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Discrimination between conventional and omega-3 fatty acids enriched eggs by FT-Raman spectroscopy and chemometric tools

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ABSTRACT

This work developed an analytical method to differentiate conventional and omega-3 fat acids enriched eggs by Raman spectroscopy and multivariate supervised classification with Partial Least Squares Discriminant Analysis (PLS-DA). Forty samples of enriched eggs and forty samples of different types of common eggs from different batches were used to build the model. Firstly, gas chromatography was employed to analyze fatty acid profiles in egg samples. Raman spectra of the yolk extracts were recorded in the range from 3100 to 990 cm^{-1}. PLS-DA model was able to correctly classify samples with nearly 100% success rate. This model was validated estimating appropriate figures of merit. Predictions uncertainties were also estimated by bootstrap resampling. The most discriminant Raman modes were identified based on VIP (variables importance in projection) scores. This method has potential to assist food industries and regulatory agencies for food quality control, allowing detecting frauds and enabling faster and reliable analyzes.

1. Introduction

Modified foods have been incorporated into the human nutrition due to their numerous health benefits. A large field in the food industry is concerned with making fortified foods mainly by incorporating vitamins and essential fatty acids (FA). Often, these modified foods have much higher commercial value than the traditional foods. Thus, two technical aspects related to this type of food have given rise to important discussions. One aspect concerns on how to incorporate specific nutrients in the foods in a suitable balance aiming to improve the human diet. The other aspect is related to the increasing demand of methods for the quality control of the end food products aiming to avoid counterfeiting and/or adulterations, which could harm the consumers.

In the case of lipids, FA of interest may be added to the processed food or even precursors of essential FA may be incorporated into the feed of animals that provide common foods to the human diet, such as cow's milk, fish and eggs. Several studies have reported the benefits of consuming foods with a proper balance of lipids as these have direct impact on diseases, such as myocardial infarction, stroke and general obesity (Bassett et al., 2009).

The traditional methodology for FA quantification utilizes gas chromatography (GC) (Seppänen-Laakso, Laakso, & Hiltunen, 2002). However, this technique requires extraction and sample derivatization, in a sequence of preparation steps that results in a tedious and laborious procedure. The running time of the analytical method is an important factor for the food industry, especially for perishables such as milk, eggs and meat. Thus, the development of new, rapid and simple methods, with minimal sample preparation, is required by the food quality control agencies.

Precursors of essential FA have been incorporated into the chicken feed for the production of enriched eggs. An example is the use of linseed oil mixed with chicken feed, in order to obtaining eggs with higher omega-3 content (Souza et al., 2008; Tesedo, Barrado, Sanz, Tesedo, & de la Rosa, 2006). The lipid composition of this food matrix has been reported by separation techniques, such as gas chromatography and capillary electrophoresis (Mazalli & Bragagnolo, 2007; Porto, de Souza, & de Oliveira, 2011). However, at present there are no studies differentiating common eggs from enriched omega-3 eggs by using spectroscopic techniques. Vibrational spectroscopic techniques, such as near infrared, mid infrared and Raman spectroscopies, in combination with chemometric tools are particularly appropriate for developing rapid and simple analytical methods involving a minimum number of sample pretreatment steps. For food analysis, Raman

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spectroscopy presents the additional advantage of not suffering interference from water molecules.

Recently, the potential of the Raman spectroscopy has been reviewed in the field of lipid analysis (Czamara et al., 2015). The literature has reported studies involving the application of this technique to lipid analysis in different matrices, such as vegetable oils (Mendes et al., 2015), meat (Olsen, Rukke, Flåtten, & Isaksson, 2007), fish (Afseth, Wold, & Segtnan, 2006) and cow's milk (Mendes et al., 2016). All of these studies have processed Raman spectra with multivariate methods, providing methods that present the rapidity of analysis as a differential, without or with a minimum sample preparation.

In spite of the potential of Raman spectroscopy for the analysis of an important food matrix such as egg, only one article has been published utilizing a more sensitive variant of this technique, surface-enhanced Raman spectroscopy (SERS), and a quantitative chemometric model for detecting trace amounts of melamine spiked in egg yolk (Cheng & Dong, 2011). Considering the multivariate nature of the problem under study in our paper, chemometric methods were used for extracting relevant information. Initially, an unsupervised model, principal component analysis (PCA), was built. Nevertheless, more relevant information was obtained by employing a supervised classification method, partial least squares discriminant analysis (PLS-DA). The obtained discriminant model was interpreted, in order to identify the most discriminant variables/wavenumbers, and validated according to the estimate of relevant figures of merit (FOM). The multivariate analytical validation of qualitative methods is an important issue aiming at their official recognition by regulatory agencies (Botelho, Reis, Oliveira, & Sena, 2015; Isabel-López, Pilar-Callao, & Ruisánchez, 2015). Another important issue addressed in our model was the estimate of confidence intervals for the predicted values, which was based on a bootstrap resampling methodology (Zoubir & Boashash, 1998).

Thus, the objective of this paper was to evaluate the potential of Raman spectroscopy associated with chemometric tools to differentiate common chicken eggs from enriched omega-3 eggs based exclusively on the analysis of their lipid composition. For this purpose, Raman spectra were obtained from the fat extracted of the egg yolk. The analyzes were not carried out directly on the egg yolk due to the presence of other interfering compounds, such as the paprika, an ingredient associated with the color shade of the egg yolk. This interference can provide undesirable background fluorescence in Raman spectra.

2. Experimental

2.1. Chemicals and solutions

All reagents and standards used were of analytical grade. Water was purified by deionization using the Milli-Q system with a resistivity of 18.5 MΩ cm (Millipore, Bedford, MA, USA). Methanol (MeOH), hexane, sodium chloride, isopropanol, acetic acid, anhydrous sodium sulfate (Na_2SO_4) and sodium hydroxide (NaOH) were purchased from Vetec (Rio de Janeiro, RJ, Brazil). A commercial fatty acids methyl esters (FAME) standard mixture with 37 components Supelco (FAME 37) was purchased from Sigma (St. Louis, MO, USA) for analysis by GC. Omega-3 FA standards for Raman analysis, such as alpha-linolenic acid (C18:3ccc), eicosapentaenoic acid (C20:5ccccc) and docosahexaenoic acid (C22:6cccccc), were purchased from Sigma (St. Louis, MO, USA). The extraction solution was prepared by adding three parts of hexane to two parts of isopropanol in a volumetric flask. To prepare the sodium methoxide solution a mass corresponding to 1.0 mol L^{-1} of NaOH was dissolved in a volumetric flask with methanol. NaCl (5%) and acetic acid (10.0 mol L^{-1}) were dissolved in water.

2.2. Samples

In this study, 80 independent egg samples were analyzed. These samples were acquired from six different egg-producing industries, consisting of eight production batches of ten samples. Each egg sample has a unique composition, which is distinctive due to the intrinsic metabolism of each chicken that originated it. These samples were acquired in the following quantities: 40 samples of common chicken eggs (20 of white eggs, 10 of brown eggs and 10 of organic brown eggs) and 40 omega-3 enriched chicken eggs (available only in brown color).

2.3. Lipid extraction method

For fat extraction, 500 mg of each yolk were weighed into 2.0 mL plastic tubes, where 0.5 mL of the extraction solution was added (Hara & Radin, 1978), and the mixture was vortexed for 30 s. In the sequence, 0.5 mL of NaCl solution were added, the mixture was vortexed for 30 s and then centrifuged at 6000 rpm for 1 min. The organic (upper) phase was transferred to a second 2.0 mL plastic tube with a Pasteur pipette. Another 0.5 mL of extraction solution was added to the first tube, then it was vortexed and centrifuged, the organic phase was transferred to the second tube and the solvent was evaporated at 40 °C under negative pressure. The second tube was previously weighed, so it was possible to obtain the fat mass extracted from each sample, which was on average 0.150 g. The extracts were stored in a freezer at −20 °C and were used for GC and Raman analysis.

2.4. GC analysis

Eight pools were prepared, one for each batch of samples, from 10 μL of each extract, which were transferred to 1.0 mL plastic tubes. The pools were vortexed and stored in a freezer at −20 °C.

The methylation of the samples was performed by basic catalysis (Christie, 1993; Christie & Han, 2012). 20 μL of each pool (in duplicate) were transferred to 2.0 mL plastic tubes. The fat extract was resuspended in 0.5 mL of methanol and 0.5 mL of sodium methoxide in methanol. The mixture was vortexed, heated in capped tubes for 10 min at 50 °C and cooled in a freezer for 5 min. 0.1 mL of acetic acid solution, 0.2 mL of sodium chloride solution and 0.3 mL of hexane were added, the mixture was vortexed for 1.0 min and centrifuged at 6000 rpm for 1.0 min. The organic (upper) phase was transferred to a second 1.0 mL plastic tube containing 0.1 g of anhydrous sodium sulfate. Another 0.2 mL of hexane was added to the first tube, the mixture was vortexed and centrifuged, the organic phase was transferred to the second tube, which was vortexed for 30 s and allowed to stand for 10 min. Finally, the FAME (fatty acid methyl ester) solution in hexane was transferred to a glass vial and analyzed by GC.

FA analysis was performed in a Shimadzu gas chromatograph equipment (GC 17A, Shimadzu, Kyoto, Japan), with split-splitless injector type and flame ionization detection (FID). A fused silica capillary column was used (DB-5 Agilent – 30 m × 0.25 mm i.d., 0.25 μm). The chromatographic conditions were: column at 80 °C, 10 °C min^{-1} to 180 °C, 7 °C min^{-1} to 330 °C; injector and detector at 250 °C; the carrier gas was hydrogen with column flow rate: 1.5 mL min^{-1}; pressure: 68 kPa; total flow rate: 42 mL min^{-1}; velocity: 42 cm s^{-1}; split ratio: 26 and 1.0 μL of sample injection. FAMEs were identified by retention time comparison with the standard solution FAME37 in GC-FID and by gas chromatograph – mass spectrometry (GC–MS) (results not shown). Fatty acids were determined by area normalization and expressed in g per 100 g of fat (AOCS, 2001).

2.5. Raman spectra

Raman spectra were obtained in a Fourier-Transform spectrometer FT-Raman model Multiram from Bruker (Billerica, USA), equipped with a germanium detector cooled by liquid nitrogen and with Nd:YAG laser, which provides the exciting radiation of wavelength 1064 nm. Spectral resolution, number of accumulations and laser power were investigated with a representative test sample. The optimized conditions of analyses were 4 cm^{-1} of resolution, 200 scans accumulation, 400 mW laser