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Evaluating the effects of the adulterants in milk using direct-infusion high-resolution mass spectrometry

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ABSTRACT

Milk is an extremely complex food, capable of providing essential nutrients as well as being an important source of energy, and high-quality proteins and fats. Due to advances in technology, and to meet the increasing demand, production costs have increased, turning milk into a target of adulterations. Routine methods usually applied to certify the quality of the milk are restricted to microbiological tests, and assays that attest the nutritional composition within the expected values. However, potentially harmful byproducts generated by adulterating substances in general are not detected through these methodologies. In this contribution, we simulated the adulteration of freshly produced milk samples with four adulterants whose use already had reported for extended shelf life: formaldehyde, hydrogen peroxide, sodium hydroxide, and sodium hypochlorite. These samples were submitted to direct-infusion high-resolution mass spectrometry analysis and multivariate statistical analysis. This approach allows the characterization of a series of molecules modified by the adulterants, what demonstrates how these species affect the nutritious characteristics of this product.

1. Introduction

Globally, there are about 7 billion consumers of milk and dairy products, most of them concentrated in developing countries (FAO, 2016). Within this market, Brazil figures as the world's fifth largest dairy producer, in a ranking that brings India as the first, accounting for 18% of global production, followed by the United States, China and Pakistan (IFCN, 2014). According to the United States Department of Agriculture (USDA), Brazilian milk production is expected to grow 1.8% in 2018, reaching 23.98 million tons, with a third of this production destined only to the inner market (USDA, 2017).

Milk is an extremely complex food, capable of providing essential nutrients as well as being an important source of energy, and high-quality proteins and fats. Cow's milk is composed on average of 87% water, 4 to 5% lactose, 3% protein, and 3 to 4% fat (Pereira, 2014). Although it is a food with high nutritional value, it has a short shelf life and therefore requires specific processing steps such as pasteurization to work around this problem. Pasteurization is a process that involves the heating followed by rapid cooling of the milk, so that the levels of any potential pathogen are reduced, thereby reducing health risks and resulting in increased shelf life (Kapaj & Deci, 2017). In addition, regarding the improvement of milk quality and safety, a government program was created in Brazil to advise and train rural producers in the structuring of their production units, implementing good agricultural practices. This program encompasses sanitary control during all stages of milk processing, i.e. from cow rearing, milking, to storage and proper transport of milk (MAPA, 2016). In this way, it is noted that milk is a food that requires care in its processing chain.

Due to advances in technology, and to meet the increasing demand, production costs have increased, turning milk into a target of adulterations (Hemme, Uddin, & Ndambi, 2014). This activity may generate not only economic losses, but also increased risk to consumers' health, commonly digestive problems (Tay, Fang, Chia, & Li, 2013). Milk adulteration involves the dilution and/or addition of lower-quality products at low concentrations and, in some cases, harmful products with the aim of masking inferior quality, increasing the volume or even replacing a substance naturally present in milk to increase the yield of the final product (Nascimento, Santos, Pereira-Filho, & Rocha, 2017). One of the simplest examples of milk adulteration is the addition of water to increase the final volume. Nonetheless, recent and more sophisticated frauds have been reported in both media and in scientific documents, and are often more difficult to detect (Nascimento et al., 2017). Some examples of recent adulterations would be the addition of urea and melamine to increase nitrogen content (Jha, Jaiswal, Borah, Gautam, & Srivastava, 2015; Lu et al., 2017), the addition of formaldehyde, hydrogen peroxide, hypochlorite, dichromate, and salicylic

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acid in order to increase shelf life (Jeong et al., 2015), and the addition of vegetable oils and surfactants to alter the fat content (Rani, Sharma, Arora, Lal, & Kumar, 2015).

Regarding these chemical adulterants, regulatory authorities such as the Food and Drug Administration (FDA) in the United States and the World Health Organization (WHO) have established maximum residue limits (MRLs) and tolerable daily intakes (TDI) to maintain food safety and health of consumers (Nascimento et al., 2017). In this way, certifying and guaranteeing aspects of milk quality is important for the population health, in addition to also increase the reliability of the products. Furthermore, consumers are increasingly demanding with respect to information on the safety, origin and composition of foods. In this way, determining food authenticity is essential to prevent substitution by cheaper, often harmful, ingredients and, consequently, adulteration.

Routine methods usually applied to ensure the quality of the milk are restricted to microbiological tests, and assays that attest the nutritional composition within the expected values. However, the effects of adulterating substances in general are not detected through these methodologies, and methods that assess the consequences of adulterations are scarce. An example of this fact would be the addition of hydrogen peroxide with the objective of activating the enzyme lactoperoxidase, naturally present in milk, increasing its antimicrobial activity, which increases the shelf life of pasteurized milk. The identification methods commonly used in this case are specific sensors for identification of hydrogen peroxide, but these are not able to evaluate the effects caused by the adulterant-milk components interaction (Reanpang, Themsirimongkon, Saipanya, Chailapakul, & Jakmunee, 2015). On the other hand, our study proposes precisely the identification of the chemical changes that can occur when the adulterant acts in milk nutrients and not only the presence of adulterant itself.

In this context, strategies have been applied and developed in recent years for the identification and quantification of adulterants in milk (Nascimento et al., 2017), but there still remains a need for methods that provide complementary results, where actual species can be determined and potentially monitored as quality control markers. Along these lines, mass spectrometry (MS) presents itself as a great analytical tool to identify adulterations in food products (De Oliveira & Catharino, 2015; Guerreiro, de Oliveira, Ferreira, & Catharino, 2014; Riccio et al., 2011), as it is a very versatile and fast technique, able to provide results with great accuracy.

MS is often coupled with separation approaches, such as liquid or gas chromatography, in the identification and quantification of adulterants (Verma & Ambatipudi, 2016). The use of these techniques, however, often implies specific sample preparation processes such as extraction and/or derivatization of compounds, which usually impair the direct analysis of a complex matrix, as in the case of milk. This fact prevent a performance of untarget high throughput analysis, which could make it more dynamic to identify the possible components of milk that have been interacted with the adulterants. High-resolution mass spectrometry (HRMS) is a technique that combines both selectivity and sensitivity, and could be used to identify a wide range of compounds in a variety of matrices. In addition, when combined with direct infusion (DI) of samples, it shows great potential to provide reliable results in just one minute (Guerreiro et al., 2018; Ibáñez, Simó, García-Cañas, Acunha, & Cifuentes, 2015; Melo et al., 2017; Vivian, Aoyagui, de Oliveira, & Catharino, 2016).

In this preliminary contribution, we simulated the adulteration of freshly produced milk samples with four adulterants that already had reported use in Brazil for extended shelf life: formaldehyde, hydrogen peroxide, sodium hydroxide, and sodium hypochlorite. These samples were submitted to DI-HRMS and multivariate statistical analysis in comparison with the unadulterated product, rendering a series of oxidized molecules that demonstrate how these species affect the nutritious characteristics of the product. The results are promising and demonstrated the versatility of DI-HRMS in providing the characterization of the main altered species in milk and, in the future, can be of great help in the development of a methodology based on direct infusion samples.

2. Materials and methods

2.1. Sample preparation

Samples of milk were obtained directly from farmers in the region of Minas Gerais, Brazil. The milk were from four different producers and a blend was made using equal amounts of milk from each producer (1:1:1:1), with the intention that the control samples were as similar to the real conditions. An aliquot of 10 μL of fresh milk blended sample was diluted in 990 μL of a solution of Methanol:Mili-Q Water (50:50). This mixture was vortexed for one minute and then filtered on PVDC membranes of 0.22 μm. This solution was split into two, each one used in a different mode of analysis, positive or negative ion mode. For positive ion mode analysis, 1 μL of formic acid (final concentration = 0.2% v/v) was added to one of these fractions of 500 μL and, for the negative ion mode analysis, 1 μL of ammonium hydroxide (final concentration = 0.2% v/v) was added to the other fraction. Methanol, ammonium hydroxide and formic acid were purchased from J. T. Baker (Xalostoc, Mexico) and used with no further purification. Deionized water was obtained from a Milli-Q system (Millipore, USA).

Adulterations with formaldehyde (15,512, Sigma-Aldrich, Saint Louis, MO), hydrogen peroxide (H3410, Sigma-Aldrich, Saint Louis, MO), sodium hydroxide (1.09137, Sigma-Aldrich, Saint Louis, MO) and sodium hypochlorite (425,044, Sigma-Aldrich, Saint Louis, MO) were performed at the concentrations of 1, 2.5, 5 and 10% v/v for each of the four adulterants in the fresh milk. The other steps of sample preparation for DI-HRMS analysis were the same as those used for the analysis of the milk profile without adulteration (control), described above. All samples were prepared in triplicates, for the control raw milk and for each different concentration of adulterants, composing a total of 51 samples: 48 adulterated (12 samples per group), and 3 controls.

2.2. High Resolution Mass Spectrometry (HRMS)

All analyses were performed on the ESI-LTQ-XL Orbitrap Discovery (Thermo Scientific, San Jose, CA, USA) with a nominal resolution of 30,000 (FWHM). Acquisitions were carried out in the mass range of 100 to 1000 m/z in both, positive and negative ion modes. The parameters used were: flow rate of 10 μL min^{-1}, capillary temperature of 280 °C, 5 kV as spray voltage and sheath gas in 10 arbitrary units. All acquisitions were obtained in quintuplicates.

2.3. Statistical analysis and biomarker identification

The method of choice to evaluate the differences between the groups was Partial least squares discriminant analysis (PLS-DA) with the variable importance in projection (VIP) score. PLS-DA is a supervised method that uses multivariate regression techniques to extract features from each group and show the existence of either differences or similarities between the analyzed samples. The selection of features that were characteristic for each sample was carried out considering the impact that each feature had in the analysis through VIP scores, which consists of the weighted average of the squares of the PLS loadings considering the amount of explained variance on the two dimension used in the model. As a cutoff threshold, only the features with a VIP score greater than 3 were analyzed. All statistical analyses were performed using the online platform MetaboAnalyst 3.0 (Xia, Sinelnikov, Han, & Wishart, 2015). For the structural elucidation of the markers, mass accuracy was the main parameter, by comparing the mass values obtained experimentally and those available in online databases, such as METLIN (Scripps Center for Metabolomics, La Jolla, CA, USA), in order to guide the choice of potential markers for adulteration in milk.