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The Potential of Matrix-assisted Laser Desorption/Ionization Mass Spectrometry in the Quality Control of Water Buffalo Mozzarella Cheese

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Adulteration by addition of bovine milk to water buffalo milk employed for mozzarella cheese production is often observed. Water buffalo milk and mozzarella cheese were analysed by matrix-assisted laser desorption/ionization mass spectrometry in order to achieve their rapid and accurate characterization and to evaluate possible fraudulence in mozzarella cheese production. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: matrix-assisted laser desorption/ionization mass spectrometry; milk proteins; cheese proteins

INTRODUCTION

Mozzarella is a typical Italian cheese usually obtained from bovine or buffalo milk. The compositive characteristics of water buffalo mozzarella cheese (fat not less than 52%, water not more than 65%) make this product of higher organoleptic and nutritional quality than the bovine product. Furthermore, water buffalo mozzarella 'Campana' obtained European Quality Certification in 1996.

Adulteration by addition of bovine milk to the water buffalo milk employed for mozzarella production is often observed and consequently the development of methods able to detect and evaluate this fraudulence are of interest. Various procedures based on separative approaches have been proposed for this purpose. In particular, gel electrofocusing[1] and high-performance liquid chromatography (HPLC)[2] have been employed for the evaluation of bovine and buffalo milk in mixtures of untreated milk and mozzarella cheese. The presence of specific proteins allowed such an evaluation in terms of differentiation of milk proteins. In particular, the HPLC of buffalo milk whey does not show any component at the same retention time as cow β-lactoglobulin A; instead, a different protein is eluted several seconds later. Quantification of cow milk in water buffalo milk and mozzarella cheese could be achieved from the relative abundance and peak height ratio of these two specific compounds.[2]

More recently, immunoblotting[3] procedures and atmospheric pressure ionization mass spectrometry (API/MS) have been used and, in the latter case, specific bovine and buffalo caseins have been easily detected.[4,5]

In previous investigations, the capabilities of matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) in the dairy industry were demonstrated.[6] The method allows valid fingerprinting of the protein profile of cow milk and was employed to test the possible thermal degradation of milk proteins during pasteurization and/or sterilization treatments. Consequently, it was considered of interest to examine the suitability of this method for the characterization of water buffalo milk and buffalo mozzarella cheese, and for the evaluation of possible fraudulence in mozzarella production.

EXPERIMENTAL

Samples

The following samples were analysed: four individual water buffalo milk samples (**1–4**); one bulk water buffalo milk sample (**5**); one bulk bovine milk sample (**6**); three bovine mozzarella cheese samples (**7–9**); three water buffalo mozzarella cheese samples (**10–12**); one commercial bovine mozzarella cheese sample (**13**); and 10 commercial water buffalo mozzarella cheese samples (**14–23**).

Samples **1–12** were purchased from small dairies and the production was carefully controlled so that any possible adulteration could be excluded. Samples **13–23** were acquired directly on the open market.

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Preparation of mozzarella whey for MALDI analysis

Mozzarella cheese was crumbled into small pieces using a sharp knife. About 30 g of crumbled sample were transferred in a polypropylene tube and centrifuged at 8000 rpm for 3 min. The supernatant was transferred into a polypropylene vial and analyzed on the same day or frozen immediately and stored at −20 °C until MALDI/MS analysis.

Mass spectrometry

MALDI measurements were performed on a Reflex time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with a Scout ion source, operating in the positive-ion linear mode. Ions formed by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated to 30 keV. The UV laser light, which had an energy of about 50 μJ, was focused on to the sample using a variable focal diameter of 100–300 μm. The laser power was attenuated by 50%.

All the milk samples described above were treated as follows: 100 μl of milk were dissolved in 1 ml of water containing 0.1% trifluoroacetic acid and diluted 1:20 (v/v) with the same solution.

All the mozzarella cheese samples were treated as described above and 100 μl of the supernatant were dissolved in 1 ml of water containing 0.1% trifluoroacetic acid and diluted 1:20 (v/v) with the same solution.

For all the samples, sinapinic acid as a saturated solution in acetonitrile–water (1:1, v/v) was used as a matrix. Volumes of 5 μl of the sample solutions obtained as described above were added to 5 μl of matrix solution.

Three independent measurements were made for each sample in order to verify the reproducibility and the mass accuracy. The latter was always in the range 0.5–0.1%. External calibration, carried out daily, was provided by the $[M + H]^+$ and $[M + 2H]^{2+}$ ion of horse myoglobin at *m/z* 16 952 and 8467, respectively.

RESULTS AND DISCUSSION

MALDI/MS has been employed previously for determining milk protein content. By direct analysis of untreated milk samples, an immediate evaluation of the protein profile can be obtained by identifying caseins and whey proteins (such as α-lactalbumin and β-lactoglobulin).[6]

In the present investigation, allowing that these main classes of milk protein show genetic polymorphism and post-translational modifications, different protein profiles of bovine and water buffalo milk would be expected. The MALDI mass spectra of the two different milks were compared in order to test this hypothesis.

The MALDI mass spectrum obtained from an untreated bovine milk sample (**6**) is reported in Fig. 1. The origin of the different peaks was assigned on the basis of published molecular mass data.[7] In the region *m/z* 8000–12 000, the protonated proteoso peptone p.p.8.1 yields an ion at *m/z* 9170 and γ_2 and γ_3 caseins give rise to signals at *m/z* 11 852 and 11 595, respectively. Further abundant peaks are due to α-lactalbumin

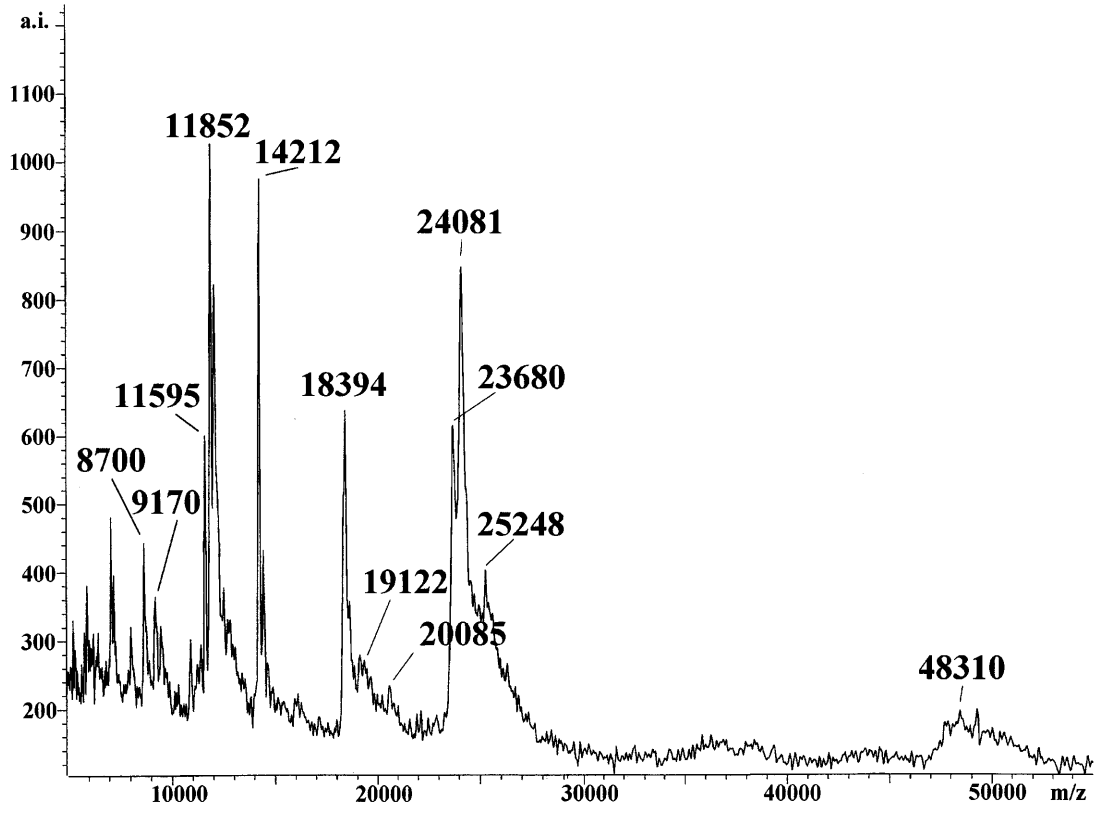

Figure 1. MALDI mass spectrum of bulk bovine milk (sample **6**).

Table 1. Literature and MALDI/MS data on molecular masses of milk and cheese proteins

Protein	Molecular mass data for bovine milk	MALDI/MS data ± SD[a] Bovine milk	Water buffalo milk	Bovine mozzarella cheese	Water buffalo mozzarella cheese
Proteoso peptone p.p.8.I	9000	9170 ± 3 (21 ± 2)	8670 ± 3 (60 ± 2)	—	8610 ± 4 (76 ± 4)
γ_3 Casein	11 500	11 591 ± 5 (42 ± 2)	11 588 ± 6 (80 ± 2)	11 595 ± 3 (50 ± 3)	11 588 ± 10 (72 ± 3)
γ_2 Casein	11 800	11 856 ± 3 (100 ± 0)	11 850 ± 5 (83 ± 3)	11 861 ± 2 (55 ± 7)	11 872 ± 10 (60 ± 3)
α-Lactalbumin	14 200	14 209 ± 8 (75 ± 2)	14 270 ± 3 (79 ± 2)	—	—
β-Lactoglobulin	18 360	18 392 ± 10 (51 ± 5)	18 306 ± 4 (34 ± 5)	18 394 ± 10 (18 ± 3)	—
κ Casein	19 000	19 125 ± 5 (10 ± 5)	19 192 ± 8 (29 ± 5)	19 120 ± 5 (20 ± 5)	—
γ_1 Casein	21 000	20 085 ± 10 (5 ± 5)	20 090 ± 5 (0.5 ± 0.1)	21 049 ± 8 (38 ± 4)	20 790 ± 10 (37 ± 3)
α_{s1} Casein	23 600	23 682 ± 10 (49 ± 7)	23 406 ± 6 (73 ± 6)	23 678 ± 10 (29 ± 3)	23 410 ± 7 (30 ± 4)
β Casein	24 000	24 085 ± 15 (83 ± 10)	24 066 ± 20 (100 ± 0)	24 081 ± 10 (100 ± 0)	24 066 ± 20 (85 ± 6)
α_{s2} Casein	25 250	25 241 ± 18 (25 ± 8)	—	25 239 ± 16 (62 ± 8)	—
Protein X	—	—	15 790 ± 3 (66 ± 3)	—	15 789 ± 4 (55 ± 3)
Unknown	—	8700 ± 2 (38 ± 2)	8270 ± 2 (85 ± 2)	8700 ± 2 (69 ± 3)	8274 ± 4 (100 ± 0)
Proteoso peptone p.p.3	22 000	—	—	22 262 ± 15 (37 ± 3)	22 170 ± 10 (33 ± 4)

[a] In parentheses: relative abundance ±SD, expressed as mean values of peak height obtained by 10 different measurements.

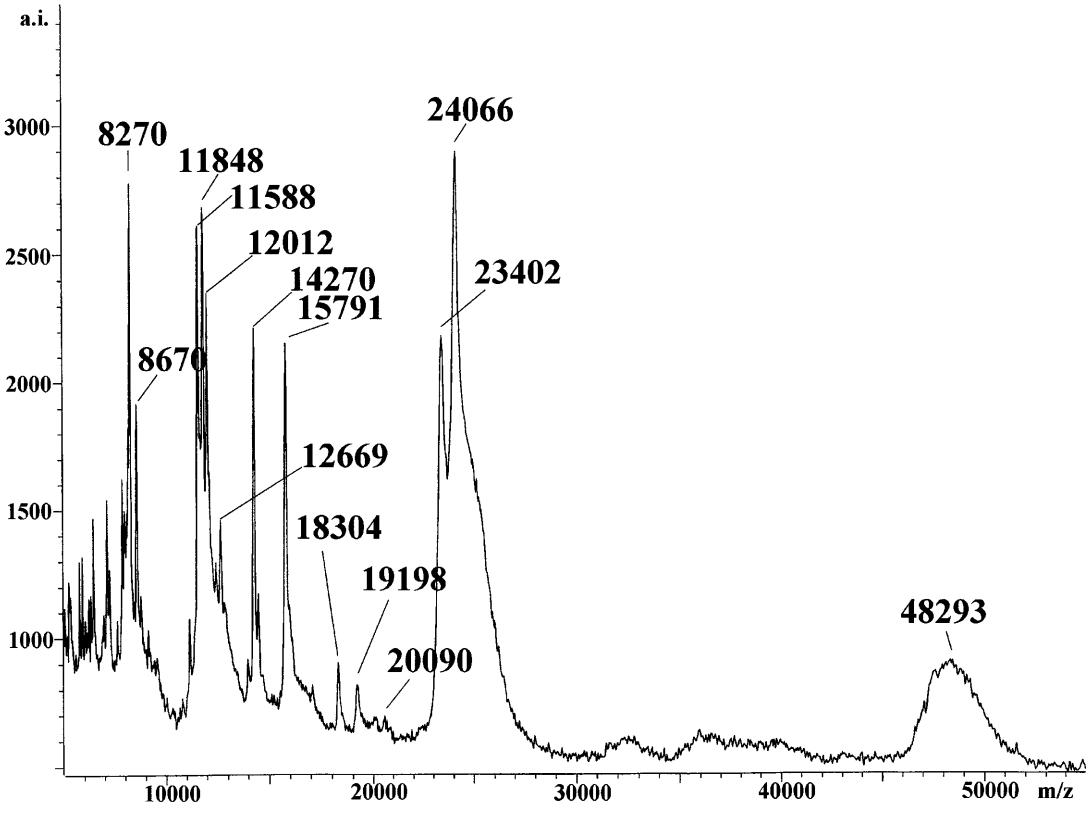

Figure 2. MALDI mass spectrum of bulk water buffalo milk (sample **5**).

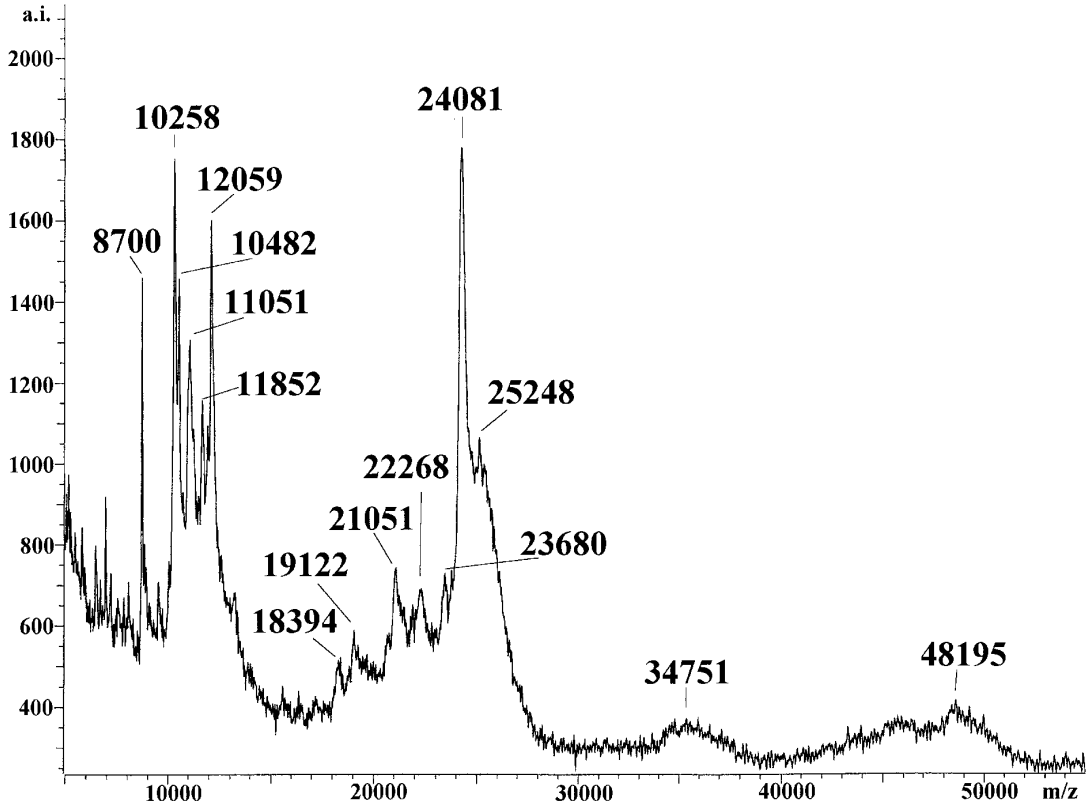

Figure 3. MALDI mass spectrum of bovine mozzarella cheese (sample **7**).

(m/z 14 212), β-lactoglobulin (m/z 18 394), α_{s1} casein (m/z 23 680) and β casein (m/z 24 081). Less abundant peaks are due to κ casein (m/z 19 122), γ_1 casein (m/z 20 085) and α_{s2} casein (m/z 25 248). A further species, not previously reported, yields a peak at m/z 8700. The proteins detected, together with the literature data on their molecular mass and the related MALDI measurements, are reported in Table 1.

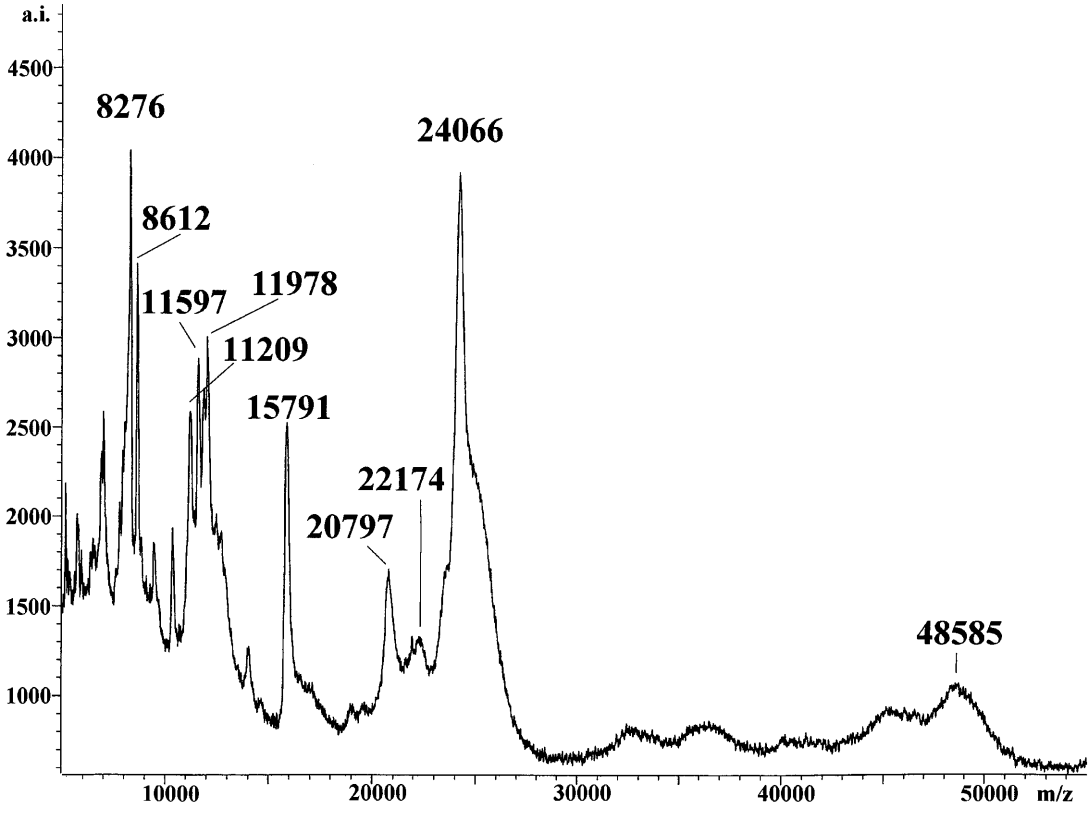

Figure 4. MALDI mass spectrum of water buffalo mozzarella cheese (sample **10**).

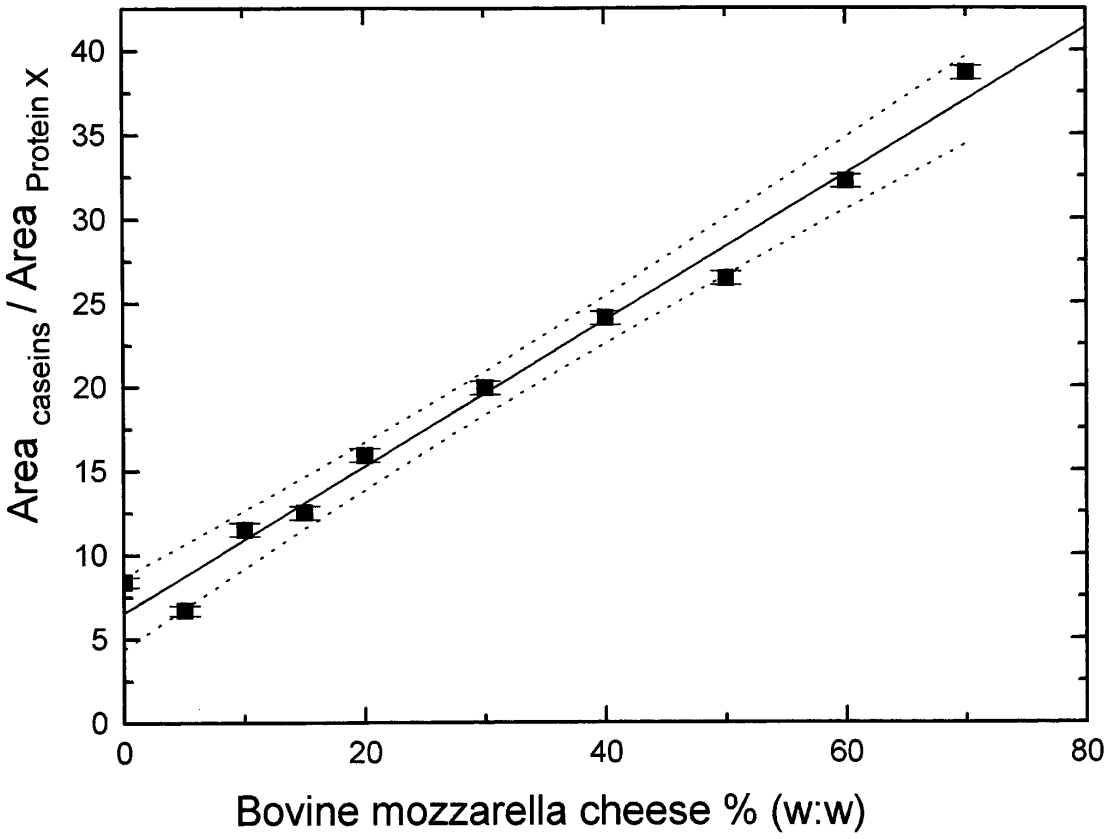

Figure 5. Plot of $area_{caseins}/area_{protein\ X}$ *vs.* mass percentage of bovine mozzarella cheese.

The protein profile of a water buffalo milk sample (**5**) obtained by MALDI/MS (Fig. 2) is very similar to that of bovine milk. In the region 8000–9000 Da, two major peaks are detected at *m/z* 8270 and 8670; the latter may be assigned to the proteoso peptone p.p.8.1, which in the case of bovine milk yields a signal at *m/z* 9170. This mass difference can be explained by the different primary structures of the two proteins. In contrast, γ_2 and γ_3 caseins exhibit, in the case of buffalo milk, molecular masses virtually identical with those of the same proteins present in bovine milk, analogously to what is observed for β casein.

In the case of buffalo milk, α_{s1} casein has a lower value (*m/z* 23 402 *vs.* 23 680) and the signal related to α_{s2} casein has almost disappeared; furthermore, the relative abundance of the peak due to β-lactoglobulin is reduced (34 *vs.* 51%).

Interestingly, a specific protein (protein X), not detected in bovine milk, yields an abundant peak at *m/z* 15 791. This species has already been detected by other analytical procedures[2] and from HPLC data it was suggested that it belonged to the β-lactoglobulin family.

Other characteristic peaks of buffalo milk are those at *m/z* 12 012 and 12 669, probably due to proteoso peptone pp.5. Further peaks are due to κ casein (*m/z* 19 198) and γ_1 casein (*m/z* 20 090).

Analogous results were obtained for samples **1–4**, with only minor variations (1–3%) in relative abundance.

In previous investigations,[6] MALDI was applied to the determination of the possible protein degradation occurring during bovine milk pasteurization (72–90 °C for 10–30 s) and sterilization (140–150 °C for 2–5 s) processes. The high specificity of the method and the high reproducibility of the data obtained allowed the detection of the sometimes extreme changes in protein profile due to thermal treatments.

In mozzarella cheese production, the milk is subjected to thermal treatment: first during the curdling phase (4–5 h at 36–40 °C) and second during the cheese paste spinning process (a few minutes at 65–75 °C), by heating with boiling water.[8] Hence possible thermal degradations of the original proteins present in milk could be expected, overlapping changes due to enzymatic proteolysis.

In practice, the MALDI mass spectra of mozzarella cheeses reveal protein profiles clearly different from those of the original milks (Figs 3 and 4). For bovine mozzarella cheeses (samples **7–9**) an increase in the ions at *m/z* 8700, 19 122 (κ casein) and 21 051 (γ_1 casein) and a decrease in those at *m/z* 18 394 (β-lactoglobulin) and *m/z* 23 680 (α_{s1} casein) are observed (see, as an example, Fig. 3); the peak at *m/z* 14 212 due to α-lactalbumin has disappeared completely. A new peak at *m/z* 22 268 is easily detectable and it can reasonably be assigned to proteoso peptone p.p.3. In the region 10 000–13 000 Da, the protein profile of bovine mozzarella cheese becomes more complicated than that observed for bovine milk: ionic species at *m/z* 10 258, 10 482 and 12 059 are detected, in addition to those at *m/z* 11 852 and 11 595 already described.

In the case of water buffalo mozzarella cheeses (samples **10–12**), the protein profiles also show clear differences with respect to that of milk, as can be seen in

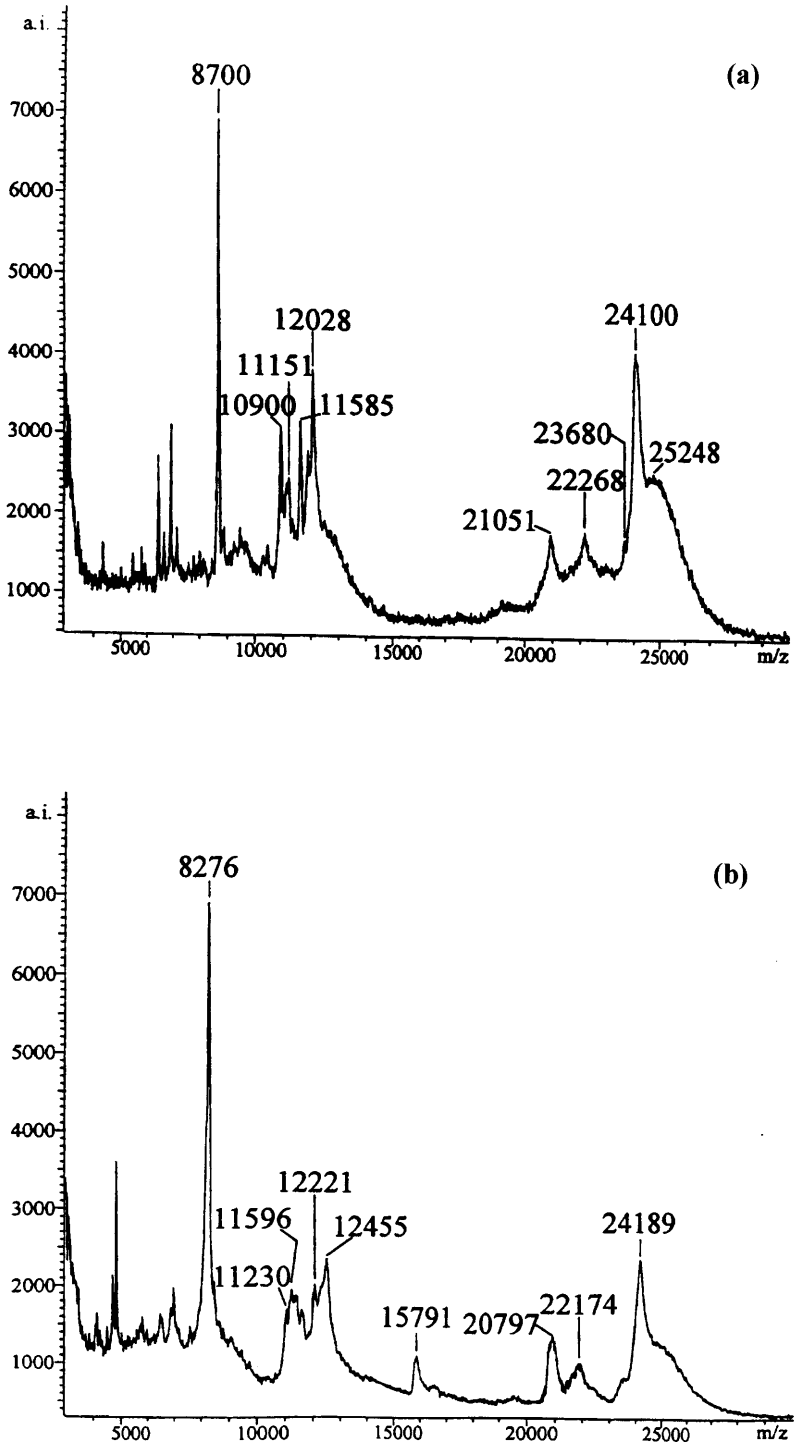

Figure 6. MALDI mass spectra of commercial mozzarella cheeses: (a) sample **13** (cow) and (b) sample **14** (water buffalo).

Fig. 4. No peaks due to α-lactalbumin and β-lactoglobulin are detected; the signal due to α_{s1} casein decreases and, as was the case for bovine mozzarella cheese, ionic species at *m/z* 20 797 and 22 174 are observed. The peak due to protein X, typical of buffalo milk, is still easily detectable at *m/z* 15 791, indicating that this compound does not decompose during thermal and/or enzymatic processes occurring in the production of mozzarella cheese, in contrast to observed effects on α-lactalbumin, β-lactoglobulin and α_{s1} casein. The peaks at *m/z* 8270 and 8600 are still present, but the latter has a higher abundance than in buffalo milk. In the region *m/z* 10 000–13 000 the MALDI mass spectrum strongly resembles that of bovine mozzarella cheese, even though differences in mass values were found among analogous species.

In order to find a parameter to evaluate the percentage of bovine milk employed in the production of the buffalo mozzarella cheese, we focused our attention on the peaks at *m/z* 15 791, due to protein X typical of buffalo milk, and on those due to α_{s1}, β and α_{s2} caseins (*m/z* 23 000–26 000). We spiked buffalo mozzarella

Table 2. Addition of bovine milk during the production of buffalo mozzarella cheese, calculated from MALDI/MS measurements

Sample	$Area_{caseins}/area_{protein\ X}$	Bovine milk (%)
14	20.04	30.98
15	14.16	17.61
16	15.40	20.40
17	17.53	25.21
18	11.92	12.56
19	10.94	10.33
20	19.39	29.42
21	14.55	18.50
22	10.71	9.81
23	13.08	15.16

cheese with different percentages by mass of bovine mozzarella cheese (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60 and 30:70). The resulting MALDI mass spectra show overlapping with those spectra reported in Figs 3 and 4, with the peaks of interest at m/z 15 791 and in the m/z 23 000–26 000 range. Using a simple integration procedure, it is possible to obtain the area of the peaks of interest. Plotting the $area_{caseins}/area_{protein\ X}$ ratio *vs.* mass percentage of bovine mozzarella cheese, the straight line $y = 6.570\,46 + 0.435\,86x$ is obtained, with a linear correlation coefficient of 0.9925 (see Fig. 5).

Commercial buffalo and bovine mozzarella cheeses were then analysed by MALDI/MS. As shown in Fig. 6, the protein profiles are similar to those found for products produced under controlled conditions, but the abundance of low molecular mass compounds is higher. Applying the integration procedure described above to the commercial buffalo mozzarella cheeses (samples **14–23**), different $area_{caseins}/area_{protein\ X}$ values were found, strictly depending on the dairies considered (see Table 2).

In conclusion, the MALDI/MS method has been demonstrated to be a valid method for the determination of possible adulteration in the production of water buffalo mozzarella cheese by addition of cow milk to the water buffalo milk employed for cheese production. The present results must be considered as preliminary; in order to be used as an authentication technique, comparisons with alternative, non-mass spectrometric methods must be made.

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