Identification of adulteration in water buffalo mozzarella and in ewe cheese by using whey proteins as biomarkers and matrix-assisted laser desorption/ionization mass spectrometry

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A rapid and accurate method to identify bovine and ewe milk adulteration of fresh water buffalo mozzarella cheese by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) is described. The differentiation among mozzarella made from water buffalo milk and from mixtures of less expensive bovine and, more recently, ewe milk with water buffalo milk is achieved using whey proteins, α-lactalbumin and β-lactoglobulins as molecular markers. It is worth noting that the method proposed here is, to our knowledge, the first strategy able to characterize possible fraudulent additions of ewe milk in samples of water buffalo milk devoted to the production of water buffalo mozzarella cheese. In addition, a linear relationship was found between the relative response of the molecular ion and the abundance of the analysed whey proteins. This demonstrates that this approach can be used to determine the amount of bovine or ovine milk added to water buffalo milk employed for mozzarella cheese production. Furthermore, this method also appears suitable for the analysis of ewe cheese. Hence these findings open the way to a new field for mass spectrometry in the evaluation of possible fraudulence in dairy industry production. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: cheese; adulteration; matrix-assisted laser desorption/ionization; whey proteins; food

INTRODUCTION

Fresh mozzarella cheese made from the milk of water buffalo is one of the greatest delicacies in Italian cuisine. To deal with the ever-increasing market demands, the production of mozzarella has grown constantly over the years. This cheese has now become an essential ingredient of a wide range of Italian recipes and for this reason one of the most sophisticated foodstuffs.1 In this context, milk adulteration remains one of the most common types of food manipulations, and the Italian Ministry of Agriculture has estimated that about 18.8% of all cheese examined in 2000 was adulterated (Inspectorate of Ministry of Agriculture Report for 2000).

In view of the fact that this cheese is sold all around the world, in 1993 an association for the safeguard of water buffalo mozzarella was founded. This association promotes initiatives which aim at safeguarding the characteristics of water buffalo mozzarella and the use of the protected brand name, in addition to constantly improving production techniques. The association monitors the production and marketing of water buffalo mozzarella in compliance with the production rules for the DOC (Certified Origin Brand, DPCM 10/05/1993) and the DOP (Certified Provenance Brand, EEC regulation No. 1107 12/06/1996).

The most common adulteration of fresh water buffalo mozzarella is performed by adding some cow milk, as cow milk is about three times cheaper than water buffalo milk. Consequently, several analytical methodologies have been developed in order to detect this kind of adulteration. These methods are mainly based on electrophoretic and/or chromatographic techniques, in particular gel electrofocusing (IEF) and high-performance liquid chromatography (HPLC), which, notwithstanding their good results, as they are able, in some cases, to detect the presence of cow milk at levels as low as 0.5–1.0% in water buffalo mozzarella samples, they can be very slow and laborious. IEF analysis, for example, is based on the conversion of β-casein to γ-caseins. Only following this conversion can the presence of bovine milk be readily identified from the differences in the isoelectric points of bovine and water buffalo γ-caseins. The presence of bovine milk in water buffalo mozzarella has been also revealed using immunoblotting.
procedures and atmospheric pressure ionization mass spectrometry (API-MS), and bovine and buffalo caseins have been studied.\textsuperscript{5,6} Compared with the large number of methods aimed at detecting the presence of cow milk in buffalo mozzarella cheese, there is no simple and standard strategy to evaluate the presence of ewe (ovine) milk. This kind of adulteration is very recent and although ovine milk is more expensive than bovine milk, the addition of the former to water buffalo mozzarella cheese has its advantages. In fact, the taste of ovine milk is more similar than that of bovine milk to the taste of water buffalo. Moreover, during the controls carried out on samples of mozzarella cheese up to now, cow milk proteins have been sought, neglecting ewe milk proteins, so their presence has not been observed.

Recently, new strategies for the structural analysis of milk proteins based on mass spectrometric methodologies have been developed.\textsuperscript{6–11} In particular, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been shown to be a powerful analytical tool in providing a valid fingerprint of the milk protein profile. The method, leading to the identification of most of the proteins present in milk samples, has been successful employed to set up some rapid and convenient protocols aimed at monitoring structural damage caused by thermal industrial processes, such as pasteurization and sterilization, and at revealing differences in the protein pattern of milk samples coming from various mammal species.\textsuperscript{7,12}

In view of these successful results, some interesting papers have shown the suitability of MALDI-MS for the evaluation of possible fraudulence in the production of marketed water buffalo mozzarella cheese and ‘Pecorino’ cheese.\textsuperscript{13,14} It is worth noting that the MALDI spectra obtained from cheese samples reported in these papers are characterized by limited resolution because they were recorded using instruments not equipped with the delayed extraction technique. The introduction of delayed extraction improved the resolution of MALDI analysis with the consequence that it is now possible to separate peaks differing by a few daltons within a mass range up to 20–30 kDa. Based on this, in a recent paper we presented a new strategy for the rapid, simple and accurate MALDI time-of-flight (TOF) MS\textsuperscript{15} determination of both the presence of bovine milk in either raw ewe or buffalo milk samples employed in industrial processes and the addition of powdered milk to a sample of fresh raw milk. Thanks to the resolution of modern MALDI-TOF instruments equipped with delayed extraction technique, we can easily separate proteins the molecular masses of which differ by just a few daltons in the mass range below 20 kDa. In our approach, the extent of adulteration was defined by evaluating the protein patterns coming from the most abundant whey proteins, \(\alpha\)-lactalbumin and \(\beta\)-lactoglobulins, these proteins being readily detectable both for water buffalo and for the other mammals studied in each analysed blend.\textsuperscript{15}

Furthermore, owing to the speed and ease of analysis of MALDI-MS, we have shown the usefulness of MALDI-MS for the determination of any possible adulteration of raw fresh milk samples that dairy industries receive every day from their producers.\textsuperscript{15} On the other hand, dairy industry staff have been found to be interested in the application of this method to marketed cheese also, so in this work we focused our attention on the final product obtained from water buffalo milk and here we present a strategy for identifying bovine and ewe milk adulteration of fresh water buffalo mozzarella cheese using MALDI-TOFMS. The results obtained demonstrate that the differentiation among mozzarella made from water buffalo milk and from mixtures of less expensive bovine and ewe milk with buffalo milk can be achieved by using whey proteins, \(\alpha\)-lactalbumin and \(\beta\)-lactoglobulins, as molecular markers. Finally, it is worth noting that the presence of the whey proteins in the spectrum of water buffalo mozzarella cheese, still easily observable as in the MALDI analysis of bulk water buffalo milk, demonstrates that these polypeptides do not completely degrade despite the thermal and enzymatic processes involved in mozzarella cheese production.

**EXPERIMENTAL**

**Samples**

The following samples were analysed: bulk water buffalo milk sample; bulk bovine milk sample; bulk ewe milk sample; bovine mozzarella cheese; water buffalo mozzarella cheese; mozzarella cheese made of water buffalo and bovine milk in ratios 50 : 50, 60 : 40, 70 : 30, 80 : 20, 90 : 10, 95 : 5 and 98 : 2; mozzarella cheese made of water buffalo and ewe milk in ratios 50 : 50, 60 : 40, 70 : 30, 80 : 20, 90 : 10, 95 : 5 and 98 : 2; and samples of ‘Pecorino’ cheese (made from ewe milk only) aged 1 month.

The water buffalo mozzarella samples for analysis were carefully controlled so that any possible adulteration could be excluded.

It is important to emphasize that mozzarella cheese samples were obtained from milk of different breeds of cows, ewes and water buffaloes in order to make the analyses independent of possible genetic modifications present in a single specimen.

**Mass spectrometry**

MALDI mass spectra were measured on a Voyager DE mass spectrometer or an STR instrument (both from Perseptive Biosystems, Framingham, MA, USA). Both instruments are fitted with a pulsed nitrogen laser (337 nm) with a 3 ns pulse duration.

All mozzarella samples were treated as follows: mozzarella cheese was crumbled into small pieces and a sample was centrifuged at 8000 rpm for 2–3 min. About 10 \(\mu\)l of the supernatant, which is the mozzarella whey, were diluted 1 : 100 (v/v) with 0.1% trifluoroacetic acid (TFA). This method of protein extraction guarantees the detection of the whey proteins in the MALDI mass spectra.

The procedure for the extraction of the protein fractions from ‘Pecorino’ cheese was as follows: about 50 g of cheese were whisked with 150 ml of Milli-Q water until a homogeneous sample was obtained. This sample was kept at 50 °C for 30 min with magnetic stirring and after cooling it was directly analysed following appropriate dilution.
For all the MALDI mass spectra, sinapinic acid at a concentration of 10 g l⁻¹ in acetonitrile–water (50:50, v/v) was used as a matrix. A 5 µl volume of the sample solution was added to 5 µl of the matrix solution, and ~1 µl of the resulting mixture was deposited on the sample holder and allowed to dry at room temperature.

Several independent measurements were made for each sample on different days in order to verify the reproducibility and the mass accuracy, which was always in the range 0.5–1.0%. Internal mass calibration, carried out daily, was performed using bovine whey proteins as calibrant.

RESULTS AND DISCUSSION

The MALDI-TOF mass spectrum of water buffalo mozzarella cheese is shown in Fig. 1. Compared with the already reported analysis of bulk water buffalo milk, the spectrum in Fig. 1 represents a nearly identical protein profile. The predominant exception is the region from 10 to 13 kDa, where the protein pattern of water buffalo mozzarella cheese exhibits more mass signals than those observed for water buffalo milk analysis. These peaks can be reasonably assigned to the thermal and/or enzymatic degradation products derived from the principal milk protein during the cheese preparation.

Concerning the rest of the spectrum in Fig. 1, it resembles the corresponding investigation conducted on water buffalo milk. In the range of the whey proteins, which is from 14 to 19 kDa, there are three signals. In addition to the peaks related to α-lactalbumin (m/z 14246.5) and β-lactoglobulin (m/z 18268.3), there is an additional peak at m/z 15755.9. This mass signal corresponds to a specific protein, called protein X, already identified by other analytical methodologies as a component of the water buffalo β-lactoglobulin family.¹⁶

The presence of whey proteins in the spectrum of the water buffalo mozzarella cheese, still easily observable as in MALDI analysis of bulk water buffalo milk, demonstrates that these proteins do not completely degrade despite the thermal and enzymatic processes involved in the mozzarella cheese preparation. These results are only apparently in contrast to what was expected according to some data recently reported.¹³ Furthermore, the existence of α-lactalbumin and β-lactoglobulin in the spectrum also suggests that regardless of their high solubility in water, they are not completely lost in the whey but can also be found in the cheese and their recovery may be due to the extraction method used (see Experimental section).

In view of this fact, and in order to find a parameter to assess the presence of cow or ewe milk in samples of water buffalo mozzarella cheese, attention was focused on the mass region of the whey proteins. Because the molecular masses of cow, ewe and water buffalo whey proteins are very different and because the MALDI resolution in this mass region (14–19 kDa) is high enough to resolve them, lactalbumins and lactoglobulins have been used as biological markers for the evaluation of possible fraudulence in water buffalo mozzarella cheese production.

The mass difference between buffalo and ewe whey proteins is ~78 Da for lactalbumins and ~116 Da for lactoglobulins, so that in this situation it is possible to use both proteins for the evaluation of ovine milk sophistication of fresh water buffalo mozzarella cheese. Seven mozzarella cheese samples made of water buffalo and ewe milk with different relative percentages by weight (2, 5, 10, 20, 30, 40 and 50 wt%) were prepared and analysed.

Figure 1. MALDI-TOF mass spectrum of water buffalo mozzarella cheese.
Figure 2. MALDI-TOF mass spectrum in the ranges m/z 14–14.5 and 18–18.5 of mozzarella cheese made of water buffalo and ewe milk in the ratio 50:50 (w/w).

Figure 3. MALDI-TOF mass spectrum in the ranges m/z 14–14.5 and 18–18.5 of mozzarella cheese made of water buffalo and ewe milk in the ratio 90:10 (w/w).
The MALDI mass spectrum in the ranges 14–14.5 and 18–18.5 kDa of mozzarella cheese made of water buffalo and ewe milk in the ratio 50:50 is presented in Fig. 2. Both mass regions clearly demonstrate the presence of ewe milk. In the mass range around 14 kDa, in addition to the expected peak at $m/z$ 14245.3 relating to water buffalo $\alpha$-lactalbumin, a signal at $m/z$ 14167.0, due to the corresponding ewe protein, is easily visible. Similarly, in Fig. 2(a), the mass range around 18 kDa is characterized by the presence of three intense peaks. That at $m/z$ 18267.3 is due to water buffalo $\beta$-lactoglobulin, and the other two at $m/z$ 18153.0 and 18177.6 correspond, respectively, to the B and A variants of ewe lactoglobulin, showing that the specific proteins are easily discernible. The situation is very similar for the MALDI

Figure 4. MALDI-TOF mass spectra in the range $m/z$ 14–16 of mozzarella cheese made of water buffalo and cow milk in the ratio (a) 50:50 (w/w) and (b) 90:10 (w/w).
Figure 5. MALDI-TOF mass spectrum of fresh 'Pecorino' cheese.

analysis of mozzarella prepared with a 90:10 mixture of water buffalo and ewe milk, which is reported in Fig. 3. Even in this case in the two mass ranges presented (around 14 and 18 kDa), the mass signals of water buffalo and ovine milk proteins are well separated and the existence of ewe milk can be readily evaluated. The study of other mozzarella cheese samples obtained from different percentages of ewe milk added to water buffalo milk demonstrate that the response of the instrument is linear up to a limit of 2%.

We also investigated the relationship between the composition of mixtures of the two milk samples and the relative peak area of the ewe and water buffalo lactalbumins in the spectra of these mixtures. For each determination, the data were an arithmetic mean of three different spectra. The relative peak area of the lactalbumins was plotted against the ratio the two different milk samples in the mixtures and a reasonably linear curve was observed. This result suggests that MALDI-MS can be utilized for the quantitative analysis of possible adulteration in the production of water buffalo mozzarella cheese by addition of ewe milk. It is worth noting that the method proposed here is, to our knowledge, the first strategy able to characterize possible fraudulent additions of ewe milk in samples of water buffalo milk devoted to the production of water buffalo mozzarella cheese.

The mass spacing between buffalo and cow whey proteins is \( \approx 58 \text{ Da} \) for lactalbumins and \( \approx 15 \text{ Da} \) for lactoglobulins. As the latter mass difference is larger than the former in this case lactalbumins were used as biological marker for detecting bovine milk adulteration of fresh water buffalo mozzarella cheese.

The MALDI mass spectrum in the range around 14 kDa for mozzarella cheese made from water buffalo and bovine milk in ratio of 50:50 (w/w) is reported in Fig. 4(a). The peaks of the cow and water buffalo \( \alpha \)-lactalbumins at \( m/z \) 14187.1 and 14245.3, respectively, are clearly separated, showing that specific lactalbumins can be easily identifiable. In a similar way, in the MALDI-MS analysis of mozzarella cheese made from a 90:10 mixture of water buffalo and bovine milk, presented in Fig. 4(b), the mass signals of the two lactalbumins are clearly discernible and the presence of cow milk can be assessed.

Analysing other mozzarella cheese samples with different percentages of bovine milk added to water buffalo milk, it was found that the response of the mass spectrometer is linear with a detection limit below 5%. This detection limit is higher than that found in the case of the addition of ewe milk. These results can be explained, as bovine milk contains a smaller amount of proteins compared with ewe milk. In addition, we found that quantitative analysis, conducted in the same manner of bovine milk sophistication, gave a linear relationship between the relative peak area of the cow and water buffalo lactalbumins and the ratio in which the two different milk samples were mixed, suggesting that even in this case, MALDI-MS can be utilized as a sensitive tool to quantify the amount of cow milk added to water buffalo milk during mozzarella production.

In view of these successful achievements, we undertook an investigation on samples of fresh 'Pecorino' cheese produced under controlled conditions. 'Pecorino' is a cheese obtained from ewe milk only. If a mixture with cow milk is used, in agreement with Italian law, the term 'ewe milk' must be listed in the ingredients and its percentage must be specified.

The cheese proteins were extracted following the procedure reported in the Experimental section and the MALDI mass spectrum of a representative whey sample is presented in Fig. 5. As can be seen, despite the thermal and enzymatic processes involved in 'Pecorino' cheese production,
α-lactalbumin and β-lactoglobulins can still be revealed, showing that also in this case it is possible to employ these proteins as molecular markers for the determination of the possible percentage of bovine milk fraudulently added to ewe milk in the production of marketed ewe cheese. It is reasonable to hypothesize that the reported MALDI-MS strategy can also be used successfully for the analysis of marketed ewe cheese. Thanks to the high sensitivity and the resolving power of MALDI-MS, these findings open the way to the possibility of using whey proteins instead of caseins as biomarkers in the evaluation of possible adulterations at expense of several types of cheese.

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