Protein-Based Animal Species Authentication in Dairy Products

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Milk is one of the most important nutritious foods, widely consumed worldwide, either in its natural form or via dairy products. Currently, several economic, health and ethical issues emphasize the need for a more frequent and rigorous quality control of dairy products and the importance of detecting adulterations in these products. For this reason, several conventional and advanced techniques have been proposed, aiming at detecting and quantifying eventual adulterations, preferentially in a rapid, cost-effective, easy to implement, sensitive and specific way. Protein-based techniques, including electrophoresis, chromatography and immunochemical assays, are considered current methodologies for assessing the authenticity of dairy products. They are generally considered fast, high throughput and cost-effective, being suitable approaches for the analysis of animal species in raw milk. However, when applied to processed foods, their reliability might be compromised due to protein denaturation and consequent epitope modification, disabling the immunorecognition of proteins. In recent years, the developments of mass spectrometry (MS) platforms for protein analysis, characterization and quantification have provided alternative approaches that rely on marker peptides instead of whole proteins, being suitable alternatives to analyze processed products.

Protein analysis | Authenticity | Dairy products

1. Electrophoretic Techniques

Different works using electrophoretic techniques have been reported so far for the detection of milk adulteration, including the use of polyacrylamide gel electrophoresis (PAGE) or, most frequently, the use of isoelectric focusing (IEF). Although PAGE is generally effective, its main limitation concerns the complex band pattern obtained, with frequent overlap of bands that can lead to an equivocal interpretation of results. Pesic et al. [1] suggested the use of a native PAGE electrophoresis for the qualitative and quantitative analysis of bovine adulteration in ovine or caprine milk based on bovine β-lactoglobulins (β-LG) and α-lactalbumins (α-LA). This method was considered a fast and convenient alternative for the detection and estimation of milk adulteration. However, its application is limited to fresh milk mixtures since heat processing and pH can cause the denaturation of whey proteins, with β-lactoglobulins being remarkably affected particularly by severe heat treatments including ultra-high temperature (UHT).

A similar approach consisting of isoelectric focusing (IEF) of γ-caseins, namely γ2-and γ3-caseins obtained by plasminolysis of β-casein, is currently the reference method in the EU for the determination of cow's milk caseins in ovine, caprine, and water buffalo cheeses [2][3][4][5][6]. In this method, the samples should be analyzed together with

reference standards containing 0% and 1% cows' milk, being considered positive if both bovine γ2-and γ3-caseins, or the corresponding peak area ratios, are equal to or greater than the level of the 1% reference standard [6]. The method can be used for detecting either raw or heat-treated cow's milk and caseinate in fresh or ripened cheeses made of ewes', goats' and buffalos' milk or their mixtures, though not being suitable for the detection of milk and cheese adulteration by heat-treated bovine whey protein concentrates [6]. It is also not adequate for species quantification, especially in ternary mixtures due to the similarities between some species, such as ovine and caprine [3][4][7]. In fact, the reference method fails in detecting goat's milk in sheep's cheese and milk. Additionally, other works demonstrated that the evaluation of cow's milk casein in water buffalo cheese by IEF is sometimes uncertain due to the presence of interfering co-migrating bands that can result in false positives [4][7][8]. Recently, Caira et al. [8] used a proteomic approach to demonstrate that this false positive result was due to the water buffalo fragment β-casein(f100-209), which was also formed after plasminolysis of buffalo's milk or dairy products and co-migrates in IEF with bovine γ2-casein. To avoid false positives due to a water buffalo casein band with an isoelectric point similar to that of bovine γ2-casein, Addeo et al. [7] proposed the use of IEF coupled to immunoblotting to detect the presence of cow's milk in water buffalo cheeses. The methodology proved to be successful in evaluating the authenticity of pure water buffalo milk and cheeses, with a limit of detection up to 0.25% bovine milk (*v/v*), which was lower than that described by the EU reference method (1%).

Capillary electrophoresis (CE) has been suggested as an alternative to gel electrophoresis-based methods for the authenticity assessment of dairy products because of its higher resolution power, low operation cost and high throughput [9][10]. Somma et al. [10] compared the efficiency of ultra-thin-layer IEF with capillary isoelectric focusing (cIEF) applied to the separation and identification of the main peptides arising from the hydrolysis of water buffalo and bovine β-caseins. Additionally, cIEF was used in combination with mass spectrometry for structural confirmation of the separated peptides. cIEF proved to be faster and more convenient because it does not require gel staining, though the cow-specific markers were only detectable at 5% cow's milk addition in water buffalo's milk, a value well above the sensitivity of the IEF method (0.5%). Nevertheless, both methods could be useful for detecting the fraudulent addition of cow's milk to buffalo's milk, which is very important in the production of Mozarella di Bufala cheese. More recently, Trimboli et al. [11] proposed the use of a routine CE method for human blood and urine protein analysis as a tool to authenticate ewe's skimmed milk. The method was based on the separation of skimmed milk proteins and the use of a characteristic peak for ewe's milk quantification in ovine/bovine skimmed milk mixtures, allowing people to detect a minimum amount of 5% of added cow's milk with good linearity, precision and accuracy. A similar approach, using a routine CE method for blood analysis, was also attempted for detecting as low as 1% cow's milk in buffalo's milk and predicting the amount of fraudulently added milk by exploiting cow's α-lactalbumin as a marker of adulteration [12]. Although most works dealing with the application of CE have been applied to milk mixtures, its use for the successful identification of animal species in cheese samples has also been demonstrated [13][14].

2. Immunochemical Techniques

Immunochemical methods are often used in the food industry for the qualitative and quantitative detection of food components and/or contaminants, being applied since the early 1980s to answer to the analytical demands in the dairy industry [3][5][15]. Essentially, an immunochemical assay consists of the reaction of an antigen with a specific antibody [5]. Therefore, immunochemical techniques provide highly specific and sensitive methods, being applied to a variety of complex food products. Compared with electrophoretic and chromatographic techniques, they are considered generally simpler, of lower cost, more sensitive and specific [3][5].

Enzyme-linked immunosorbent assay (ELISA) is the immunochemical technique most frequently used in dairy product analysis with diverse formats, including direct, indirect, sandwich and competitive, being applied to detect whey proteins and caseins. ELISA are frequently used in the analysis of milk and dairy products because of their easy application in routine analysis, low-cost, speed and sensitivity. However, the selected antisera influences the specificity and sensitivity of the method, thus requiring specific antibodies capable of differentiating species, without providing false positives due to cross-reactivity with non-target species or other food ingredients [15][16][17]. This could be achieved by the use of novel immunoreagents obtained by antipeptide antibody technology, suitable for milk species identification [18]. The characteristics, advantages and limitations of antibody-based techniques for the assessment of dairy products authenticity have been reviewed by Pizzano et al. [5].

ELISA has been used for species authentication in milk and dairy products since the late 1980s [5]. Hurley et al. [19] described the development of an indirect competitive ELISA, using bovine immunoglobin G (IgG) as a target, due to its high immunogenicity, to detect the presence of cow's milk in other types of milk. The sensitivity of this technique was assayed using raw, pasteurized and previously frozen cow's milk, concluding that high temperatures caused specific epitope modification. The detection limit in this method was 1 µg/mL of bovine IgG (0.1%), highlighting its high sensitivity without cross-reactivity with other species. Another study aiming at detecting cheese adulterations also targeting bovine IgG, but applying a sandwich ELISA, was performed by the researchers [16]. This methodology allowed further lowering the sensitivity to 0.001% of bovine milk in goat soft cheese and 0.01% of bovine milk in sheep and buffalo soft cheese.

ELISA targeting fairly thermostable proteins, such as caseins, has been proposed as a feasible alternative to detect adulterations in heat-treated milk and dairy products. Among caseins, bovine β-caseins present a high specific antigenicity, not being affected by heat treatment and having a concentration more or less stable and independent of season, climatic and feeding conditions [20][21][22]. Therefore, different ELISA have become available in the format of commercial kits for routine surveillance tests. The performance of such kits has been evaluated in different studies showing their usefulness for qualitative purposes but exhibiting inconsistencies in quantitative determinations of cheese adulteration. In 2008, Costa et al. [23] evaluated two specific commercial ELISA kits to quantify the amount of cow's and goat's milk added to sheep's milk and cheese and concluded that they were more successful in detecting the adulteration in milk than in cheeses. More recently, Zeleňáková et al. [24] tested the reliability of a commercial ELISA (RC-bovino from Zeu-Inmunotec, Spain), concluding that the quantification of cow's milk in sheep's cheese was not exact, possibly due to modifications in the cheese matrix that take place during the manufacturing process. The same commercial ELISA kit was also used by Stanciuc et al. [25] to qualitatively detect the presence of cow's milk in goat's and sheep's cheeses for confirmation of positive results

obtained with a immunochromatographic method. From 73 tested samples from Romania, 67.3% of sheep's cheeses and 79.7% of goat's cheeses were adulterated by the addition of cow's milk, suggesting the need to improve the quality control in the cheese industry. Another commercial kit (Casein ELISA set, SEDIUM R&D) was used by Zeleňáková et al. [26] to detect and quantify cow's milk caseins in sheep's milk and cheese, obtaining a calibration curve in the range of 0.5–50% using different mixtures of heat-treated milks. When applied to cheeses, the kit did not provide any relation between the presence of caseins and the increase in the cow's milk proportion in the mixture, either using raw or pasteurized milk, concluding its inadequacy for cheese analysis. By contrary, the use of a sandwich ELISA kit (β-Lactoglobulin ELISA Set, SEDIUM R&D) targeting bovine β-lactoglobulin to detect adulterations in sheep's milk and cheese was able to provide a quantitative analysis within 0.2–20 mg/kg [27].

Lateral flow immunoassays (LFIA) are alternative tools very easy to handle by non-expert workers. Thus, they can be applied in-field for screening purposes and are appropriate to be used by the cheese industry to quickly check and control the genuineness of the milk used along its production chain. Recently, Galan-Malo et al. [28] developed and validated a rapid test based on LFIA able to detect down to 0.5% of cow's milk in goat's, sheep's or buffalo's milk without identifying any false-positives among over 146 negative assayed samples.

Although most available immunochemical assays concern the authentication of sheep's, goat's and buffalo's milk and/or cheeses, some studies have addressed other animal species. Pizanno et al. [18] developed an ELISA based on the use of antipeptide antibodies raised against the 1–18 sequence stretch of cow's β-casein to successfully detect the presence of low levels (0.5%, *v/v*) of cow's milk fraudulently blended with high-valued donkey's milk. An indirect competitive ELISA to detect cow's milk in yak's milk using a specific monoclonal antibody for bovine β-casein (mAb 1-9B) was developed by Ren et al. [29]. The method allowed detecting 10 µg/mL of bovine milk in yak's milk and was not affected by any external factors such as temperature and milk treatment.

3. Chromatographic and Mass Spectrometry Techniques

Up until now, different chromatographic techniques, including either gas or liquid chromatography, have been applied to authenticate dairy products because of their relative simplicity and speed, as well as possibility of automation [3][15]. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was firstly used for the separation of the different casein fractions, relying on both normal (NP) or reverse-phase (RP) columns to identify cow's milk in goat's and sheep's milk [30][31][32][33]. However, UV detection has drawbacks related to low specificity in the presence of co-eluting peaks or interferents. Thus, during the past decade, the technological advances, mainly in the area of mass spectrometry (MS) detection, have steadily replaced UV detectors, whenever the detection of food frauds is concerned. Soft-ionization techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), have made possible to accurately analyze proteins and peptides, therefore allowing their use as reliable biomarkers for dairy product authentication. Peptides as biomarkers present advantages over proteins, which are affected by thermal processing [34]. Owing to the specificity, fastness, sensitivity and high reproducibility of the mass spectra, several methodologies based on MALDI time-of-flight mass spectrometry (TOF MS) have been developed, so far, to obtain informative fingerprints of milk proteins towards dairy product authentication [35].

Based on MALDI-TOF MS analysis of intact proteins of different milk species, Cozzolino et al. [36] suggested α-lactalbumin and β-lactoglobulin as markers for detecting cow's milk added to sheep's and buffalo's milk or cheese. Researchers also demonstrated the usefulness of the method in detecting the addition of powdered to fresh milk based on the presence of lactosylated forms originated by heat processing. The analysis of entire proteins by direct MALDI-TOF MS coupled to unsupervised statistical analysis was also successfully proposed for milk authentication by Di Girolamo et al. [35] and Nicolaou and Goodacre [37]. Identical results were obtained by Kuckova et al. [38] regarding the identification of the species of origin in milk, though the same was not verified when the method was applied to analyze commercial cheeses, which could be attributed either to protein profile modifications or to adulteration of ovine and caprine cheeses. Recently, Rau et al. [39] demonstrated the feasibility of MALDI-TOF MS combined with a small in-house validated database, containing more than 150 reference spectra of milk and cheese, as a rapid, easy and robust method to identify the species of origin in mozzarella and white brined cheeses. The direct protein extraction without applying a tryptic digestion step allowed performing the analysis in less than 30 min with reduced analytical costs.

Other approaches have relied on a bottom-up proteomic strategy, based on MS analysis of peptides obtained after enzymatic digestion [39][40][41][42]. Calvano et al. [40] reported several bovine-specific peptide markers in milk tryptic digests that can be useful for detecting adulterations by cow's milk addition to goat's or sheep's milk. Since the detection of sheep's milk adulterated with goat's milk is a difficult task because of their similar protein profiles, two goat-specific peptide markers assigned to κ-casein were identified [40]. Caira et al. [41] used a MALDI-TOF MS method to simultaneously determine the presence of water buffalo's and cow's milk in Italian water buffalo's mozzarella cheese. Since crossbreeding with other water buffalo breeds has been avoided in indigenous Mediterranean Italian buffalo, these animals generally exhibit reduced milk protein polymorphisms when compared to other international breeds. Therefore, hundreds of milk samples (Italian and from several other countries) were analyzed, aiming at identifying signature peptides associated with water buffalo origin for the authentication of PDO products [41]. Caseins were the target proteins owing to the identified differences between indigenous and international breeds, namely the unique presence of a β-CN A variant and an internally deleted αs1-CN (f35-42) variant in international water buffalo milk samples. The peptidomic approach allowed the identification of several tryptic signature peptides as molecular marker candidates to detect the addition of imported water buffalo's milk in Italian PDO products, as well as adulterations with cow's milk blending. The proposed methodology enabled the specific detection of international water buffalo and bovine caseins down to 2% and 0.78%, respectively. MALDI-TOF MS has also been proposed to detect the adulteration of water buffalo's ricotta with bovine milk based on a specific peptide marker, corresponding to the region 149–162 of β-lactoglobulin, enabling its detection down to 5% [42]. Nardiello et al. [43] proposed the use of a nano LC−ESI-ion-trap tandem mass spectrometry (nano LC-ESI-IT-MS/MS) methodology combined with a database post-processing to validate peptide sequence assignments and determine the species of origin in milk samples. Bovine species-specific peptides originated from αS1-casein and β-lactoglobulin were identified as suitable authenticity markers with detection levels as low as 1%.

MALDI-TOF MS has also been referred to as a tool for selecting the most suitable peptide makers in further analysis by liquid chromatography coupled to mass spectrometry (LC-MS) [44][45][46]. In fact, LC-MS has been increasingly applied in food analysis owing to its powerful capacity in detecting and quantifying specific analytes in

complex mixtures, offering particularly enhanced selectivity and sensitivity when multiple reaction monitoring (MRM) scanning is applied [47][48]. Cuollo et al. [44] used two techniques, namely MALDI-TOF MS and LC-ESI/MS, to detect specific signature peptides to differentiate cow's, sheep's, goat's and water buffalo's milks, with both approaches providing similar sensitivities (1% for caprine and 0.5% for the other species). αs1-CN (f8-22) peptide was selected as a convenient marker for cow's, sheep's and water buffalo's milk, while αs1-CN (f8-22) was for goat's milk. MALDI-TOF MS data were tentatively used to perform quantitative analysis based on synthetically modified proteotypic peptides as internal standards, but accurate evaluation of caprine milk in quaternary mixtures was only achieved by LC-ESI-MS.

Sforza et al. [49] described an LC-MS method to evaluate the presence of cow's milk in fresh sheep's milk cheese targeting short marker peptides, namely αs1-CN (f1-23) and αs1-CN (f1-14), generated from proteolytic activities of the rennet enzyme chymosin and starter lactic acid bacteria, respectively. While the first peptide was degraded over time, thus being undetectable after long ageing periods, the second was frequently observed in cow's milk cheeses. Despite this occurrence, the researchers referred to its detection in hard cheeses aged for more than 30 months. Moreover, the degradation of αs1-CN (f1-23) peptide also led to other fragments that could be detected. The method allowed the detection of cows' milk down to 1% in all the analyzed cheeses, demonstrating the usefulness of these two candidate biomarkers to assess the addition of cow's milk in fresh sheep's cheese [49]. Czerwenka et al. [50] developed an LC-MS method to detect the adulteration of cow's milk in water buffalo's milk and mozzarella cheese, targeting the whey β-lactoglobulin as an adulteration marker. Since this water-soluble protein is mainly present in the whey fraction and not in the cheese, the analyzed parts were the brine in which this type of cheese is usually sold, or in the exudate obtained after cheese centrifugation. Researchers showed that sufficient amounts of β-lactoglobulin were present either in the brine or exudate, allowing the detection of adulterations with cow's milk. The application of this method to assess 18 commercial samples of water buffalo mozzarella cheese allowed detecting three adulterated products. However, quantitative determination presented several pitfalls because of the variability the target the analyte between and within the two blended milks and the lack of an internal standard. Quantification of the fraudulent addition of bovine milk in the production of buffalo mozzarella PDO cheese was claimed by Russo et al. [47], based on UPLC-MS/MS exploiting the MRM mode, though the protein level in the studied cheeses was not taken into consideration. The use of MRM allowed a highly selective and sensitive detection and quantification of the chosen proteotypic marker, even in complex matrices, by simultaneously monitoring both their parent and one or more product ions. The selection of the species-specific proteotypic marker—phosphorylated β-CN (f33-48) tryptic peptide—was performed by an untargeted LC-MS/MS analysis by means of a quadrupole TOF MS equipped with an ESI source (ESI-Q-q-TOF). Additionally, to select the best conditions for trypsin digestion, a preliminary study was conducted by MALDI-TOF MS. Overall, the method allowed targeting the marker peptides with high specificity, thus being adequate for the authentication of complex matrices such as dairy products [47].

Despite the claimed advantage of quantitative analysis by LC-MS methods, it must be referred that it mainly gives an estimation of the fraud extent since the protein content of milk is known to vary with different factors, with the breed and season being of most relevance [45][46][47][48][49][50][51]. Trying to overcome this aspect, Gunning et al. [48] proposed the use of MRM MS-targeting αS1-casein to detect the addition of cow's milk to buffalo mozzarella

cheese. The relative amounts of each species in binary mixtures were determined based on corresponding peptides arising from a corresponding protein strategy and the ratios of transition peak areas. Moreover, identical peptides with the same sequences in both species were used to establish the relative levels of both species of αS1-casein in the component mixtures. The method was applied in a survey of 28 products sold in UK retail and restaurants, enabling researchers to verify that almost 2/3 were suspicious of being adulterated with cow's milk. An UHPLC-MS/MS method also exploiting MRM mode, using at least two transitions for each compound, has recently been reported by Ke et al. [52] to quantify cow's whey and whole milk powder in goat's and sheep's milk products, including infant formula. This method allowed the simultaneous quantification of four caseins (β-CN, αs1-CN, αs2-CN, and κ-CN) and two whey proteins (α-lactalbumin, β-lactoglobulin) based on the detection of their signature peptides. Isotopic labeled signature peptides were used as internal standards to compensate the matrix effect. The method was successfully validated regarding several parameters. Calibration curves for the tryptic signature peptides presented good linearity, the limits of quantification were between 0.01–0.05 g/100 g for the target proteins and the method showed high precision, reproducibility and recovery rates. The analysis of 11 commercial samples of goat infant formula milk powder revealed some adulterations among the evaluated products [52].

Although proteomic approaches developed so far mostly rely on the target identification of marker peptides, recently an untargeted UHPLC−MS/MS high resolution MS (HRMS) combined with chemometrics, was proposed to discriminate among cow's, goat's and buffalo's milk samples [53]. The approach allowed the identification of different marker compounds, suggesting β-carotene and ergocalciferol for cow's and water buffalo's milk identification, respectively. Moreover, the levels of octanoic, nonanoic and decanoic acids were found to be higher in goat's than in cow's and buffalo's milk [53].

Recently, the development of ambient ionization techniques, such as direct analysis in real time (DART), enabled a high-throughput and easy analysis of food. The potential of this ionization technique coupled to HRMS and chemometrics was exploited for dairy product authentication, including the discrimination of cow's, goat's and sheep's milk. Results showed that DART-HRMS analysis of the non-polar fraction of milk had a limited discrimination potential, probably due to the high variability in triacylglycerols (TAG) among each group of samples [54].

Although the application of both chromatographic and mass spectrometry techniques to dairy product authentication mainly relies on protein analysis, other compounds such as fatty acids and TAG have also been addressed for this purpose [55][56][57]. Bratu et al. [58] used GC-MS analysis of fatty acid methyl esters coupled to principal component analysis (PCA) to differentiate 25 different cheeses (including cow, goat and sheep). Although sample discrimination in 3 groups was achieved using 12 components, more studies should be performed comprising a higher number of samples, also including model cheeses made with mixtures of milk besides pure milk cheeses. Vieitez et al. [59] showed that the addition of cow's milk to pure goat's milk influences the TAG profile by determining the partition number (PN), which characterizes the molecular structure of TAG. The analysis of blends containing 10, 20 and 50% of cow's milk showed that the addition of cow's milk to goat's milk affects the TAG profile by decreasing TAG with PN between 38 and 42, while increasing it with PN between 46 and 50. Of the

15 commercial samples evaluated, 3 presented a different TAG profile, suggesting their possible adulteration with cow's milk.

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