxidant and steak flavor, were analyzed. The results of carbon and nitrogen deltas indicated that these ingredients do not interfere in the $\delta^{13}C$ and $\delta^{15}N$ of added soy protein in the beef burgers (Ducatti, 2014).

To confirm that the control samples 100% beef were hamburgers composed of meat without addition of soy protein, we analyzed samples of cuts of tenderloin and thin skirt. The values of tenderloin ($\delta^{13}C = -13.28$ and $\delta^{15}N = 5.78$) and thin skirt ($\delta^{13}C = -12.99$ and $\delta^{15}N = 7.13$) were close to the values of the control samples 100% beef (L) ($\delta^{13}C = -12.99$ and $\delta^{15}N = 5.84$) (Table 1). Similarly, when compared, the soy deltas ($\delta^{13}C = -25.07$ and $\delta^{15}N = 1.96$) were similar to the hamburger containing only soy protein and to the 100% soy control (M) ($\delta^{13}C = -26.02$ and $\delta^{15}N = -0.54$).

To evaluate the isotopic profile and verify if in the beef hamburgers containing >4% soy protein, 10 commercial brands (with 10 replicates of each brand) were analyzed and nominated as brands A, B, C, D, E, F, G, H, I and J, for a total of 100 commercial hamburgers samples. Referred to as the L samples were the control 100% meat, whereas the M samples were the control 100% soy.

Besides that, the isotopic analyzes were performed separately with 10 replicates of control 100% meat (L) and 10 of control 100% soy (M) to stay for that standards.

The samples were collected in the supermarkets of state of São Paulo and forwarded under refrigeration for the Stable Isotope Center of the Biosciences Institute, UNESP/Botucatu-Brasil. Then, to perform the isotopic analysis of the hamburger meat, 25 g samples were thawed and dried in a forced-air oven at 56 °C for 48 h.

Since lipids are relatively poor in ^{13}C the amount of fat in the samples had to be considered. Fat was extracted from the hamburger samples with diethyl ether (Soxhlet method) and kept at 55–65 °C for 4 h. According to Tieszen, Boutton, Tesdahl, and Slade (1983), the fractions that have a high value of lipids are relatively poor in ^{13}C compared to fractions that have a small amount of fat.

Posteriorly, the hamburger samples were ground in a liquid nitrogen cryogenic mill at −196 °C for 3 min at the maximum frequency to obtain a homogeneous, microscopic material of very fine particle size (particles <60 μg) (Rosa, Moraes, Gomes Neto, Nobrega, & Nogueira, 2002).

Then, the ground and defatted burgers were weighed in high precision scales (Mettler Toledo AG, MX5, Excellence Plus), 50–60 μg for carbon and 500–600 μg for nitrogen in tin capsules, to define the isotopic ratios of carbon and nitrogen (Ducatti, 2014).

Soon after weighing, the samples were analyzed by a Delta-S mass spectrometer (Delta V Advantage Isotope Ratio MS, Thermo Scientific) coupled to an elemental analyzer (Flash 2000 Organic Elemental Analyzer EA for IRMS) to determine the isotopic composition of the products (Ducatti, 2014). The samples were transformed into gaseous form to make readings as well as the reference standard.

The gaseous form most commonly used for the analysis of carbon is CO_2, and for nitrogen is N_2. In the spectrometer, the gas molecules are

Table 1
Average data of $\delta^{13}C$ and $\delta^{15}N$ and their standard deviation of commercial brands of beef hamburgers.

	$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
Brand	Average	STDEV	Average	STDEV
(A)	−18,17d	0,85	2,94d	0,24
(B)	−19,98f	0,52	1,87e	0,14
(C)	−19,40ef	0,48	2,21e	0,28
(D)	−19,70ef	0,36	1,68e	0,23
(E)	−17,12c	0,73	4,10c	0,22
(F)	−15,85b	0,35	4,45bc	0,08
(G)	−13,34a	0,38	4,75b	0,53
(H)	−15,73b	0,34	3,27d	0,25
(I)	−18,78de	0,43	3,20d	0,19
(J)	−18,91de	0,88	3,02d	0,36
Control samples 100% beef (L)	−12,99a	0,32	5,84a	0,47
Control samples 100% soy (M)	−26,02g	1,24	−0,54f	0,80
Tenderloin	−13,28	–	5,78	–
Thin skirt	−12,99	–	7,13	
Soy	−25,07	–	1,96	–

A–J = Brazilian commercial brand beef hamburger; L = control samples 100% meat; M = control samples 100% soy; $\delta^{13}C$ and $\delta^{15}N$ = enrichment isotopic relative to ^{13}C and ^{15}N; STDEV = standard deviation; average followed by different letters in the column, differ by Tukey test ($p < 0.05$).

ionized and then, these ions are separated into a spectrum according to the ratio of mass/charge (m/z) using magnetic and electric fields (Barrie & Prosser, 1996).

Already in the detection system, the characteristic ion beams of each isotope beam are collected in Faraday cups. The neutralization of these ions results in electric currents. These electric currents are amplified, recorded and at last occurs the data acquisition on the computer (Boutton, 1996; Ducatti, 2007).

The results were expressed as values of $\delta^{13}C$ based on the *Peedee Belemnite* (PDB) standard, which has a 0.2‰ margin of error, and values of $\delta^{15}N$ relative to the atmospheric N_2 standard, which has a 0.3‰ margin of error and were calculated by Eq. (1):

$$\delta(\text{sample, standard}) = [(\text{Rsample}/\text{Rstandard}) - 1] \quad (1)$$

where δ = enrichment of heavier isotope of the chemical element X (C or N) of the sample relative to its international standard. Dimensionless; R = represents the isotope ratio between the least and the most abundant isotope, $^{13}C/^{12}C$ and $^{15}N/^{14}N$. Dimensionless.

The isotopic results were submitted to multivariate analysis of variance (MANOVA) with the GLM procedure of SAS (1996), compiled into standard isotopic straight, using the Origin 6.0 Professional program (MicroCal Software Original 6.0 Professional, 1999), also being evaluated by ANOVA and Tukey test.

The legal limit (4%) was obtained by theoretical measurement of the amount of source of C_3 and C_4, based on the principle of isotope dilution. The basic principle of isotope dilution method is based on mixture of two isotopically different sources with each other, generating a product whose isotopic composition reflects the contribution of these two sources, as well as the relative amount of each of them (Oliveira, 2002).

To determine the legality of commercial beef hamburgers, a system of equations was used (Eqs. (2) and (3)) with two sources (S1 and S2) and two isotopes proposed by Philips (2001).

The system of equations is a quantitative method according to the isotopic mass balance, which enables measuring the participatory indices of the sources in the product (Ducatti, 2007).

$$\text{Product} = \text{Source S1} + \text{Source S2}$$

$$\delta^{13}C = a\,\delta S1 + b\,\delta S2 \quad (2)$$

$$\delta^{15}N = a\,\delta' S1 + b\,\delta' S2 \quad (3)$$

where S1 = soy; S2 = meat; Product = hamburger; δS1 and δS2 = relative isotopic enrichment of carbon from S1 of C_3 carbon (soy) and S2 of C_4 carbon (meat), respectively; δ'S1 and δ'S2 = relative isotopic enrichment of nitrogen from S1 of C_3 nitrogen (soy) and S2 of C_4 nitrogen (meat), respectively; $\delta^{13}C$ and $\delta^{15}N$ = relative isotopic enrichment of the product (hamburger) in ^{13}C and ^{15}N, respectively; a = relative contribution from S1; b = relative contribution from S2.

Isolating the value (b) of Eq. (2) and of Eq. (3) and equating each other, obtain the value of (a), in the other words the participatory index of soy source.

$$\frac{\delta^{13}C - a\delta F_1}{\delta F_2} = \frac{\delta^{15}N - a\delta' F_1}{\delta' F_2}$$
$$a = \frac{\delta^{13}C\delta' F_2 - \delta^{15}N\delta F_2}{\delta F_1\delta' F_2 - \delta' F_1\delta F_2} \quad (4)$$

Substituting the value of (a) in Eq. (2) or in Eq. (3) obtains the value of (b), in the other words the participatory index of meat source.

$$b = \frac{\delta^{15}N\delta F_1 - \delta^{13}C\delta' F_1}{\delta F_1\delta' F_2 - \delta' F_1\delta F_2} \quad (5)$$

Obtaining a + b = 1, since there are only two participatory sources and one product.

3. Results and discussion

Average data of $\delta^{13}C$ and $\delta^{15}N$ and their standard deviations of 10 commercial brands beef burgers and the control samples are shown in Table 1. The statistical analysis revealed significant ($p < 0.01$) differences between tested samples.

The control samples 100% beef (○) (Fig. 1) showed the average value of −12.99‰ for $\delta^{13}C$, characteristic of tissues of C_4 plants (−12‰), like tropical grasses and fodder, which are commonly used to feed cattle (matter of raw burgers analyzed). The high average value of $\delta^{15}N$ (5.84‰) of the control samples 100% beef is based on the fact that the cattle are close to the top of the food chain.

In contrast to the values of control samples of meat samples, control samples 100% soy had an average value of −26.02‰ for $\delta^{13}C$ and −0.54‰ for $\delta^{15}N$, values peculiar to the plant of C_3 photosynthetic cycle (soy) and nitrogen fixing plants.

The brands B (41.9%), C (46.7%) and D (41.3%) (calculated by Eqs. (2) and (3)) showed the least amount of meat in its composition, namely 57.3, 57.1 and 55.4%. In other words, more than half the burgers were composed of soy protein. The other brands present intermediate percentages of meat: A (57.3%), H (69.1%), I (57.1%) and J (55.4%). The brand G was the brand with the highest quantity of meat (89.8%), followed by the brand F (78.1%) and the brand E (70.5%).

Isotope ratios of the samples and controls are visualized in Fig. 1, with a confidence interval of 95%.

Eqs. (1) and (2) were used to calculate the isotopic pair ($\delta^{13}C = -13.5$‰ and $\delta^{15}N = 5.58$‰) corresponding to the 4% (▲) addition of soy protein permitted by law (Brasil, 2000) (Fig. 1).

For the data of δ C and δ N of controls (soy and meat), a linear regression was performed. This straight represents the possible values that these products can take in the mix. In Fig. 1, the upper and lower lines indicate the 95% reliability area for the regression line (central line).

By linear regression and the coefficient of determination (0.95), the straight line is well adjusted to the selected points (Fig. 1). The function obtained by linear regression was $\delta^{13}C = -24{,}71$ and $\delta^{15}N = 1{,}97$.

Fig. 1 clearly shows the decrease in the $\delta^{13}C$ value of the hamburgers with the increasing percentage of soy protein. Therefore, as companies add soy protein to their food products, the isotopic enrichment value of hamburgers tends to become closer to that of soy protein $\delta^{13}C$ (−26.02‰) and further from that of meat $\delta^{13}C$ (−12.99‰). The same trend occurred with the nitrogen values, with greater amounts of added soy protein resulting in smaller $\delta^{15}C$ values, with the isotope values closer to −0.54‰ (100% soy) and farther from 5.84‰ (100%

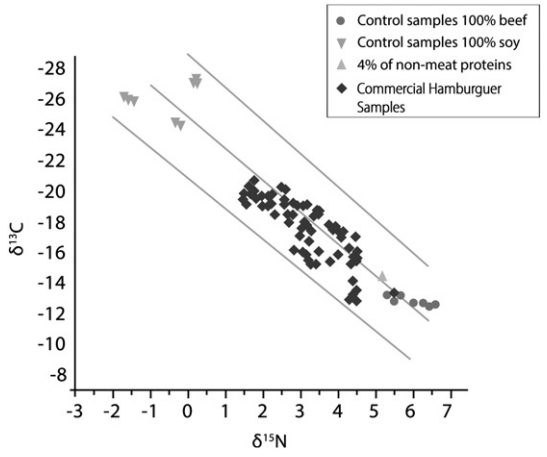

Fig. 1. Standard straight based on the carbon and nitrogen isotope data from the meat and soy control samples and analysis of the commercial hamburger samples.

beef). Only the 10 control samples 100% beef (○) and 3 samples (◊) (symbols on overlap in Fig. 1) of the G brand were found to be below the 4% threshold for soy allowed by the law.

The addition of >4% soy protein is associated with the fraudulent practices of food producers who employ unauthorized additives, outside the permitted limits or incorrectly, usually targeting the best competitive conditions and, above all, profit.

For this reason food labeling must be clear and informative for the consumer. Thus, quantitative methods are needed to test if the specification on the label is in conformity with the composition of the food. As support to the citizen, isotope analysis offers possibilities for quantitative food analysis and can help to uncover fraudulent practices or adulterated foods.

4. Conclusions

The stable isotopes technique certified that only three beef hamburger samples of the G brand showed values within the permitted range of added soy by the Normative Instruction no. 20 of 07/31/2000 (Brasil, 2000). The other 97 samples from 10 commercial brands showed >4% soy protein; therefore, they are in violation of the specific current national legislation.

The inedited technique of stable isotopes can guarantee the authenticity of the information on the label and provide a possible certification of standards for the hamburgers worldwide.

Author contributions

Rhani Ducatti: execution and writing the manuscript.

José Paes de Almeida Nogueira Pinto: conception of the study.

Maria Marcia Pereira Sartori: statistical analysis and drafted the figure.

Carlos Ducatti: planning and interpretation of results.

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