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Analytical Methods

Mass spectrometry detection of fraudulent use of cow whey in water buffalo, sheep, or goat Italian ricotta cheese

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ABSTRACT

Ricotta cheese is a typical Italian product, made with whey from various species, including cow, buffalo, sheep, and goat. Ricotta cheese nominally manufactured from the last three species may be fraudulently produced using the comparatively cheaper cow whey. Exposing such food frauds requires a reliable analytical method. Despite the extensive similarities shared by whey proteins of the four species, a mass spectrometry-based analytical method was developed that exploits three species-specific peptides derived from β-lactoglobulin and α-lactalbumin. This method can detect as little as 0.5% bovine whey in ricotta cheese from the other three species. Furthermore, a tight correlation was found ($R^2 > 0.99$) between cow whey percentages and mass spectrometry measurements throughout the 1–50% range. Thus, this method can be used for forensic detection of ricotta cheese adulteration and, if properly validated, to provide quantitative evaluations.

1. Introduction

Ricotta cheese (ricotta) is a typical Italian unripened cheese variety (literally meaning "cooked again") obtained through heat-induced (85–90 °C) coagulation of whey proteins, after addition of acidifying agents (e.g., lemon juice or vinegar) (Fox & McSweeney, 2004). Ricotta curd is transferred to molds, eventually surrounded by ice, where drainage occurs for 12–14 h (Coni & Bocca, 1999). Usually 5–20% whole or skimmed milk or cream is added to the whey resulting from cheese production, to improve yield (Shahani, 1979) or taste. According to an old, but currently in force, Italian law (Italian Decree, 1925), ricotta cannot be classified as a cheese but as a by-product of cheese-making, while in the European custom code (European Parliament and Council Regulation, 2008), it is classified as "whey cheese", within the class of dairy products.

Ricotta or similar whey cheeses, widely spread in the Mediterranean area (Manouri, Myzithra, Brocciu, Serac) and Northern Europe (Ekte geitost, Floteost, Gudbrandsdalsost), can be made from almost any kind of sweet whey resulting from bovine, water buffalo, ovine and caprine cheese manufacturing. Although regarded as a secondary dairy product for a long time, ricotta is a bestselling product on the Italian market, because of its variety of uses.

The lower availability and the higher price of water buffalo, caprine and ovine milk, in comparison with bovine milk, may induce the fraudulent addition of bovine whey during the production of ricotta cheese from other species. This is an illegal practice for both commercial reasons and medical and ethical reasons, because consumers may be allergic or intolerant to particular bovine whey components (Lara-Villoslada, Olivares, & Xaus, 2005).

From a commercial point of view, the authenticity of whey origin is even more relevant for high-quality dairy products, such as "Ricotta di bufala campana" (100% water buffalo whey) (European commission, 2010) and "Ricotta romana DOP" (100% ovine whey) (European commission, 2005), approved as European Protected Denomination of Origin (PDO).

The addition of bovine whey during the preparation of ricotta cheeses nominally from other species is not just of theoretical interest and could give rise to an illegal procedure. Indeed, instances of such practice were uncovered in the course of control activities carried out in our laboratories, when some commercial ovine ricotta samples were found to contain cow caseins, although bovine components were not declared on the label.

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The prevention of this kind of fraud is necessary to protect both consumers and producers from illegal behaviors, but no official method for detecting the addition of undeclared bovine whey in water buffalo, caprine or ovine ricotta cheese, is currently available. Reviews on this topic may be found in De La Fuente and Juarez (2005) and Fuselli and Tidona (2013). Most approaches focus on protein components, using electrophoretic (IEF, PAGE), chromatographic (HPLC), or immunological (ELISA) methods. All these methods, primarily based on physical–chemical properties of specific proteins almost in their native form, need a specific validation for both ripened cheeses and dairy products made with heat-treated milk or whey, such as ricotta. In fact, during ripening, new potentially interfering proteolysis products appear, the degradation of some critical proteins may occur, or heat-sensitive native proteins may become unfolded after the high temperature treatment.

Di Luccia developed two methods based on polyacrylamide gel isoelectric focusing and SDS-pore gradient gel electrophoresis (Di Luccia et al., 1994) to characterize the origin and protein composition of ricotta cheese made from mixtures of whey from different species or from mixtures of whey and milk, respectively. Fuselli developed a method for the detection of the addition of bovine whey to water buffalo ricotta above 5% (v/v), based on extraction and purification of denatured whey proteins and polyacrylamide gel separation by isoelectric focusing (Fuselli et al., 2015). These methods require a preliminary separation of whey proteins from caseins, high technical skills and the use of polyacrylamide gels. Moreover, they are only able to recognize fraudulent addition of bovine milk during ricotta manufacturing at percentages equal to or greater than 5%.

Approaches based on mass-spectrometry analysis of protein pattern, mainly MALDI-TOF and LC–MS/MS, have been also applied to species identification in dairy products, with the aim of guaranteeing the authenticity of PDO products, such as water buffalo mozzarella (Russo et al., 2012).

In the last decade, several studies based on PCR techniques, which ensure high specificity and sensitivity and can also be quantitative (real-time PCR) were carried out (Drummond et al., 2013; Reale, Campanella, Merigioli, & Pilla, 2008), but their reliability depends on the number of somatic cells actually present in milk or cheese samples. Although some of the above mentioned methods might be potentially used for the aim of this study, they need a specific validation to verify their reliability, when applied to ricotta, because of the hard heat treatment its preparation entails. Other methods, based on casein (Addeo, Pizzano, Nicolai, Caira, & Chianese, 2009; Bramanti, Sortino, Onor, Beni, & Raspi, 2003; Russo et al., 2012; Veloso, Teixeira, & Ferreira, 2002) or native whey proteins, cannot be used.

The aim of this study is to develop and evaluate the applicability of a method based on LC–MS/MS to specifically reveal the fraudulent addition of bovine whey to water buffalo, ovine, or caprine ricotta.

For control purposes, the illegal addition of bovine milk to ovine, caprine and water buffalo cheeses may currently be revealed by an official European reference method (European commission, 2008), based on the detection of specific peptides from bovine caseins. This is a qualitative method, whose response is a simple declaration of presence or absence of bovine milk (above 1%). It should be emphasized that, in control activities, one is often requested by the court to give at least a rough indication or estimate of the extent of the fraud. Moreover, for ovine and caprine cheeses, the detection is carried out against European reference samples, without regard to the protein content and composition of the original milk used to produce the sample under investigation.

Thus, it would be desirable to develop a method capable of giving an indication of the percentage of fraudulently added bovine whey. However, it should be also taken into account that, given the fact that the protein content of milk shows variability as a function of, at least, breed and season (Fuselli and Tidona, 2013), approaches based on the evaluation of specific protein markers can provide at the most rough quantitative, or semi-quantitative, information.

The aim of this study is to develop a method for specifically detecting the addition of undeclared bovine whey to ricotta and providing a semi-quantitative evaluation of the added amount.

2. Materials and methods

2.1. Ricotta cheese samples

Pure raw pooled milk samples were collected at the farm from the following animals and each sample's protein content was determined by Kjeldahl method. Cow (*Italian Frisian* and *Italian Pezzata Rossa* breeds), 3.2%; Mediterranean water buffalo, 4.6%; *Lacaune*-sheep, 5.3%; *Camosciate delle Alpi* goats, 3.6%. The pure milk samples were rennet coagulated, according to the procedure described in the EU official method (European commission, 2008) and the curd was drained off. The resulting whey was used to prepare standard ricotta samples, by heating to 80–85 °C until the coagulated proteins floated to the surface and were collected. Both pure and mixed (1%, 5%, 10%, 20%, 30%, or 50% v/v cow/other species whey) ricotta reference standards were prepared. More than 40 ricotta reference samples were prepared in house and 3 commercial ricotta samples were purchased at retail markets. In house ricotta samples, used to test the semi-quantitative evaluation model, were prepared from pooled milk samples different from that used for the model.

Standard or commercial ricotta (10 g) was suspended in 30 ml of water, using an Ultra Turrax T25 (IKA-Werke GmbH & Co., Staufen, Germany) for 1 min, at 20,000 rpm. The suspension was centrifuged for 5 min at 3100×*g*, the pellet was washed twice with 30 ml of acetone to remove fats and finally dried in air.

2.2. Protein extraction and digestion

Dried bovine, goat, sheep, or buffalo ricotta cheese (10 mg) was dissolved in 4 M urea (ICN Biomedicals), 2 mg/ml dithiothreitol (DTT, Sigma–Aldrich), and 10% acetonitrile (ACN, Sigma–Aldrich) to a final concentration of 10 mg/ml and then immersed in ultrasonic bath for 10 min. Bovine β-lactoglobulin B (β-LGB) by Sigma–Aldrich was used as standard reference.

Protein extract (30 μg) was separated on a 1D, 4–12% polyacrylamide NuPAGE gel (Novex, Invitrogen) with MES running buffer (Novex, Invitrogen) and the gel was stained with the Colloidal Blue Staining kit (Invitrogen). Bands of interest were excised, reduced, alkylated, and digested with trypsin (Promega) as described elsewhere (Shevchenko, Wilm, Vorm, & Mann, 1996).

Ricotta cheese proteins were dissolved in 0.4 M urea, 10 mM DTT, 1% ACN and 50 mM ammonium bicarbonate (ICN Biomedicals) to a final concentration of 1 mg/ml. This protein solution was reduced at 37 °C for 1 h, alkylated with 55 mM iodoacetamide (Sigma–Aldrich) in the dark for 30 min, and incubated with trypsin or AspN at 1:100 (w/w) enzyme/substrate ratio at 37 °C overnight. Enzyme activity was blocked by adding 0.1% trifluoroacetic acid (TFA, Carlo Erba). The peptide solution was desalted using C-18 Zip Tip pipet tips (Millipore), dried in a vacuum concentrator (Speed-Vac, Thermo-Savant), and dissolved in 0.1% formic acid (FoA, JT Baker).