

# MALDI-TOF mass spectrometry for the monitoring of she-donkey's milk contamination or adulteration

Vincenzo Cunsolo,\* Vera Muccilli, Rosaria Saletti and Salvatore Foti



Donkey's milk (DM), representing a safe and alternative food in both IgE-mediated and non-IgE-mediated cow's milk protein allergy, can be categorized as precious pharma-food. Moreover, an economically relevant interest for the use of DM in cosmetology is also developing. The detection of adulterations and contaminations of DM is a matter of fundamental importance from both an economic and allergenic standpoint, and, to this aim, fast and efficient analytical approaches to assess the authenticity of this precious nutrient are desirable. Here, a rapid matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)-based method aimed to the detection of bovine or caprine milk in raw DM is reported. The presence of the extraneous milks was revealed by monitoring the protein profiles of the most abundant whey proteins,  $\alpha$ -lactalbumin ( $\alpha$ -LA) and  $\beta$ -lactoglobulin, used as molecular markers. The possibility of obtaining a quantitative analysis of the level of cow or goat milk in DM based on the MALDI-TOF peak areas of  $\alpha$ -LAs was also explored. The results showed that the experimental quantitative values were in good agreement with the real composition of each mixture. As pretreatment of the milk samples is not required, and owing to the speed and the high sensitivity of MALDI-MS, the protocol here reported could represent a reliable method for routine analyses aimed to assess the absence of contamination in raw fresh DM samples. Copyright © 2013 John Wiley & Sons, Ltd.

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**Keywords:** donkey's milk; milk allergy; contamination; adulteration; MALDI-TOF MS

## Introduction

Confirming the popular tradition, recent clinical studies have clearly demonstrated that donkey's milk (DM) can be categorized among the best mother milk substitute for the great majority of newborns allergic to cow's milk proteins (CMP), due to its much reduced or absent allergenic properties coupled to excellent palatability and nutritional value.<sup>[1]</sup> For the use of this aliment in the diet of allergenic children, it is indispensable to avoid contamination by other milk allergens, due to insufficient cleaning of production system and instruments. Moreover, since an economically relevant interest for the use of DM in cosmetology is also developing, the production of DM-based cosmetics could represent a potential target for adulteration, such as the fraudulent addition of cheaper and more readily available milks from other species as cow or goat. On the light of these considerations, the detection of adulterations and contaminations of DM is a matter of fundamental importance from both economic and allergenic standpoints, and, to this aim, fast and efficient analytical approaches to assess the authenticity of this precious nutrient are desirable. Currently, both immunological and chemical methods are used to detect dairy product contamination or adulteration. However, immunological methods, such as enzyme-linked immunosorbent assay and immunoblotting, are troubled with false-positive results and inadequate quantification. On the other hand, chemical methods, mainly based on electrophoretic and/or chromatographic techniques,<sup>[2,3]</sup> although able, in some cases, to detect the presence of the adulterant at levels as low as 0.5–1.0%, are laborious and time-consuming. The present European Community reference method for detection of cow's milk and caseinate in cheeses

made from ewe's milk, goat's milk, buffalo milk or mixtures of ewe, goat and buffalo milk is isoelectric focusing of  $\gamma$ -caseins after plasminolysis.<sup>[4]</sup> This method allows only a qualitative detection of cow's milk.

Recently, mass spectrometry (MS), and in particular matrix-assisted laser desorption/ionization MS (MALDI-MS), has been successfully employed for dairy authentication purposes, and it has been proposed as tool for rapid dairy controls.<sup>[5]</sup> In particular, taking into account that the primary structure of homologous proteins shows species-specific amino acid differences which affect both their molecular masses and peptide mass fingerprinting, MS-based methods, thanks to the resolution power and to the high sensitivity of modern instruments, are able to assess milk authenticity by characterizing the protein or peptide profile present in a specific product. It has been demonstrated that MS-based approaches (i.e. reversed-phase-high-performance liquid chromatography/electrospray ionization (HPLC/ESI MS) and MALDI-MS) may represent suitable methods for detecting milk adulteration by monitoring the profile of the most abundant whey proteins (i.e.  $\alpha$ -lactalbumin ( $\alpha$ -LA) and  $\beta$ -lactoglobulin ( $\beta$ -LG)).<sup>[6–8]</sup> It is worth noting that, even if LC-ESI MS and MALDI-MS could be considered two complementary approaches, a great advantage of MALDI-MS over LC-ESI MS is the speed of analysis. Thus, MALDI-MS may provide a fast and reliable method for routine analyses of raw fresh milk samples.

\* Correspondence to: Vincenzo Cunsolo, Department of Chemical Science, University of Catania, Viale A. Doria, 6, I-95125 Catania, Italy. E-mail: vcunsolo@unict.it

Department of Chemical Science, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

In the present study, we report the use of MALDI MS in the detection of adulterations or accidental contaminations of DM by milk of ruminants (e.g. goat or cow's milk), by monitoring the protein profile corresponding to the most abundant whey proteins (i.e.  $\alpha$ -LA and  $\beta$ -LG). The possibility of obtaining a quantitative analysis of the level of goat or cow's milk in DM based on the MALDI-time of flight (TOF) peak areas of  $\alpha$ -LAs was also explored.

## Experimental

### Materials and methods

Acetic acid, trifluoroacetic acid (TFA), sinapinic acid (SA), cytochrome C from bovine heart, myoglobin from horse skeletal muscle and trypsinogen from bovine pancreas were obtained from Aldrich (St. Louis, Missouri, USA). Sodium acetate, HPLC grade H<sub>2</sub>O and CH<sub>3</sub>CN were provided by Carlo Erba (Milan, Italy). The General Protein/Mass Analysis for Windows (GPMW) software (<http://welcome.to/gpmaw>) was used for all sequence handling. The ClustalW2 tool (<http://www.clustal.org/>) was used for sequence alignment.

### Milk samples preparation

Bulk DM samples were collected at middle lactation stage from she-donkeys belonging to two Sicilian autochthonous donkey breeds (Ragusano and Grigio Siciliano) (118 she-donkeys from five different farms of Sicily). Bulk goat's milk (Argentata breed) and bulk cow's milk (Modicana and Frisona breed) were obtained by two different farms of Southern of Sicily. After milking, the samples were immediately frozen and stored at  $-20^{\circ}\text{C}$  until used. The milk was defatted by centrifugation at 5500 rpm,  $4^{\circ}\text{C}$  for 10 min. The fat layer was carefully removed and casein fraction precipitated from the skimmed milk with sodium acetate/acid acetic buffer at pH 4.4. Complete separation of the sodium caseinate precipitate from the supernatant (i.e. whey proteins) was obtained by centrifugation at 8500 rpm,  $4^{\circ}\text{C}$  for 30 min. The whey protein solutions were subsequently characterized by MALDI-TOF MS as reported in the next paragraph.

The whey fractions of bulk donkey, goat and cow milks were first analyzed. Then, simulating fraudulent addition or unintentional contamination of DM by goat's or cow's milk, the following mixtures were prepared: mixtures of goat's milk and DM in volume ratios 0.5/95.5; 1/99; 2/98; 5/95; 10/90; 20/80; 30/70, 40/60 and 50/50; mixtures of cow's milk and DM in volume ratios 0.5/95.5; 1/99; 2/98; 5/95; 10/90; 20/80; 30/70, 40/60 and 50/50. Each mixture was prepared in triplicate. The whey proteins fractions of these milk mixtures were obtained as above described and then characterized by MALDI-TOF MS. Each whey fraction was analyzed in triplicate, and nine independent spectra for each mixture were thus obtained.

### MALDI-TOF MS analysis

MALDI mass spectra were acquired using a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a UV nitrogen laser (337 nm). Data acquisition and data analysis were performed using the *Voyager v. 5.1* and *Data Explorer v. 5.1* software (Applied Biosystems), respectively. SA at a concentration of  $10\text{ }\mu\text{g}/\mu\text{l}$  (in acetonitrile + 0.1% TFA/water + 0.1% TFA, 70:30, v/v) was used as matrix. About  $10\text{ }\mu\text{l}$  of the whey protein solution of each sample was diluted 1:100 (v/v) with 0.1% aqueous

TFA.  $5\text{ }\mu\text{l}$  of sample solution was added to  $5\text{ }\mu\text{l}$  of matrix solution. This mixture was gently shaken, and  $1\text{ }\mu\text{l}$  was directly loaded on the MALDI sample plate.<sup>[9]</sup> Each spectrum was obtained in linear positive ion mode over a  $m/z$  range 10 000–30 000 and averaged by 150 single-shot spectra from several positions within a given sample spot in order to obtain representative sample data. Then, three sample data were averaged to achieve the final spectrum (Accumulated Spectrum). Mass calibration was made using a mixture (1:1:5 v/v/v; conc.:  $0.1\text{ }\mu\text{g}/\mu\text{l}$ ) of cytochrome C ( $m/z$  12 232), myoglobin ( $m/z$  16 952) and trypsinogen ( $m/z$  23 982) as external standards. Three independent measurements were carried out for each sample on different days in order to control the reproducibility and the mass accuracy, which was in all cases below 0.02%.

### Data handling for quantitative analysis

In order to attempt a quantitative evaluation of the level of extraneous milk in DM,  $\alpha$ -LAs from the different species were chosen as reference milk protein. The peak areas (assumed proportional to the protein concentration in milk) of the  $\alpha$ -LAs signals in the accumulated spectra were obtained by integrated Gaussian curve fitting generated by the Origin software v. 7.0. Taking into account that the average content of  $\alpha$ -LA is about 1.8, 2.2 and  $1.2\text{ mg/ml}$  in donkey, goat and cow's milk, respectively, the experimental peak areas were normalized by a correction factor. Therefore, the experimental peak area of donkey's  $\alpha$ -LA was correct by the factor:

$$f = \frac{[\alpha\text{LA}]_{\text{goat}}}{[\alpha\text{LA}]_{\text{donkey}}} = 1.2$$

in the mixtures with goat's milk, and by the factor:

$$f = \frac{[\alpha\text{LA}]_{\text{cow}}}{[\alpha\text{LA}]_{\text{donkey}}} = 0.66$$

in the mixture with cow's milk.

The peak area ratio for bovine or caprine  $\alpha$ -LA to that for total  $\alpha$ -LAs in milk (extraneous plus equine) was then plotted against the percentage of goat or cow's milk to obtain calibration functions to be used for quantification. The plotted values for each mixture were obtained as average of nine independent measurements.

## Results and discussion

### MALDI-TOF MS of bulk DM samples

Compared with bovine milk, the characterization at molecular level of DM proteins is at a relatively early stage, and limited data are available for their genetic polymorphism.<sup>[10–15]</sup> At present time, only few genetic variant are known for the three most abundant whey proteins (i.e.  $\alpha$ -LA,  $\beta$ -LG and lysozyme (LYS)). Particularly, a unique sequence of  $\alpha$ -LA (UniProtKB/Swiss-Prot Acc. No. P28546)<sup>[16]</sup> and two variants, named A<sup>[17]</sup> and B<sup>[18]</sup> of LYS (UniProtKB/Swiss-Prot Acc. No. P11375) are reported. Two molecular forms of  $\beta$ -LG, named  $\beta$ -LG type I and  $\beta$ -LG type II, have been detected in DM. Two variants of  $\beta$ -LG I (UniProtKB/Swiss-Prot Acc. No. P13613), named A<sup>[19]</sup> and B<sup>[18]</sup> are known, whereas four variants of  $\beta$ -LG II (UniProtKB/Swiss-Prot Acc. No. P19647), named A,<sup>[20]</sup> B,<sup>[18]</sup> C<sup>[18]</sup> and D<sup>[21]</sup> have been identified and characterized (Table 1). Furthermore, nothing is known about the incidence of each genetic variant of these three proteins in bulk DM. Therefore, on the light of these considerations, five bulk DM samples obtained from different farms of Sicily and

**Table 1.** List of the genetic variants of the most abundant donkey's milk whey proteins. The variants detected in the samples here investigated are reported in bold

| Protein                                     | Variant  | Primary accession number <sup>a</sup> | Amino acid residues | Theoretical molecular mass (Da) <sup>b</sup> |
|---------------------------------------------|----------|---------------------------------------|---------------------|----------------------------------------------|
| $\alpha$ -lactalbumin ( $\alpha$ -LA)       | <b>A</b> | <b>P28546</b>                         | <b>123</b>          | <b>14 214.3</b>                              |
| lysozyme (LYS)                              | A        | P11375                                | 129                 | 14 679.6                                     |
|                                             | <b>B</b> | <b>P11375</b>                         | <b>129</b>          | <b>14 631.5</b>                              |
| $\beta$ -lactoglobulin II ( $\beta$ -LG II) | A        | P19647                                | 163                 | 18 258.7                                     |
|                                             | <b>B</b> | <b>D6QX31</b>                         | <b>163</b>          | <b>18 222.6</b>                              |
|                                             | C        | D6QX32                                | 163                 | 18 236.6                                     |
|                                             | D        | P19647                                | 163                 | 18 310.7                                     |
| $\beta$ -lactoglobulin I ( $\beta$ -LG I)   | A        | P13613                                | 162                 | 15 524.3                                     |
|                                             | <b>B</b> | <b>P13613</b>                         | <b>162</b>          | <b>18 510.3</b>                              |

<sup>a</sup> Primary accession number for the protein in UniProtKB/Swiss-Prot database.

<sup>b</sup> Theoretical molecular mass calculated by the GPMW software from the amino acid sequences of the mature proteins considering cysteine residues in oxidized form.

belonging to different breeds were characterized by MALDI-TOF MS in order to investigate the whey protein profile in terms of genetic variants occurrence. MALDI-MS analysis of these samples showed that the protein pattern of the most abundant whey proteins is always identical. A typical MALDI-TOF mass spectrum of these DM samples (Fig. 1) is dominated by three main peaks at  $m/z$  14 215, 14 632 and 18 511. The comparison of these  $m/z$  values with the molecular masses of the major donkey's whey milk proteins (Table 1) reveals that: (1) the signal at  $m/z$  14 215 is attributable to the unique genetic variant actually identified of  $\alpha$ -LA (theoretical  $M_r$  14 214.3 Da); (2) the signal at  $m/z$  14 632 arises from the variant B of LYS ( $M_r$  14 631.5 Da); and (3) the signal at  $m/z$  18 511 corresponds to the variant B of  $\beta$ -LG I ( $M_r$  18 510.3 Da). In addition, the mass spectrum shows a weak signal at  $m/z$  18 223 which is attributable to the variant B of the  $\beta$ -LG type II ( $M_r$  18 222.6 Da). These findings, even if restricted only to five bulk milk samples, reveal for the first time the most commonly expressed variants of the major whey proteins in bulk DM. On the light of these data and in order to find a parameter to detect the presence of goat or cow's milk in DM samples, attention was focused on these whey components, and the MALDI mass spectrum reported in Fig. 1 was taken as reference whey protein profile of a genuine bulk DM.

### MALDI-TOF MS of bulk goat and cow's milk samples

Because  $\alpha$ -LA and  $\beta$ -LGs represent the most abundant whey proteins in milk of ruminants such as cow and goat, these two components were chosen as biological markers for the evaluation of the possible presence of goat or cow's milk in DM samples. In Fig. 2 are reported the MALDI-TOF mass spectra of the whey

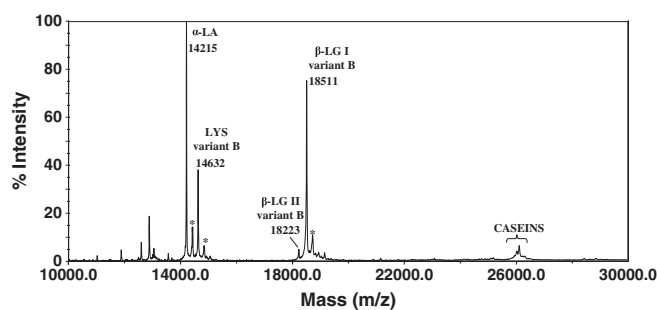


**Figure 1.** MALDI-TOF mass spectrum of the genuine bulk donkey's milk sample. Asterisks indicate protein-matrix adducts.

protein fraction obtained by raw goat (Fig. 2(a)) and cow (Fig. 2(b)) bulk milk samples. In both spectra, the different peaks can be easily assigned on the basis of the molecular masses reported in the databases (see Table 2). In particular, in the MALDI mass spectrum of goat's milk (Fig. 2(a)), two main signals are present. The peak at  $m/z$  14 187 corresponds to the  $\alpha$ -LA, whereas the second signal presents at  $m/z$  18 188 can be attributed to the  $\beta$ -LG. The MALDI mass spectrum of cow's milk (Fig. 2(b)) shows a protein profile in which the three most intense peaks are attributable to the most commonly expressed variants of bovine  $\alpha$ -LA and  $\beta$ -LGs. Particularly, the signal at  $m/z$  14 179 is due to the variant B of  $\alpha$ -LA, whereas the two peaks at  $m/z$  18 278 and 18 364 correspond to the variants B and A of  $\beta$ -LG, respectively. It can be noted that these proteins show molecular masses that can be completely distinguished from that of the homologous donkey's whey proteins (Table 2). Therefore, by monitoring the MALDI-TOF MS profile of a donkey milk sample adulterated or contaminated by goat or cow milk, it is possible to reveal all the proteins present and thus rapidly identify the extraneous milks.

### MALDI-TOF MS analysis of the mixtures of goat and DM

Nine mixtures of goat and DM with different volumetric ratios (0.5/99.5, 1/99, 2/98, 5/95, 10/90, 20/80, 30/70, 40/60 and 50/50,

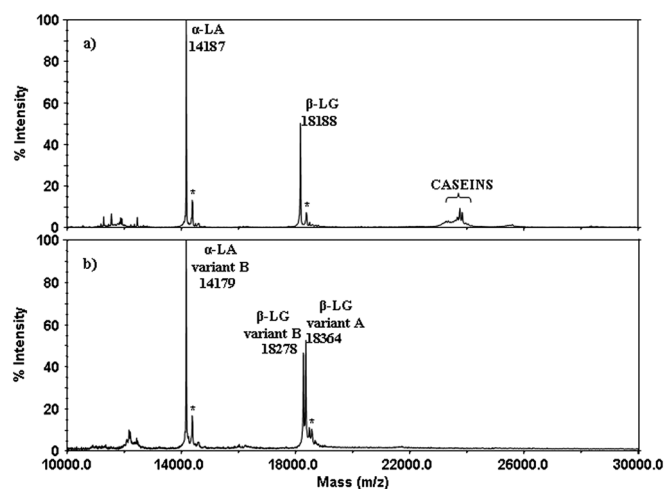


**Figure 2.** MALDI-TOF mass spectra of raw bulk goat (a) and cow (b) milk samples. Asterisks indicate protein-matrix adducts.

**Table 2.** Molecular mass of the most commonly expressed variants of  $\alpha$ -lactalbumins and  $\beta$ -lactoglobulins from cow and goat, and relative mass difference with respect to the corresponding proteins from donkey

| Protein                               | Variant | Primary accession number <sup>a</sup> | Theoretical molecular mass <sup>b</sup> (Da) | Mass difference with respect to the homologous protein from donkey (Da) <sup>c</sup> |
|---------------------------------------|---------|---------------------------------------|----------------------------------------------|--------------------------------------------------------------------------------------|
| <i>Goat</i>                           |         |                                       |                                              |                                                                                      |
| $\alpha$ -lactalbumin ( $\alpha$ -LA) | A       | P00712                                | 14 186.0                                     | −28.3                                                                                |
| $\beta$ -lactoglobulin ( $\beta$ -LG) | A       | P02756                                | 18 187.2 <sup>d</sup>                        | −323.1; −35.4                                                                        |
| <i>Cow</i>                            |         |                                       |                                              |                                                                                      |
| $\alpha$ -lactalbumin ( $\alpha$ -LA) | B       | P00711                                | 14 178.0                                     | −36.3                                                                                |
| $\beta$ -lactoglobulin ( $\beta$ -LG) | A       | P02754                                | 18 363.3 <sup>d</sup>                        | −147.0; +140.7                                                                       |
|                                       | B       | P02754                                | 18 277.2 <sup>d</sup>                        | −233.1; +54.6                                                                        |

<sup>a</sup> Primary accession number for the protein in UniProtKB/Swiss-Prot database.

<sup>b</sup> Theoretical molecular mass calculated by the GPMW software from the amino acid sequences of the mature proteins considering cysteine residues in oxidized form.

<sup>c</sup> Mass difference for  $\alpha$ -LAs is calculated with respect to the variant B from donkey. Mass difference for  $\beta$ -LGs is calculated with respect to the variants I B and II B from donkey, respectively.

<sup>d</sup> These  $\beta$ -LG variants present five cysteine residues. Theoretical molecular mass are calculated considering four of these residues in the oxidized form and ones in the reduced form.

goat/donkey: v/v) were prepared and analyzed. The MALDI mass spectrum in the  $m/z$  range 13–20 kDa relative to the mixture containing 50% of goat's milk is shown in Fig. 3(a). The comparison with MALDI-MS of the genuine bulk DM (Fig. 1) reveals the presence of two new peaks that clearly demonstrate the presence of goat milk in the sample investigated. Indeed, in addition to the expected peak at  $m/z$  14 215 corresponding to donkey  $\alpha$ -LA, a strong signal at  $m/z$  14 187 due to goat  $\alpha$ -LA is also present. Another intense additional peak, clearly attributable to goat  $\beta$ -LG, is present at  $m/z$  18 188. An analogous profile is shown by the MALDI-MS analyses of mixtures prepared with percentages of goat milk in donkey milk up to 10% v/v, where the signals of both goat  $\alpha$ -LA and  $\beta$ -LG proteins are easily visible (MALDI-MS of all mixtures analyzed are available on the supplementary material online). In the MALDI mass spectra of the mixtures obtained with lower percentages (5, 2, 1 and 0.5% v/v) of goat's milk added to DM, the signal corresponding to the  $\alpha$ -LA from goat was not detectable, whereas the goat  $\beta$ -LG peak was clearly discernible up to a limit of 0.5% (Fig. 3(b)), allowing to detect the presence of goat's milk. It

should be emphasized that this detection limit is comparable to that of the more laborious and time-consuming currently used methods. Moreover, because fraudulent additions of goat's milk at these levels are barely probable to be found as they are uneconomical, this finding shows that the proposed method could be also useful to identify accidental cross-contaminations at very low concentration. Indeed, even if it is not easy to define the risk threshold limit, sensitive subjects could react to very low amount of allergens, and anaphylactic reactions to goat's milk in children with CMP allergy have been reported.<sup>[22,23]</sup>

### MALDI-TOF MS analysis of the mixtures of cow and DM

In order to investigate the feasibility of detecting the presence of cow's milk in DM by MALDI-TOF MS analysis, nine mixtures with different volumetric ratios (0.5/99.5, 1/99, 2/98, 5/95, 10/90, 20/80, 30/70, 40/60 and 50/50, cow/donkey: v/v) of bovine milk added to DM were prepared and analyzed. In Fig. 4(a) is reported the MALDI mass spectrum of the mixture containing 50% of cow

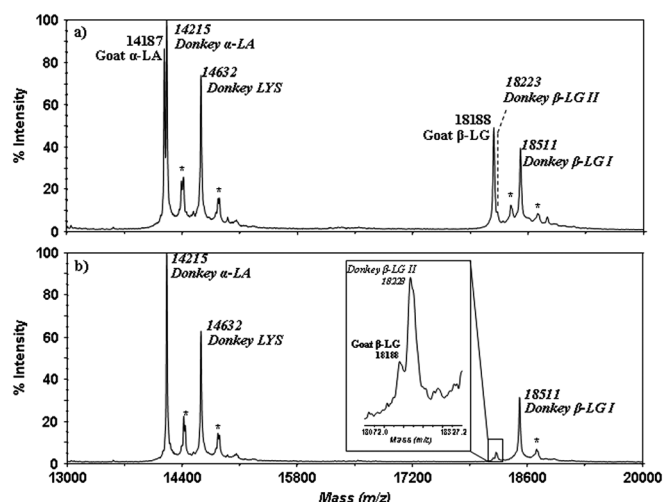


**Figure 3.** MALDI-TOF mass spectra in the  $m/z$  range 13–20 kDa of donkey's milk samples adulterated by 50% (a) and 0.5% (b) of goat's milk. Asterisks indicate protein-matrix adducts.

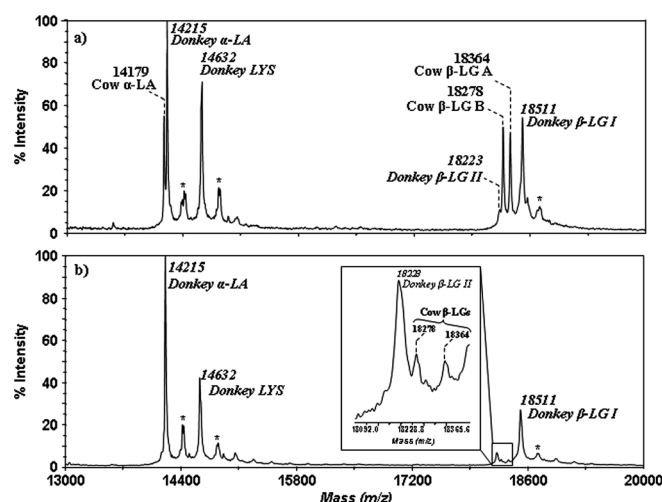


**Figure 4.** MALDI-TOF mass spectra in the  $m/z$  range 13–20 kDa of donkey's milk samples adulterated by 50% (a) and 2% (b) of cow's milk. Asterisks indicate protein-matrix adducts.

milk in the  $m/z$  range 13–20 kDa. The signals of cow and donkey  $\alpha$ -LAs at  $m/z$  14 179 and 14 215, respectively, are clearly discernible. Analogously, in the mass range of 18–19 kDa are present two intense peaks at  $m/z$  18 278 and 18 364, which are clearly attributable to the B and A variants of bovine  $\beta$ -LGs, respectively. The mass signals of cow  $\alpha$ -LA and  $\beta$ -LGs are clearly discernible in the MALDI-MS analyses of DM samples containing percentages of bovine milk up to a limit of 10% v/v (MALDI-MS of all mixtures analyzed are available on the supplementary material online). At lower percentage, the signal of cow  $\alpha$ -LA was not detectable, whereas the peaks of cow  $\beta$ -LGs, even if with very low intensity, were visible up to a limit of 2% (Fig. 4(b)). This percentage is higher than that found for goat's milk addition, probably because bovine milk presents a lower concentration of  $\alpha$ -LA and  $\beta$ -LGs with respect to goat's milk.

Finally, it should be evidenced that although the data here reported have been limited to bovine or caprine species, the same procedure can be in principle easily extended to milks from other species, such as ovine or bubaline. Indeed,  $\alpha$ -LA (Acc. N. P09462;  $M_r$  14 247.1 Da) and  $\beta$ -LG (Acc. N. P67976;  $M_r$  18 147.1 Da) from sheep, and bubaline  $\alpha$ -LA (Acc. N. Q9TSN6;  $M_r$  14 277.1 Da) and  $\beta$ -LG (Acc. N. P02755;  $M_r$  18 263.2 Da) show molecular mass differences with respect to homologous donkey's proteins that are clearly discernible by MALDI-TOF analyses.

### Quantitative analysis

Because the data obtained showed that MALDI-MS analysis provide a rapid tool for DM authenticity assessment, we also investigated the possibility to obtain quantitative information. It is well known that, although MALDI-MS represents one of the key technologies for the identification of proteins, its application for quantitative purposes is hampered by several intrinsic factors. A major issue is the non-homogeneous distribution of the analyte during the co-crystallization with the matrix, especially when the dried droplet preparation method is used.<sup>[24]</sup> This circumstance determines that at some points of the sample, no analyte signals can be obtained, whereas at other positions, strong signals can be produced (hot-spot formation). As a consequence, variations in ion current are observed with consecutive laser shots fired at the same position on the target surface (shot-to-shot reproducibility), across different locations on the target surface (region-to-region reproducibility) and between identical loadings of the same sample onto different targets (sample-to-sample reproducibility). In order to minimize these pitfalls and to obtain reliable measures for a possible quantitation, the following protocol for sample preparation and mass data acquisition was employed: (1) each sample was pre-mixed in a microtube with the matrix solution immediately before the deposition on the MALDI sample plate; (2) the matrix solution was prepared in a volatile solvent (70% acetonitrile), and the target was dried by warm air in order to obtain a faster crystallization time. Indeed, faster crystallization generates smaller crystals, and a much more uniform incorporation of the analytes into the crystalline lattice.<sup>[25]</sup> In addition, with the aim to reduce, at least in part, the problem of the region-to-region reproducibility of the same sample, multiple spectra were acquired from different locations across the target surface and accumulated to yield a more representative spectrum. Finally, the quantitative data of each sample was achieved by an average of nine independent measures in order to minimize the pitfall related to the sample-to-sample reproducibility.

In order to determine the percentage of goat or cow's milk present in DM sample, the attention was focused on the peaks

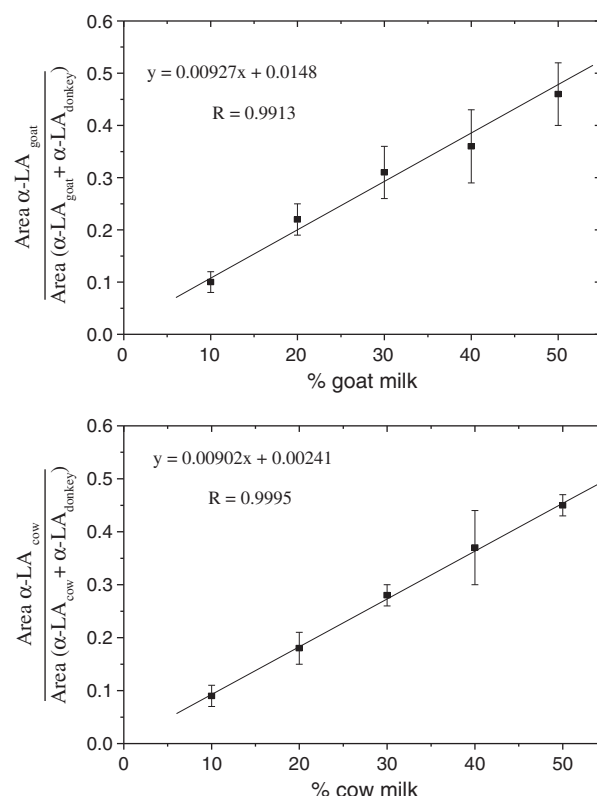


**Figure 5.** Calibration plots for different mixtures of goat's milk and donkey's milk (a) and for cow's milk and donkey's milk (b). Experimental data were plotted taking into account different protein concentrations in the components making up the mixture, as reported in the experimental section. Data were fitted to a linear regression function  $y = ax + b$ .

corresponding to the species-specific  $\alpha$ -LAs. Assuming that the peak area of  $\alpha$ -LAs is proportional to their relative concentration in milk, experimental data were corrected taking into account that the  $\alpha$ -LA content in milk vary from species to species (see experimental section). Then, plotting the peak area ratio for goat  $\alpha$ -LA to that for total  $\alpha$ -LAs in milk (goat plus donkey) versus volume percentage of added goat milk in donkey milk gave a regression line:

$$y = 0.00927x + 0.0148$$

with a linear regression coefficient of 0.9913 (Fig. 5a). Analogously, cow milk in donkey milk gave a regression line:

$$y = 0.00902x + 0.00241$$

with a linear regression coefficient of 0.9995 (Fig. 5b). These plots seem to indicate that a linear regression would provide a reasonable calibration function. Indeed, the slope and the intercept of the both linear functions are in good agreement with the theoretical values (slope: 0.0100, intercept: 0.000) and the correlation factors are also satisfactory.

### Conclusions

Identification of unintended contaminations or fraudulent additions of other milks to DM is of major interest from both allergenic and economic standpoints, and fast and efficient approaches to assess its authenticity are desirable. In the present paper a MALDI-based method aimed to the identification of goat or cow's

milk in DM samples is reported. Our results demonstrate that MALDI-MS may represent a suitable method for a rapid characterization of DM samples in order to assess their authenticity. In particular, by monitoring the MS profile of the two most abundant whey proteins (i.e.  $\alpha$ -LA and  $\beta$ -LGs), MALDI-MS is able to identify the fraudulent addition of milk from different species in DM and also to recognize unintended contaminations up to a limit of 0.5–2.0%. It should be emphasized that this detection limit is comparable to that of the more laborious and time-consuming currently used methods. Moreover, as pretreatment of the milk samples is not required and owing to the speed and the high sensitivity of MALDI-MS, the protocol here reported represents a reliable method for routine analyses aimed to the identification of adulterations or even contaminations of raw fresh DM samples. Finally, although MALDI-MS application for quantitative purposes is hampered by several intrinsic factors, a quantitative analysis on the level of goat or cow's milk in DM from mass spectrometric data was attempted. The results showed that, by careful optimization of the experimental conditions to reduce the intrinsic pitfalls of MALDI for quantitative analyses, the experimental quantitative values were in good agreement with the real composition of each mixture.

Although in the present study, the identification of extraneous milk has been limited to bovine or caprine species, the same procedure can be in principle easily extended to milks from other species, such as ovine and bubaline, because their  $\alpha$ -LA and  $\beta$ -LGs have molecular masses that can be completely resolved from that of the homologous donkey's proteins. Therefore, the MALDI-MS approach may provide a fast and reliable response for the presence of extraneous milk in DM.

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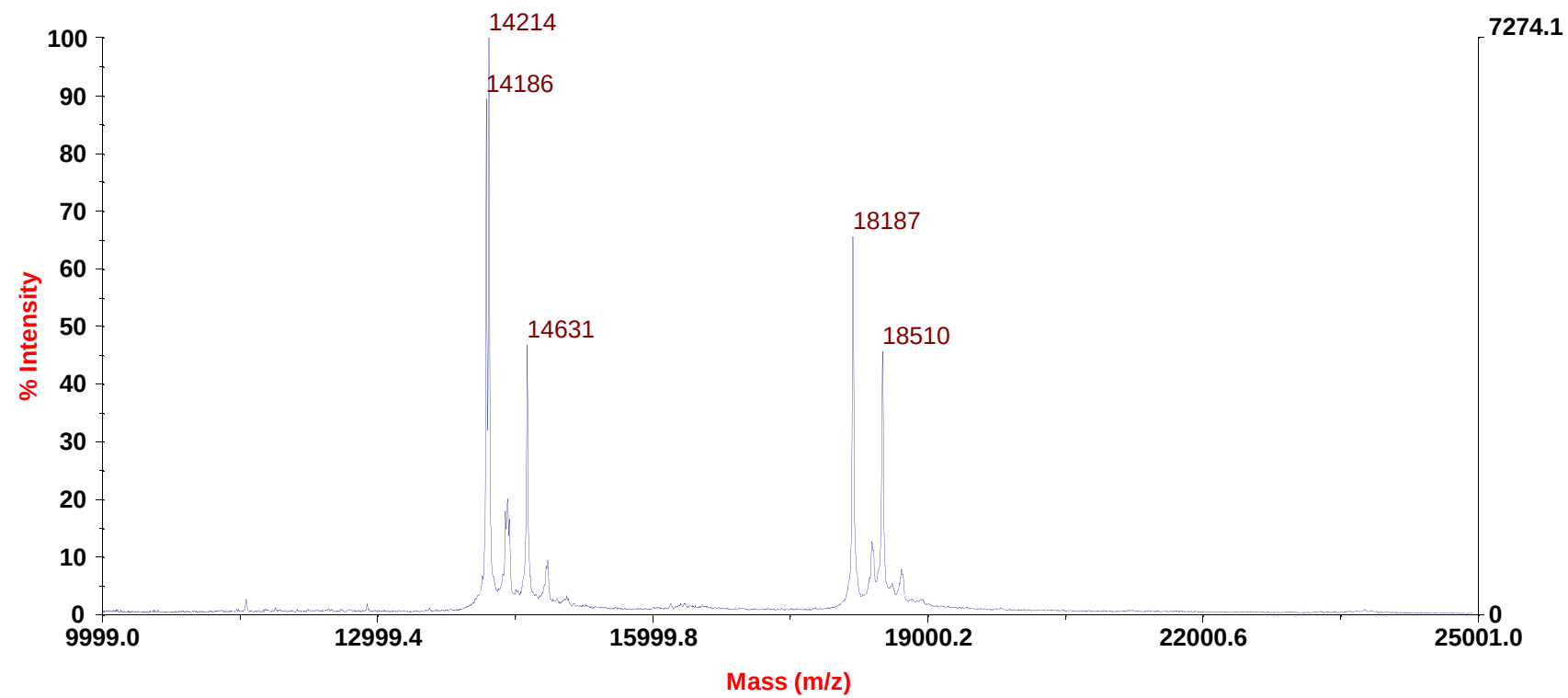
### Supporting information

Supporting information may be found in the online version of this article.

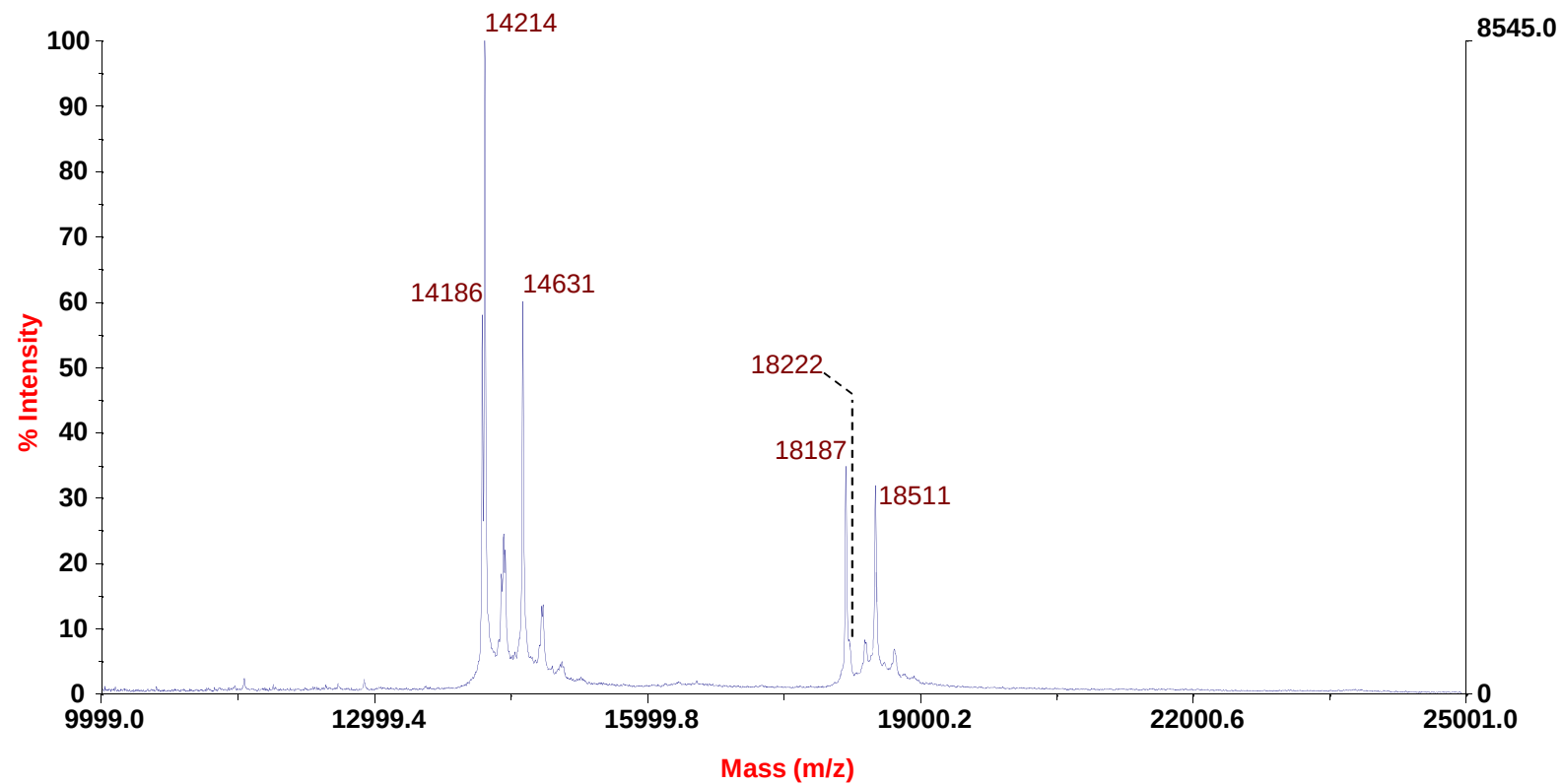
### References

- [1] E. Salimei, F. Fantuz. Equid Milk for human consumption. *Int. Dairy J.* **2012**, *24*, 130.
- [2] F. Addeo, R. Pizzano, M. A. Nicolai, S. Caira, L. Chianese. Fast isoelectric focusing and antipeptide antibodies for detecting bovine casein in adulterated water buffalo milk and derived mozzarella cheese. *J. Agric. Food Chem.* **2009**, *57*, 10063.
- [3] G. Enne, D. Elez, F. Fondrini, I. Bonizzi, M. Feligini, R. Aleandri. High-performance liquid chromatography of governing liquid to detect illegal bovine milk's addition in water buffalo mozzarella: Comparison with results from raw milk and cheese matrix. *J. Chromatography A* **2005**, *1094*, 169.
- [4] European Commission. Commission regulation (EC) No 273/2008 of 5 March 2008 laying down detailed rules for the application of council regulation (EC) no. 1255/1999 as regards methods for the analysis and quality evaluation of milk and milk products. *OJEC*, **2008**, *L88*, 1–115.
- [5] V. Cunsolo, V. Muccilli, R. Saletti, S. Foti. Applications of mass spectrometry techniques in the investigation of milk proteome. *Eur. J. Mass Spectrom.* **2011**, *17*, 305.
- [6] R. Cozzolino, S. Passalacqua, S. Salemi, D. Garozzo. 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. *J. Mass Spectrom.* **2002**, *37*, 985.
- [7] C. Czerwenka, L. Muller, W. Lindner. Detection of the adulteration of water buffalo milk and mozzarella with cow's milk by liquid chromatography–mass spectrometry analysis of  $\beta$ -lactoglobulin variants. *Food Chem.* **2010**, *122*, 901.
- [8] R. K. Chen, L. W. Chang, Y. Y. Chung, M. H. Lee, Y. C. Ling. Quantification of cow milk adulteration in goat milk using high-performance liquid chromatography with electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1167.
- [9] M. Kussmann, E. Nordhoff, H. Rahbek-Nielsen, S. Haebel, M. Rossel-Larsen, L. Jakobsen, J. Gobom, E. Mirgorodskaya, A. Kroll-Kristensen, L. Palm, P. Roepstorff. Matrix-assisted Laser Desorption/Ionization Mass Spectrometry Sample Preparation Techniques Designed for Various Peptide and Protein Analytes. *J. Mass Spectrom.* **1997**, *32*, 593.
- [10] V. Cunsolo, R. Saletti, V. Muccilli, S. Foti. Characterization of the Protein Profile of Donkey's Milk Whey Fraction. *J. Mass Spectrom.* **2007**, *42*, 1162.
- [11] A. Criscione, V. Cunsolo, S. Bordonaro, A. M. Guastella, R. Saletti, A. Zuccaro, G. D'Urso, D. Marletta. Donkey's Milk Protein Fraction Investigated by Electrophoretic Methods and Mass Spectrometric Analysis. *Int. Dairy J.* **2009**, *19*, 190.
- [12] V. Cunsolo, E. Cairone, V. Muccilli, R. Saletti, S. Foti. Sequence and Phosphorylation level determination of two donkey  $\beta$ -Caseins by mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *13*, 1907.
- [13] V. Cunsolo, E. Cairone, D. Fontanini, A. Criscione, V. Muccilli, R. Saletti, S. Foti. Sequence determination of  $\alpha_{s1}$ -casein isoforms from donkey by mass spectrometric methods. *J. Mass Spectrom.* **2009**, *44*, 1742.
- [14] V. Cunsolo, V. Muccilli, E. Fasoli, R. Saletti, P. G. Righetti, S. Foti. Poppea's bath liquor: the secret proteome of she-donkey's milk. *J. Proteomics* **2011**, *74*, 2083.
- [15] R. Saletti, V. Muccilli, V. Cunsolo, D. Fontanini, A. Capocchi, S. Foti. MS-Based Characterization of  $\alpha_{s2}$ -casein isoforms in donkey's milk. *J. Mass Spectrom.* **2012**, *47*, 1150.
- [16] M. G. Giuffrida, A. Cantisani, L. Napolitano, A. Conti, J. Godovac-Zimmermann. The amino acid sequence of two isoforms of alpha-lactalbumin from donkey (*Equus asinus*) milk is identical. *Biol. Chem. Hoppe Seyler* **1992**, *373*, 931.
- [17] J. Godovac-Zimmermann, A. Conti, L. Napolitano. The primary structure of Donkey (*Equus asinus*) lysozyme contains the Ca(II) binding site of alpha-lactalbumin. *Biol. Chem. Hoppe Seyler* **1988**, *369*, 1109.
- [18] M. Herrouin, D. Mollé, J. Fauquant, F. Ballestra, J. L. Maubois, J. Leonil. New genetic variants identified in donkey's milk whey proteins. *J. Protein Chem.* **2000**, *19*, 105.
- [19] J. Godovac-Zimmermann, A. Conti, L. James, L. Napolitano. Micro-analysis of the Amino-Acid Sequence of Monomeric beta-Lactoglobulin I from Donkey (*Equus asinus*) milk. *Biol. Chem. Hoppe Seyler* **1988**, *369*, 171.
- [20] J. Godovac-Zimmermann, A. Conti, M. Sheil, L. Napolitano. Covalent structure of the minor monomeric beta-lactoglobulin II component from donkey milk. *Biol. Chem. Hoppe Seyler* **1990**, *371*, 871.
- [21] V. Cunsolo, A. Costa, R. Saletti, V. Muccilli, S. Foti. Detection and sequence determination of a new variant of Donkey's  $\beta$ -Lactoglobulin II. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1438.
- [22] F. Pessler, M. Nejat. Anaphylactic reaction to goat's milk in a cow's milk-allergic infant. *Pediatric Allergy Immunol.* **2004**, *15*, 183.
- [23] B. Belloni-Businco, R. Paganelli, P. Lucenti, P. G. Giampietro, H. Perborn, L. Businco. Allergenicity of goat's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* **1999**, *103*, 1191.
- [24] F. Hillenkamp, M. Karas. *The MALDI Process and Method, in MALDI MS: A Practical Guide to Instrumentation, Methods and Applications*, F. Hillenkamp, J. Peter-Katalinić (Eds). Wiley-VCH, Weinheim: Germany, **2007**.
- [25] A. J. Nicola, A. I. Gusev, A. Proctor, E. K. Jackson, D.M. Hercules. Application of the fast-evaporation sample preparation method for improving quantification of angiotensin II by matrix-assisted laser desorption/ionization. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1164.

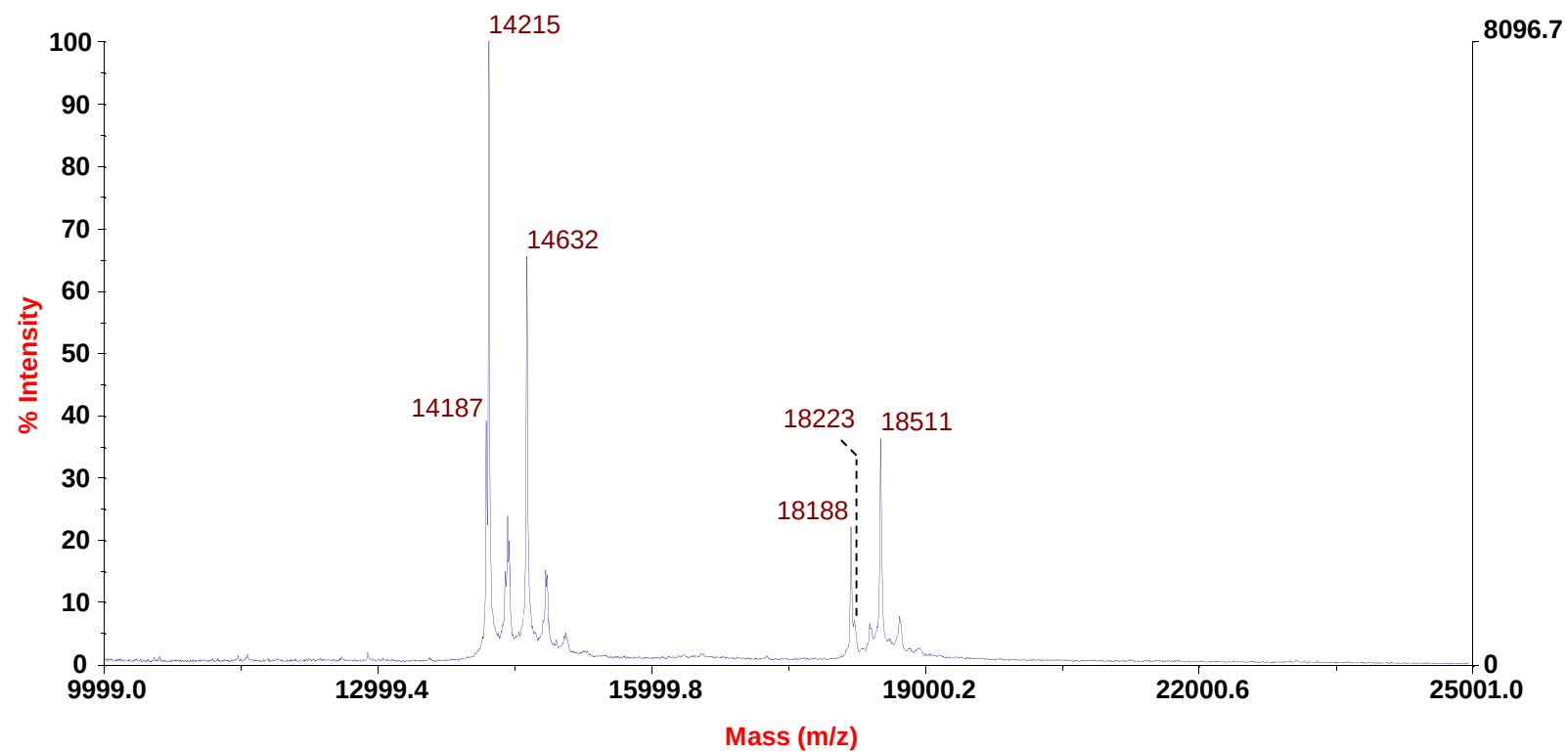
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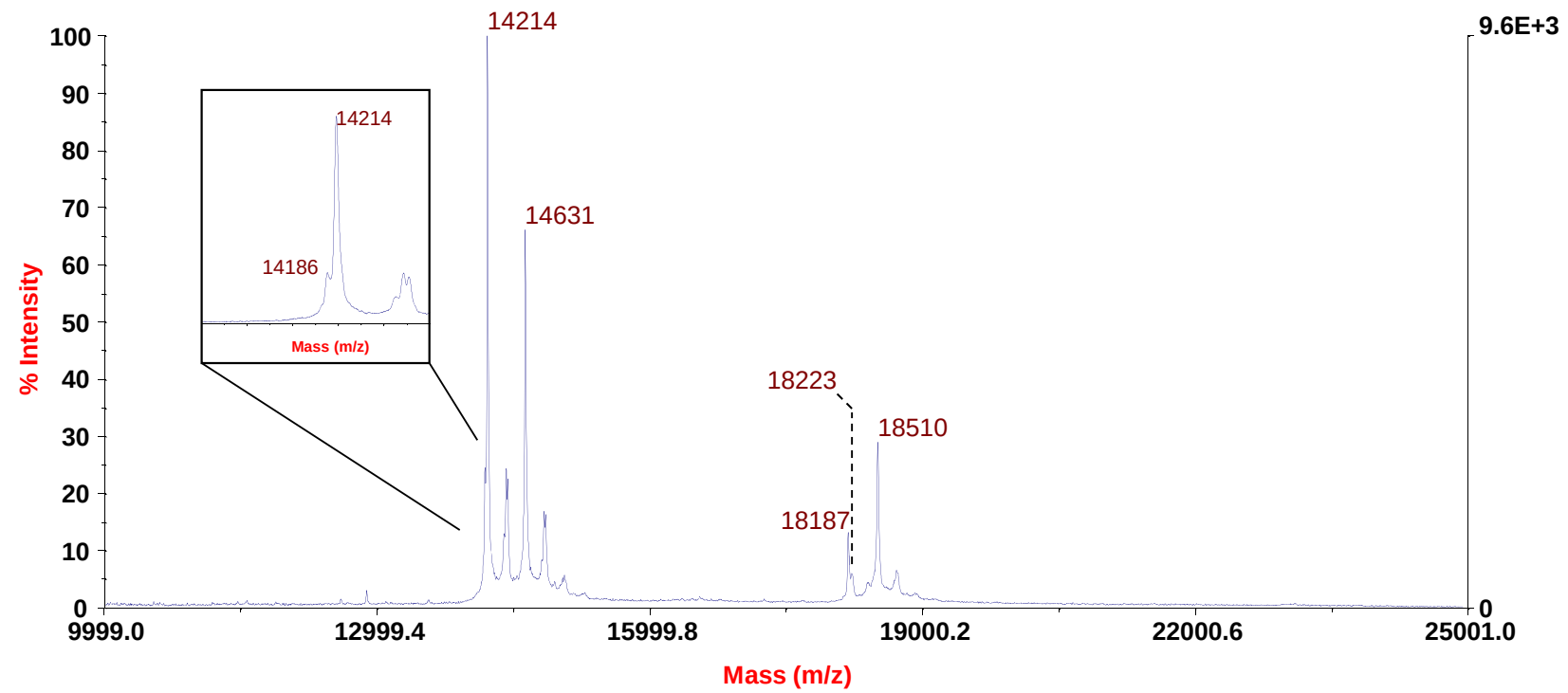
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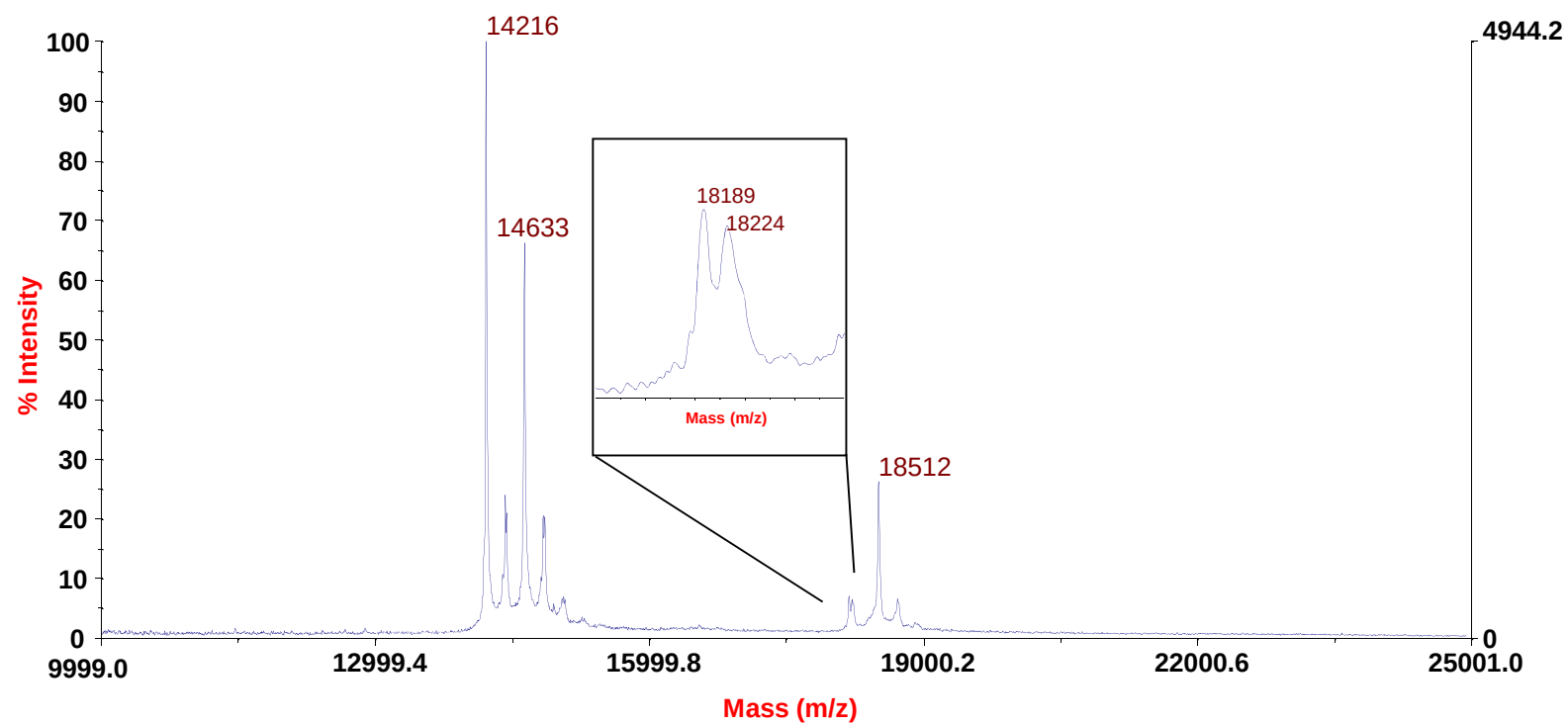
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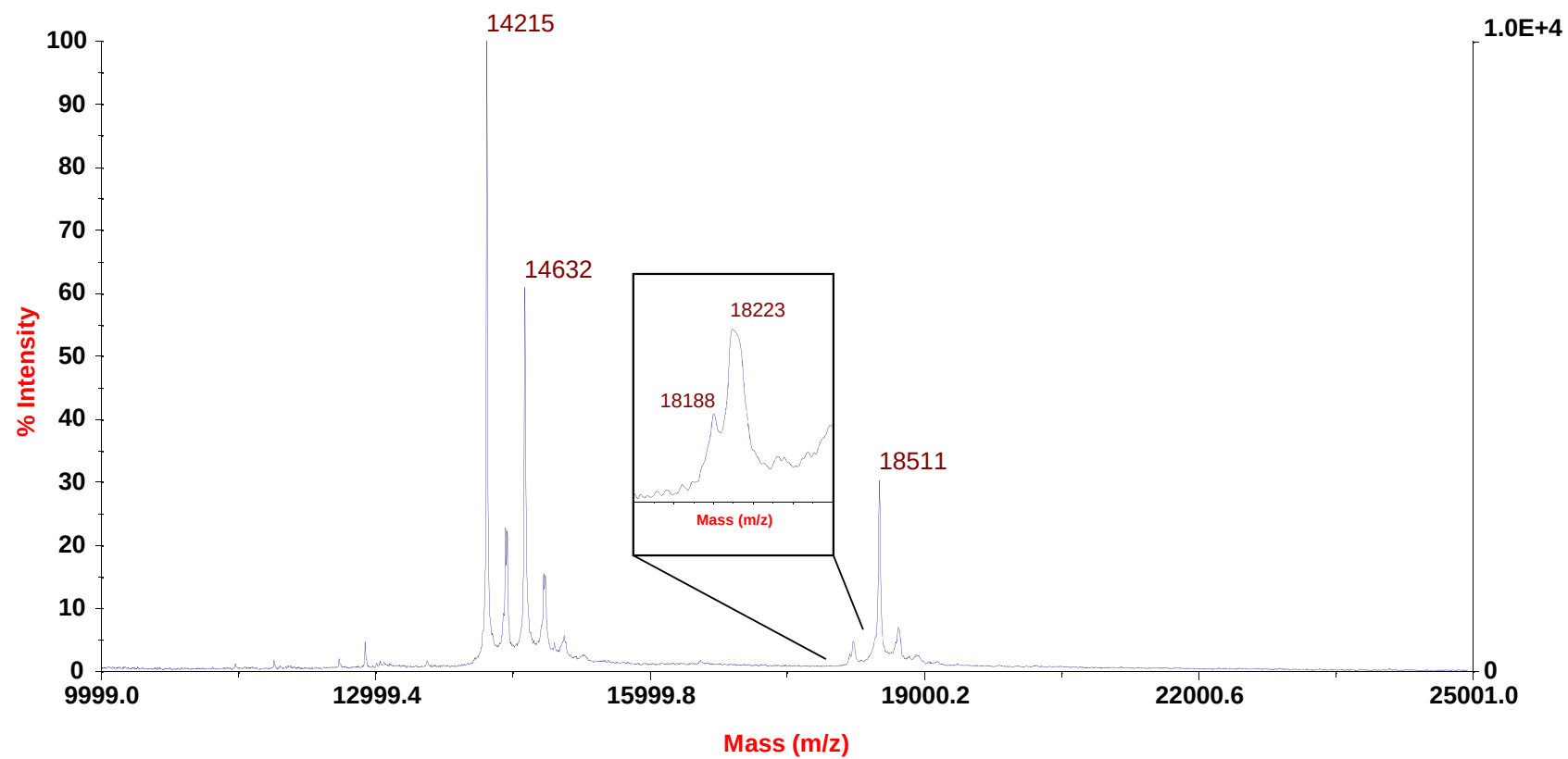
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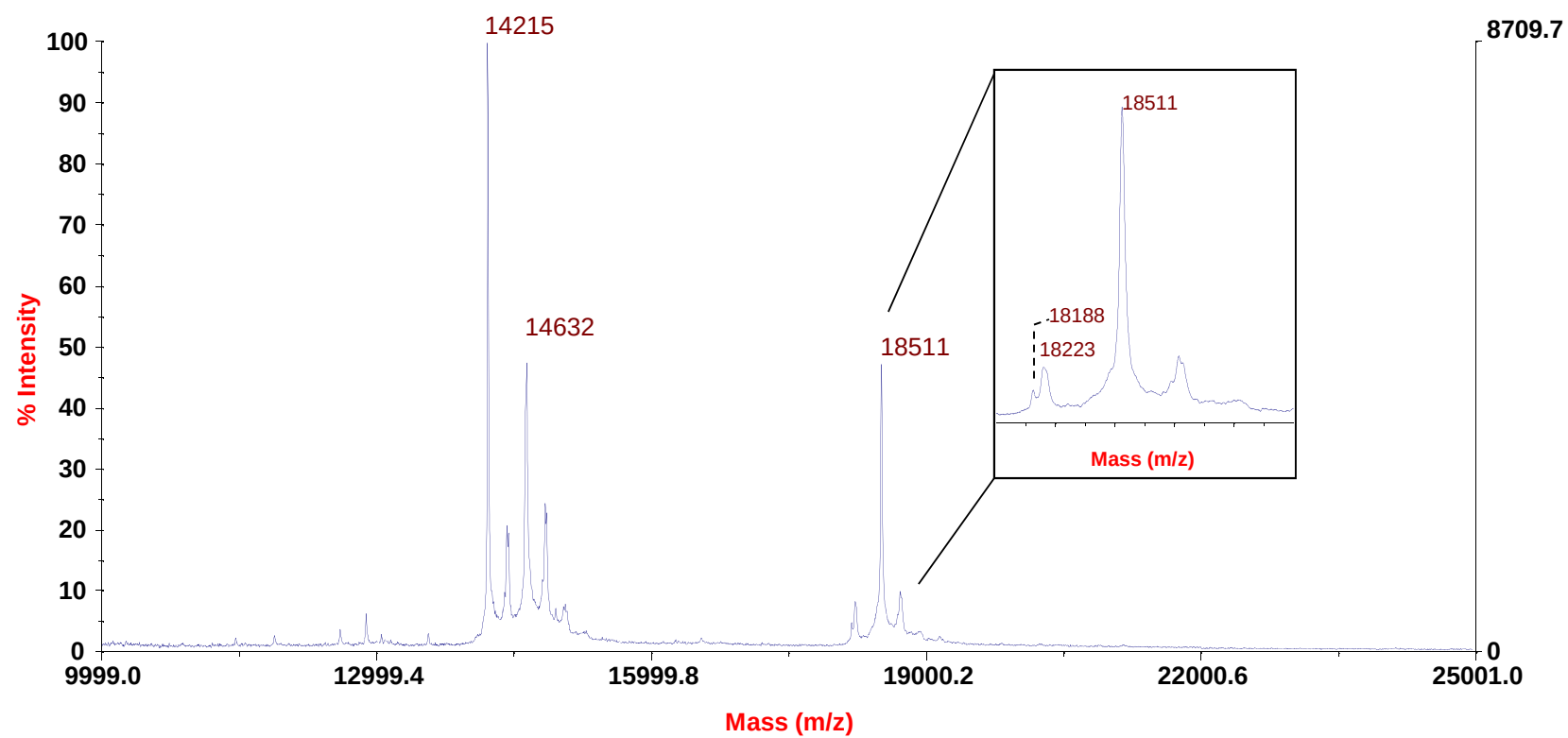
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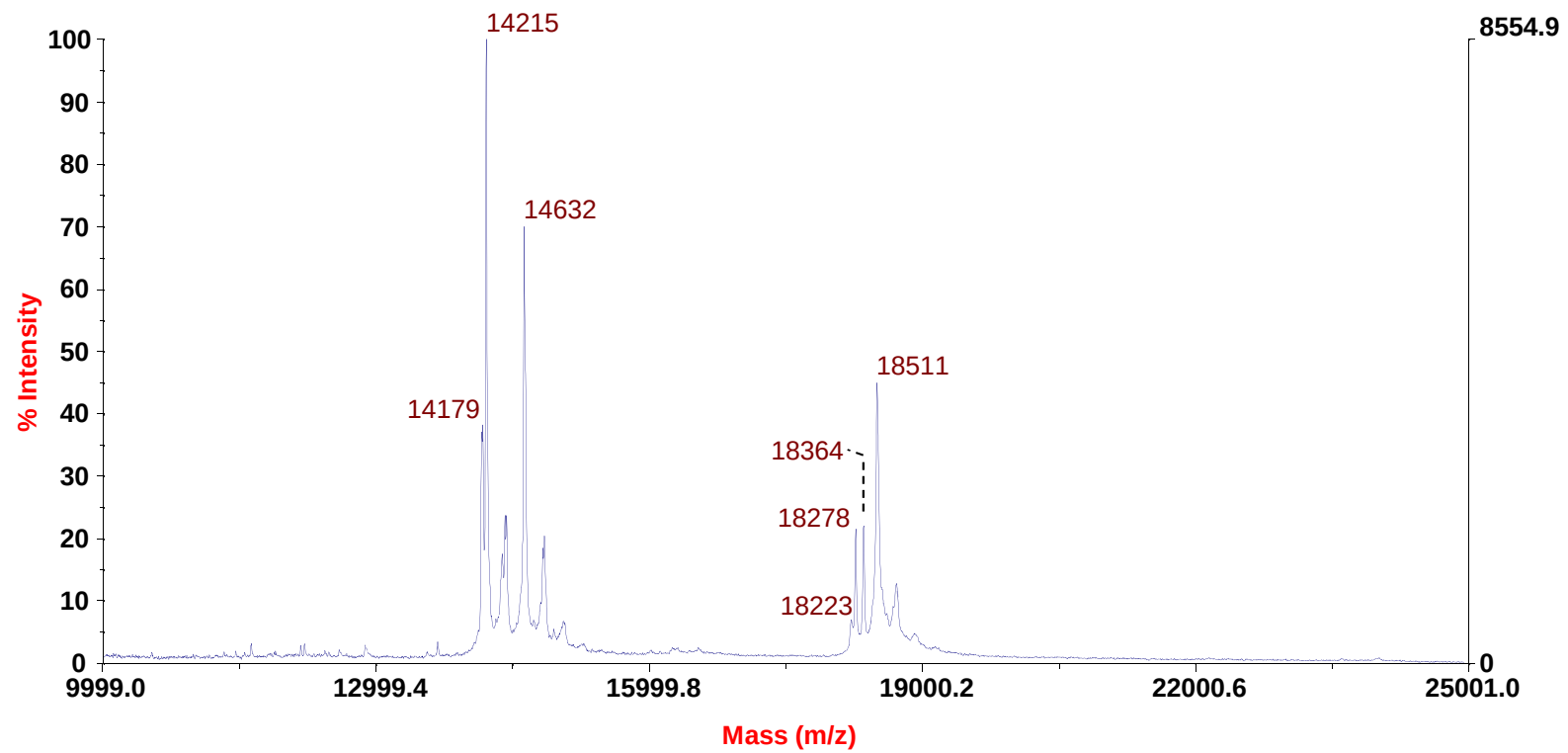
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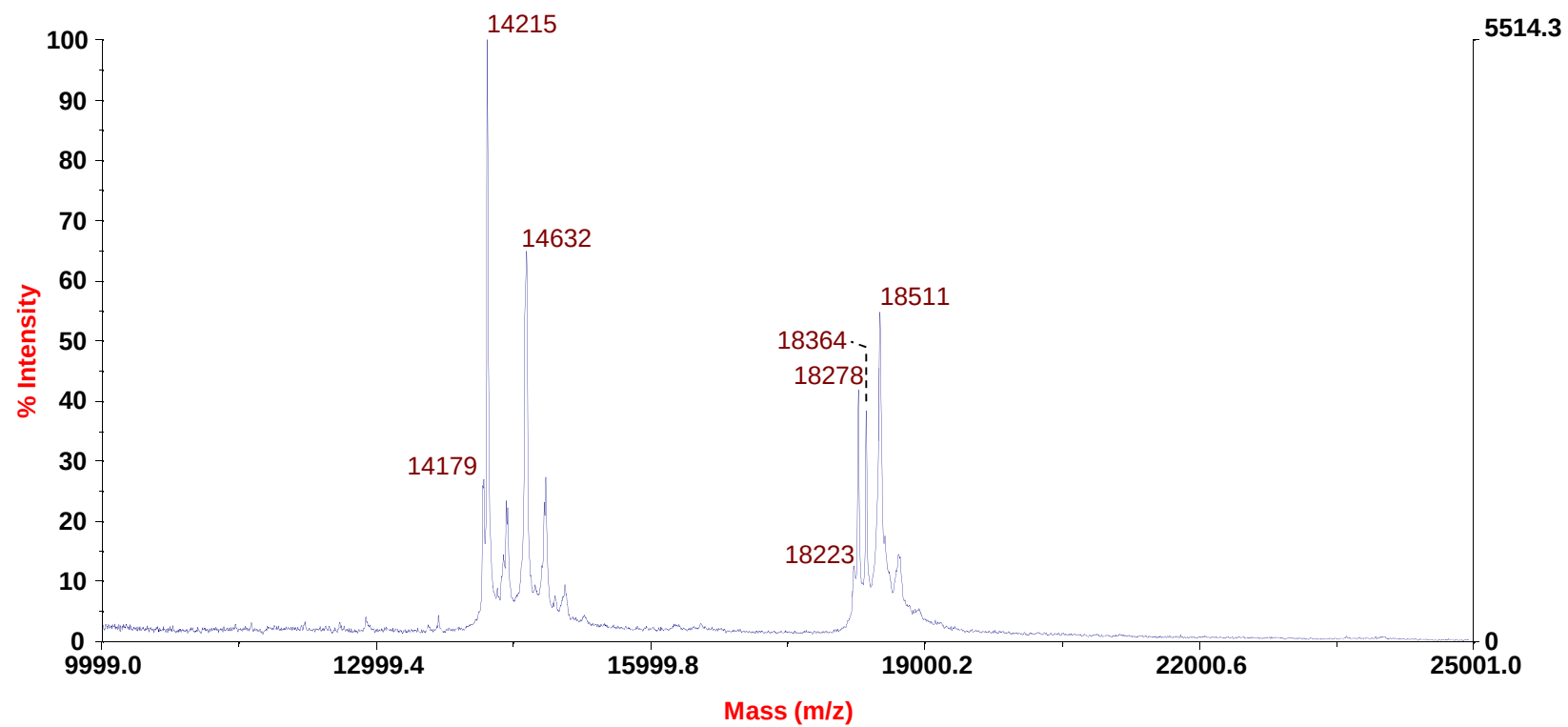
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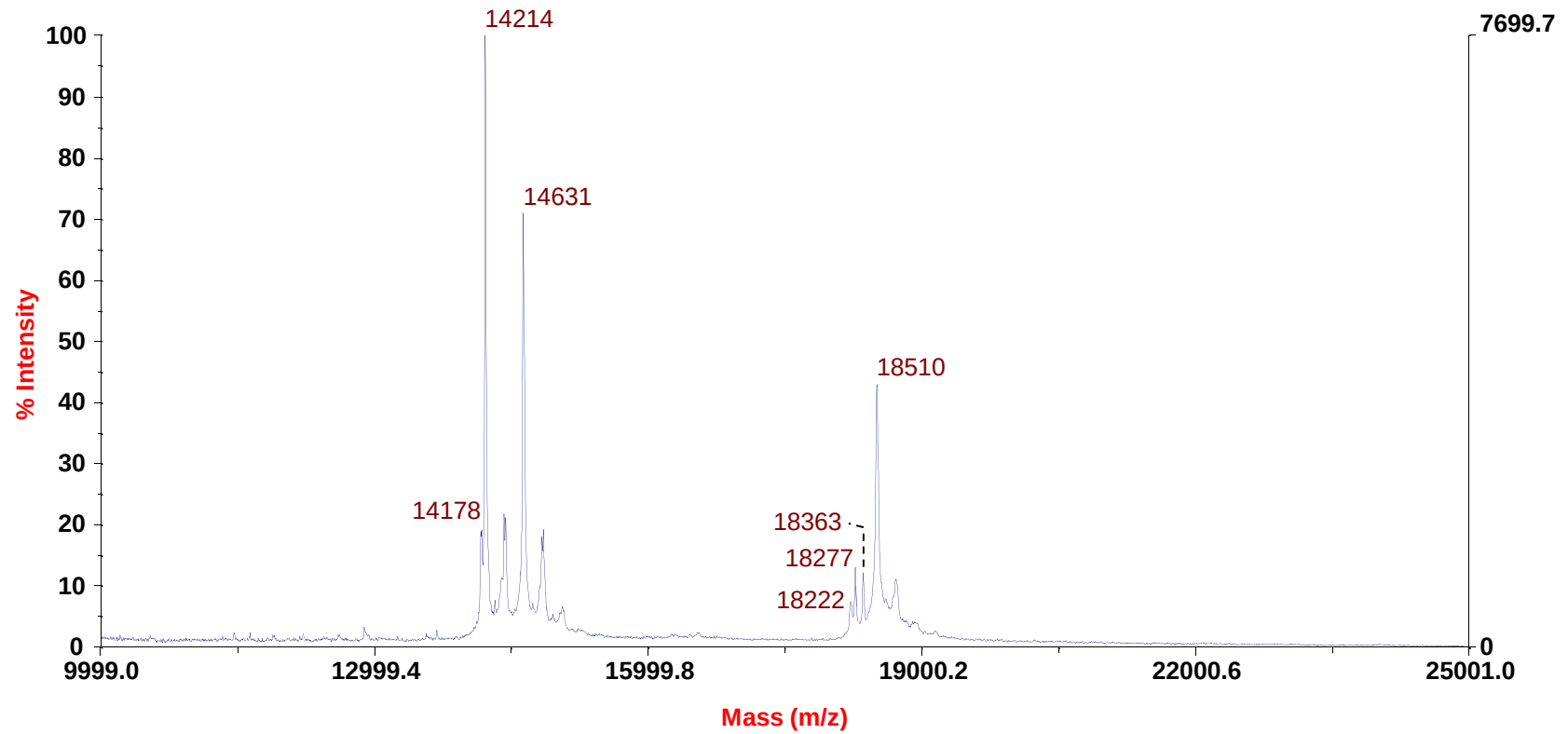
# Mixture donkey's milk / cow's milk 60/40 (v/v)



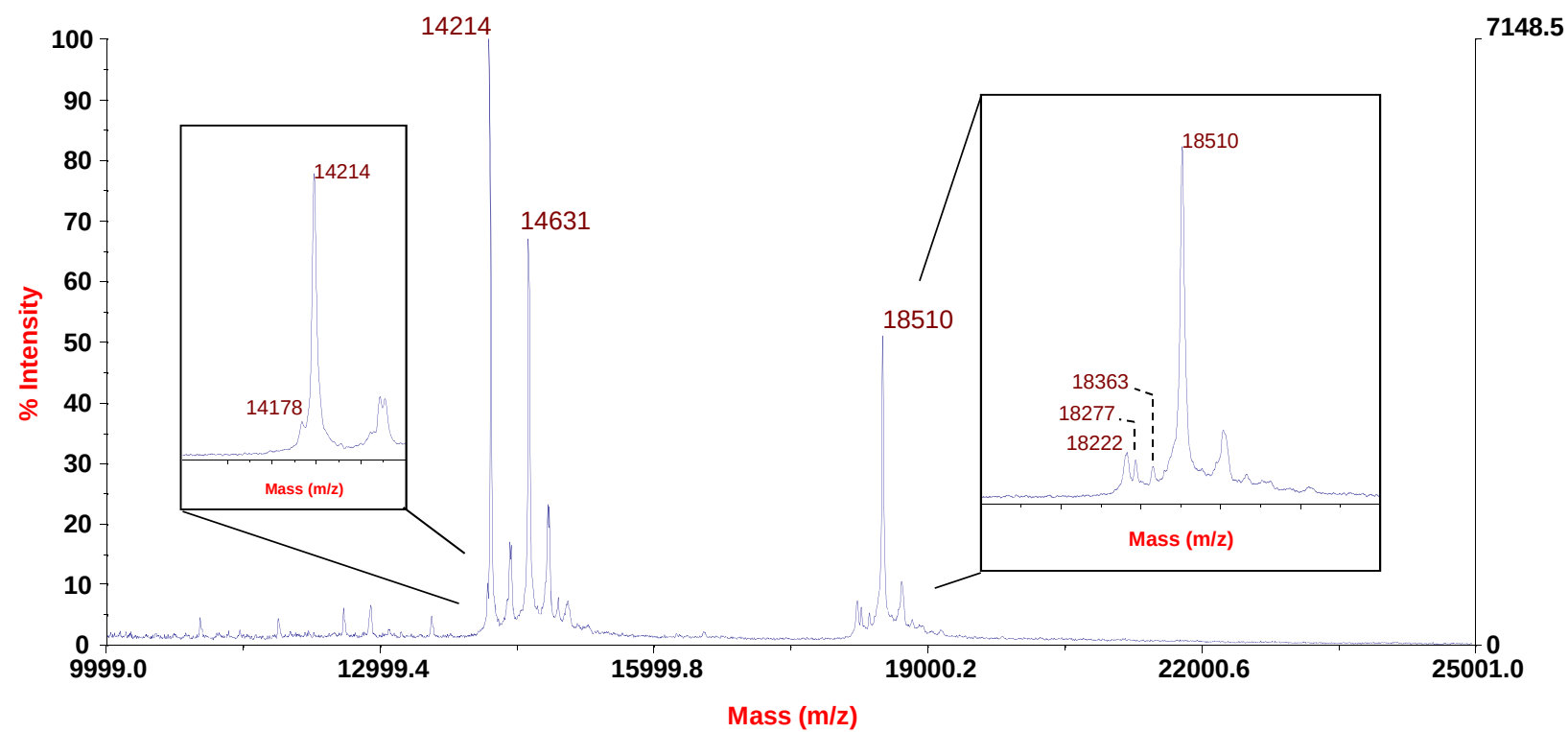
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# Mixture donkey's milk / cow's milk 80/20 (v/v)



## Mixture donkey's milk / cow's milk 90/10 (v/v)



## Mixture donkey's milk / cow's milk 95/5 (v/v)

