

MALDI-TOF mass spectrometric determination of intact phospholipids as markers of illegal bovine milk adulteration of high-quality milk

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Abstract In the dairy industry one of the most common frauds is mixing high-value milk (sheep's and goats') with milk of lower value (cows'). This illegal practice has commercial, ethical, and serious sanitary consequences because consumers can be exposed to hidden allergens contained in the undeclared cows' milk. Here, we investigated the possibility of using matrix-assisted laser-desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) as a rapid, sensitive, and accurate technique for detection of milk adulteration by analysis of phospholipid profiles. Lipid extracts of pure raw milk, commercial milk, and binary mixtures of cows' and goats' milk and cows' and sheep's milk (the concentrations of each milk varied from 0 % to 50 %) were analyzed with α -cyano-4-chlorocinnamic acid as matrix. The abundance ratio of the ions at m/z 703 and m/z 706 was found to be species-correlated and was used as marker of cows' milk in sheep's and goats' milk. Furthermore, the procedure could potentially be applied to cheese samples, because peaks at m/z 703 and 706 were also found in several commercial cheese samples. This approach proved to be an efficient, rapid, and inexpensive method of detecting milk fraud.

Keywords Milk adulteration · Goat · Cow · Sheep · MALDI · Lipid

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Introduction

The dairy industry is subjected to a growing number of frauds, because of the high nutritional value of milk and cheeses, that have an effect on the prices of these products. In Italy, the most common illegal practices in the dairy industry are selling skimmed milk and semi-skimmed milk instead of whole milk (http://old.politicheagricole.it/SicurezzaAlimentare/ConoscereGliAlimenti/040_Latte.htm), addition of anhydrous milk products (caseins and caseinate, milk protein concentrate, whey proteins) to liquid milk (<http://www.pnragrobio.unina.it/SIFORTI.html>), milk dilution [1], and the mixing of high-quality milk, for example buffalo's, sheep's, and goats' milk with cows' milk [2].

Sometimes, because of the fluctuation in milk availability and price, it could be very convenient to adulterate goats' milk with sheep's milk [3]; usually, however, cows' is the most common adulterant because of its high abundance and low price. Adulteration could have severe sanitary consequences because consumers are exposed to hidden allergens, for example cows' milk proteins, especially α -s-2-casein and β -lactoglobulin [4]. Goats' milk is often regarded as an alternative to cows' milk, especially for children, because of its high digestibility and its minor allergenic properties [5, 6], although cases of allergic reactions in children after consumption of sheep's or goats' milk have also been reported [7, 8].

The milk lipid components of dairy products could reflect the difference among species and thus be very informative [9–11]. Milk fat is a globule (size ranging from 0.1 to 15 nm in diameter) mainly composed of triglycerides (TAGs) and minor percentages of diglycerides, monoglycerides, phospholipids (phosphatidylcholines, phosphatidylethanolamine, and sphingomyelins), glycolipids, sterols, and free fatty acids. Cows', goats', and sheep's milk contains almost the same TAGs [12, 13] even if some, e.g. C42, C50, C52, and C54

[12, 14–16], are present at different concentrations. Recent studies [9, 17, 18] have found that sheep's milk has twice the lipid content of cows' and goats' milk, and that sheep's and goats' milk are rich in essential fatty acids (linoleic and arachidonic) and contain more medium and short-chain saturated fatty acids than cows' milk. Goats' milk also contains large amounts of caproic, caprylic, and capric acids [19]. Phosphatidylcholines, phosphatidylethanolamines, and sphingomyelins are the principal polar lipids in goats', cows', and sheep's milk [20], followed by phosphatidylinositol and phosphatidylserine. It has also been reported [21] that the main difference between the phospholipid content of goats' and bovine milk is related to sphingolipids.

Many analytical methods have been developed and improved in recent years to assess milk authenticity, trying to increase the sensitivity, rapidity, and simplicity of the analyses, to reduce sample-preparation time, and to overcome some limitations of the official methods. Research methods are based on the DNA study [22], sensory analyses [23], protein analysis by liquid chromatography [24, 25] and mass spectrometry (MS) [3, 26], immuno-enzymatic assays [27], and electrophoresis [28, 29]. The European Community official method [30] for detection of adulteration of cows' milk fat by foreign fats (for example vegetable fats) entails determination of TAGs by gas chromatography (GC). However, the use of lipid profiles for determination of milk authenticity is restricted to few studies; most are based on analysis of TAGs by GC [13, 31], reversed-phase HPLC–MS [24, 32], and high-resolution gas chromatography (HRGC) [33]. For instance, Iverson et al. [34] investigated the distribution of fatty acids in cows', goats', and sheep's cheeses by gas–liquid chromatography. For the first time, a marker ratio based on lauric acid (C12) and capric acid (C10) intensities was proposed for determination of cows' milk in cheeses labelled as goats' or sheep's. More recently, Antonilli and coworkers [33] analyzed the methyl esters of the fatty acids of cows' milk, goats' milk, and their mixtures, and showed that the main quantitative difference between the two types of milk is related to the concentration of capric acid (C10). They also proposed other marker ratios (C14/C10, C10/13MeC14, C10/C15, C10/14MeC15, C10/C16:1, C16/C10, and C10/15MeC16) that could be useful for discriminating cows' from goats' milk down to 5 % adulteration.

More recently, MALDI-TOF MS has been increasingly applied [35–39] to the determination of TAGs, because of advantages compared with other analytical methods, e.g. rapid and easy sample preparation and no need for analyte derivatization. A very recent paper [40] shows that MALDI can be a useful technique for detection of vegetable oils or fats in bovine milk. Other recent papers [41, 42] report direct MALDI analysis of milk tryptic digests for detection of milk adulteration by evaluation of species-specific markers in the peptide profiles. As far as we know, no data

have been reported for MALDI analysis of lipids as markers of adulteration of milk with milk from different animal species.

Here, we report a rapid and sensitive method for extraction and MALDI-TOF MS determination of phospholipids in milk, to detect lipid markers of cows' milk in sheep's and goats' milk. Pure raw and commercial milk samples, and binary mixtures containing cows' and goats' milk and cows' and sheep's milk were analyzed. Samples were subjected to Bligh–Dyer extraction and the organic fraction was analyzed by MS using the new matrix α -cyano-4-chlorocinnamic acid (CCICA) [43]. The method was also applied to analysis of commercial cows', goats', and sheep's cheese samples to demonstrate its potential for detection of the same markers in a processed food.

Experimental section: Materials and methods

Materials

Lipids (trilaurin, trimyristin, tripalmitin, and tristearin) were obtained from Supelco (Bellefonte, PA, USA). All solvents used (Sigma–Aldrich, St Louis, MO, USA) were LC–MS grade; 2,5-dihydroxybenzoic acid (DHB) was from Sigma–Aldrich, α -cyano-4-chlorocinnamic acid (CCICA) was synthesized by the procedure reported below and re-crystallized four times before use. Commercial milk, yoghurt, and cheese samples were purchased from local supermarkets. Raw cows', goats', and sheep's milk samples were obtained from local farmers.

Synthesis of CCICA

CCICA was synthesized by standard Knoevenagel condensation of cyanoacetic acid and *p*-chlorobenzaldehyde [44]. Ammonium acetate was used as a catalyst. Cyanoacetic acid (2 g, 1 equiv), 0.9 equiv *p*-chlorobenzaldehyde, and 0.15 equiv ammonium acetate were heated under reflux with stirring in a sufficient amount of toluene (ca 50 mL). After quantitative separation of the reaction water by use of a Dean–Stark apparatus (ca 3 h), the reaction mixture was cooled to 50 °C and filtered. The crude product was washed with sufficient amounts of distilled water and purified by repeated recrystallization from 70:30 (%v/v) acetonitrile–water. A saturated solution of the solid in 70:30 (%v/v) acetonitrile–water was heated until boiling. When the solid dissolved completely, the solution was cooled to room temperature, then on ice, causing precipitation. The precipitate was isolated by filtration and the procedure was repeated in triplicate.

Samples

Pure commercial and raw cows' milk, goats' milk, and raw sheep's milk (purchased from local supermarkets) and binary

mixtures of these were analyzed. In particular, commercial cows' milk samples ($n=24$) of six different brands and various types (UHT, pasteurized, microfiltered, and high digestibility) were tested. For commercial goats' milks, samples from three different brands were processed ($n=12$). Use of commercial milk samples of different brands (Italian, French, and German) takes into account the effect of geography and season. All raw samples ($n=18$, six for each species) were collected from three different farms during different stages of lactation. The procedure was repeated at least in triplicate for all pure samples and binary mixtures. Moreover, samples intentionally adulterated with 5 %, 20 %, and 50 % of cows' milk in sheep's and goats' milk were prepared. The analyses were also performed on cow and goat yoghurt samples (at least in triplicate) and on cows' cheese slices. The method was eventually used for analysis of phospholipids from the cheese samples Grana Padano DOP, Pecorino Romano DOP, and goats' ricotta cheese purchased from different markets and of different age of seasoning.

Sample preparation

Total lipids were extracted from milk samples by the Bligh–Dyer method [45]. Briefly, 1.87 mL CHCl_3 –MeOH 1:2 were added to 500 μL milk. CHCl_3 (0.62 mL) and water (0.62 mL) were then added and the resulting solution vortexed mixed and centrifuged. The lower, organic, phase obtained was transferred to a clean vial and concentrated under a nitrogen stream.

Yoghurt, cheese slices, and cheese samples (2 g) were whisked with 6 mL water until a homogenous mixture was obtained and kept at 50 °C for 30 min with magnetic stirring. The supernatant was transferred to a clean vial and subjected to the lipid-extraction procedure described above. All extracts were mixed 1:1 (v/v) with the CCICA matrix (5 mg mL^{-1} MeOH with 0.1 % TFA) and analyzed by MALDI-TOF-MS.

Mass spectrometry

MALDI-TOF MS analysis was performed with a Micromass M@LDI-LR time-of-flight mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with a nitrogen UV laser (337 nm wavelength), a precision flat target plate sample-introduction system bearing a micro-titre target plate, reflectron optics, and a fast dual micro-channel plate (MCP) detector.

Positive ion spectra were acquired in reflectron mode. The potentials applied were: pulse, 2,610 V; source, 15,000 V; reflectron, 2,000 V; and MCP 1,900 V. The laser firing rate was 5 Hz, and, unless otherwise specified, 60 laser shots were used for each well. The 60 resulting spectra were averaged, the background was subtracted, and the spectra were smoothed by use of a Savitzky–Golay algorithm.

For lipid analysis, the m/z range investigated was 200–2000 and external mass calibration was performed by using

a lipid mixture composed of trilaurin, trimyristin, tripalmitin, and tristearin.

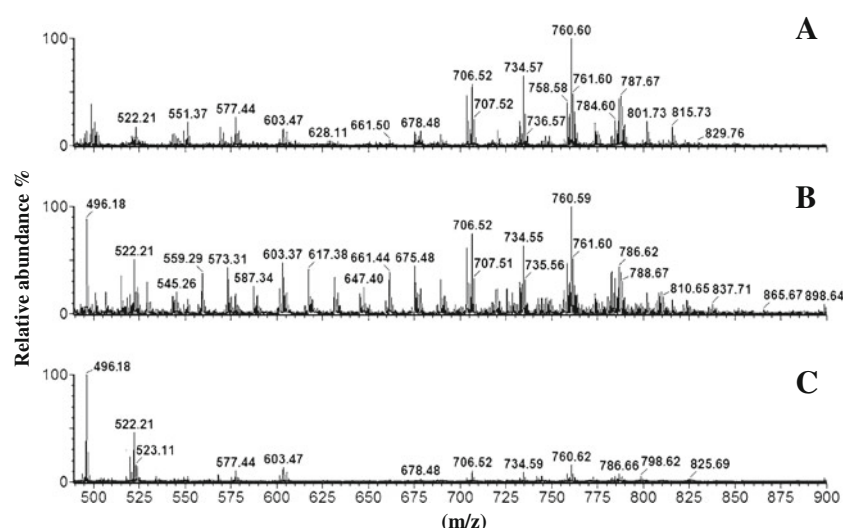
Statistical analysis

Statistical analysis of the data was performed with Microsoft Excel Software (Microsoft Office 2003). Intra and inter-assay precision was assessed by the measurement of the relative standard deviation (RSD) of three replicates per sample performed by the same operator. For biological variability, comparisons were performed by use of the two-sample t -test, calculating the means, sample sizes, variances, and degrees of freedom of different batches of raw and commercial milk samples of bovine origin. Moreover, the two-sample t -test was also used to discriminate among pure cows', goats', and sheep's milk and adulterated milk samples.

Results and discussion

It has recently been reported [46], in a paper dealing with the analysis of phospholipids in hazelnut oil, that the ionization of phospholipids at the expense of triacylglycerols can be substantially affected by the particular MALDI matrix used. Because our objective was to investigate milk phospholipid profiles searching for characteristic differences between species, preliminary experiments were devoted to selection of an appropriate MALDI matrix. Figure 1 shows MALDI spectra obtained from analysis of raw cows' milk using CCICA (a), DHB (b), and CHCA (c) as matrix. When DHB was used inhomogeneous spots and complicated spectra (both phospholipids and triacylglycerols were present) were observed, and when CHCA was used hydrolysis of intact PLs was favoured (high-intensity ions from lysophosphatidylcholines). Because our objective was to detect differences between PL profiles, the matrix CCICA was the most useful for obtaining reliable ionization efficiency and ion abundance information and was therefore used throughout the work. Figure 2 shows MALDI spectra obtained from analysis of raw cows' (a), goats' (b), and sheep's milk (c) lipid extracts. A complete list of the compounds observed in Fig. 2, with their most probable attributions, is given in Table 1. As is apparent, the use of the CCICA matrix enabled acquisition of mass spectra of, mainly, phospholipids (PLs). Lipid assignments were aided by use of the LIPID MAPS database (<http://www.lipidmaps.org/>) and literature data [19–21, 47]. The most abundant peaks present in all the samples were those of phosphatidylcholine (PC) adducts. In particular, the ions at m/z 760.6 and 782.6 can be attributed to protonated and sodiated molecules of PC (34:1), respectively, and the ions at m/z 758.6 and 780.6 to protonated and sodiated molecules of PC (34:2), respectively. Less

Fig. 1 MALDI mass spectra obtained from lipid extracts of pure cows' milk analyzed by using CICCA (A), DHB (B) and CHCA (C) as matrix



abundant signals at the m/z values 786.6, 808.6, 788.6, and 810.7 were assigned to the H^+ and Na^+ adducts of PC (36:2) and PC (36:1), respectively. The spectra also contain signals attributable to sodiated adducts of phosphatidylethanolamines (PEs), i.e. PE (34:4) (m/z 734.6), PE (36:2) (m/z 766.5), PE (36:1) (m/z 768.6), protonated and sodiated adducts of sphingomyelin (SMs), i.e. [SM (16:0)+ H] $^+$ (m/z 703.6), [SM (16:0)+ Na] $^+$ (m/z 725.6), [SM (22:0)+ H] $^+$ (m/z 787.6), and sodiated adducts of phosphatidylinositols (PIs), i.e. [PI (28:2)+ Na] $^+$ (m/z 773.7), [PI (30:2)+ Na] $^+$ (m/z 801.8), [PI (31:2)+ Na] $^+$ (m/z 815.8). Moreover, in the low-mass region, peaks attributable to the fragmentation of the most abundant PLs or TAGs, corresponding to losses of $RCOO^-Na^+$ or $RCOO^-K^+$ molecules, are clearly distinguishable at m/z values 577.4, 601.5, and 603.5. At first

sight, raw milk samples from cows, goats, and sheep produced similar lipid profiles. However, a careful inspection of Fig. 2 (spectra processed using the overlapping function of mMass software) suggested that some peaks present at different intensities in the three spectra could be potentially useful for detection of cows' milk in goats' or sheep's milk. In fact, a substantial difference was observed in the relative intensities of the signals at m/z values 706.6 ([PC (30:0)+ H] $^+$) and 703.6 ([SM(16:0)+ H] $^+$); in the spectra obtained from pure goats' and sheep's milk samples the relative intensity of the m/z 706.6 ion is lower than the relative abundance of the m/z 703.6 ion whereas for the pure cows' milk sample the opposite behaviour was observed. This evidence was confirmed for all the milk samples analyzed (Experimental section). The precision of the proposed

Fig. 2 MALDI mass spectra obtained from lipid extracts of pure cows' (a), goats' (b), and sheep's (c) milk

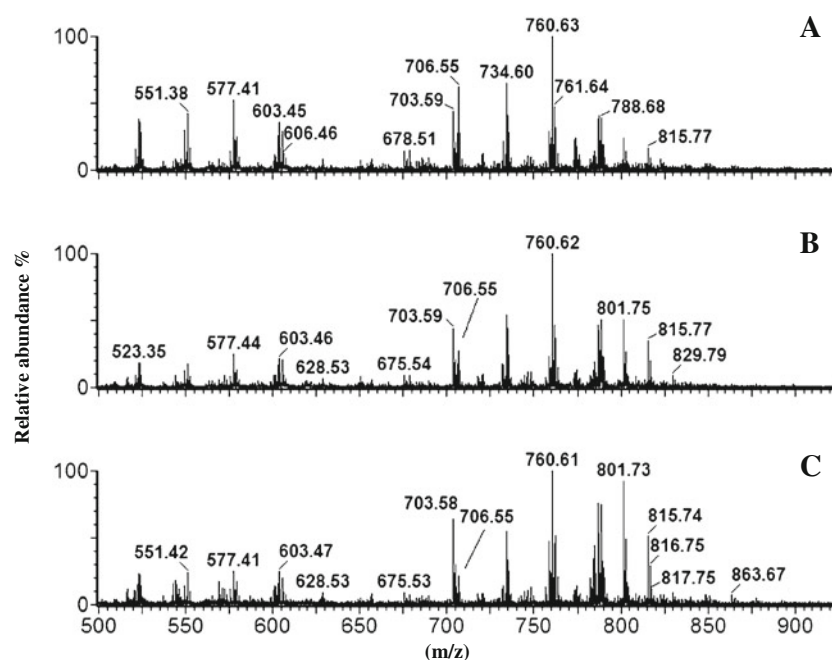


Table 1 Probable attribution of the signals observable in the spectra of Fig. 2

<i>m/z</i>	Probable attribution	Cow	Sheep	Goat
520.16	[LPC (18:2)+H] ⁺		x	
522.18	[LPC (18:1)+H] ⁺		x	x
524.21	[LPC (18:0)+H] ⁺		x	
549.37	[MLn] ⁺	x	x	x
551.38	[MLn] ⁺	x	x	x
575.41	[PL] ⁺	x	x	x
577.41	[PO] ⁺	x	x	x
579.43	[PS] ⁺	x	x	x
601.54	[OL] ⁺	x	x	x
603.46	[OO] ⁺	x	x	x
605.46	[OS] ⁺	x	x	x
675.53	[SM(14:0)+H] ⁺	x	x	x
678.51	[PC(28:0)+H] ⁺	x	x	x
703.57	[SM(16:0)+H] ⁺	x	x	x
706.55	[PC(30:0)+H] ⁺	x	x	x
720.56	[PE(33:6)+Na] ⁺	x	x	x
725.55	[SM(16:0)+Na] ⁺	x	x	x
731.58	[SM(18:0)+H] ⁺	x	x	x
732.58	[PE(34:5)+Na] ⁺	x	x	x
734.57	[PE(34:4)+Na] ⁺	x	x	x
744.55	[PE(36:2)+H] ⁺	x	x	x
746.56	[PE(36:1)+H] ⁺	x	x	x
748.57	[PE(36:0)+H] ⁺	x	x	x
758.60	[PC(34:2)+H] ⁺	x	x	x
759.60	[SM(20:0)+H] ⁺	x	x	x
760.63	[PC(34:1)+H] ⁺	x	x	x
766.54	[PE (36:2)+Na] ⁺	x	x	x
768.56	[PE (36:1)+Na] ⁺	x	x	x
772.60	[PC(34:6)+Na] ⁺	x	x	x
773.69	[PI(28:2)+Na] ⁺	x	x	x
774.67	[PC(34:5)+Na] ⁺	x	x	x
780.58	[PC (34:2)+Na] ⁺	x	x	x
782.63	[PC(34:1)+Na] ⁺	x	x	x
783.61	[SM(22:2)+H] ⁺	x	x	x
784.60	[PC(36:3)+H] ⁺	x	x	x
785.63	[SM(22:1)+H] ⁺	x	x	x
786.64	[PC(36:2)+H] ⁺	x	x	x
787.63	[SM(22:0)+H] ⁺	x	x	x
788.65	[PC(36:1)+H] ⁺	x	x	x
801.75	[PI(30:2)+Na] ⁺	x	x	x
808.62	[PC (36:2)+Na] ⁺	x	x	x
809.63	[SM(22:0)+Na] ⁺	x	x	x
810.67	[PC (36:1)+Na] ⁺	x	x	x
815.77	[PI(31:2)+Na] ⁺	x	x	x
829.78	[PI(32:2)+Na] ⁺	x	x	x

PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; O, oleic acid; P, palmitic acid; M, myristic acid; L, linoleic acid; Ln, linolenic acid; S, stearic acid

method was tested by determination of the intra-assay (measured on the same day) and inter-assay (different days) coefficients of variation (CV, %) for each kind of sample (Table 2); for all the compounds analyzed, values were less than the acceptable value of 10 %. This good precision in terms of repeatability of intensity ratio responses indicates that the method could be suitable for milk species discrimination.

To confirm this hypothesis, binary mixtures containing cows' and goats' milk and cows' and sheep's milk were prepared, subjected to the extraction procedure described in the Experimental section and analysed by MALDI-TOF MS. The MALDI spectra obtained from the lipid extracts from raw goats' milk, raw goats' milk adulterated at 5 %, 20 %, and 50 % with raw cows' milk, and raw cows' milk are reported in Fig. 3a. The *m/z* range shown is limited to 700–710, in which the diagnostic ions have been labelled. In the spectra obtained from goats' milk adulterated at 5 %, 20 %, and 50 % with cows' milk, the ratio between the relative intensities of the ions at *m/z* 706.6 and 703.6 gradually increases passing from 5 % to 50 % cows' milk. As can be seen in Fig. 3b, which reports MALDI spectra obtained from lipid extracts of commercial goats' milk, commercial goats' milk adulterated at 5 %, 20 %, and 50 % with commercial cows' milk, and commercial cows' milk, respectively, the same results were obtained, indicating that the phospholipid markers are still present and informative even after milk processing. This experimental evidence was not surprising, because, as mentioned in the Introduction, it has recently been reported that the main difference between the phospholipid content of goats' and bovine milk is related to sphingolipids and phosphatidylethanolamine [19–21]. In fact, as reported by Rodríguez-Alcalá et al. [21], the PE content of cows' milk is higher than that of goats' milk whereas the sphingomyelin content is higher in goats' milk. Thus, the ratio of the relative intensities of the ions at *m/z* 706.6 and 703.6 can be reasonably used to detect milk adulteration, because it magnifies the main differences between the milk from the two species. The phospholipid content of sheep's milk is similar to that of goats' milk, thus confirming the previous findings.

MALDI spectra obtained from the lipid extracts of raw sheep's milk, raw sheep's milk adulterated at 5 %, 20 %, and 50 % with raw cows' milk, and raw cows' milk are reported in Fig. 4. Again, the ratio between the two diagnostic peaks increases with the progressive addition of the adulterant milk.

Experiments to assess the statistical significance of the approach were then performed. Several (see Experimental section for details) samples of commercial and raw cows', goats', and sheep's milk were analyzed to check the possibility of variability among samples of

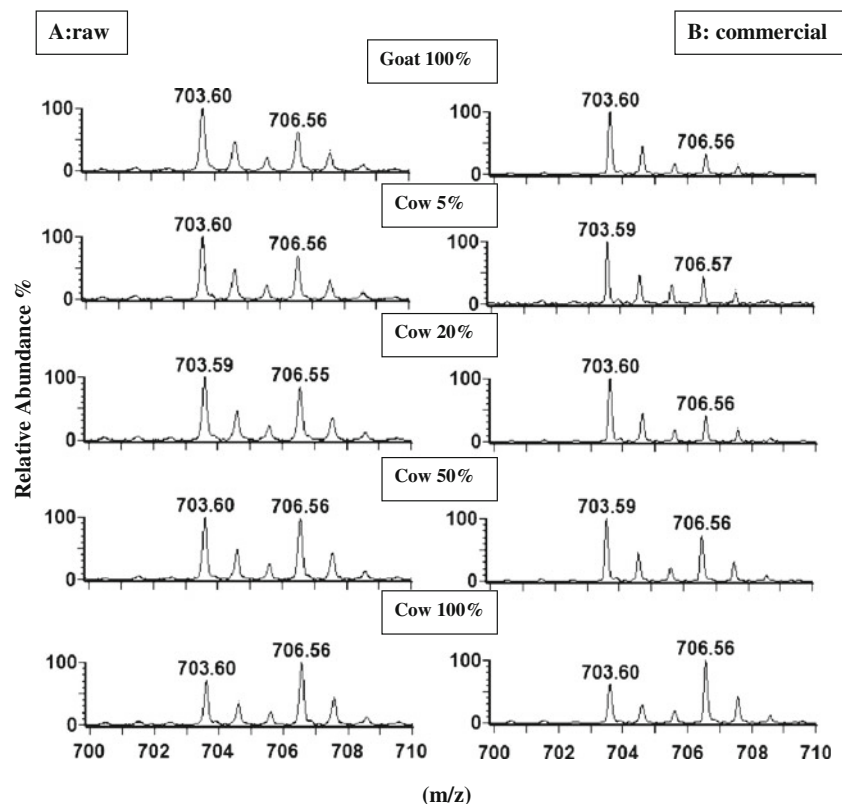
Table 2 Mean values (for three replicates) and coefficient of variation (CV, %) of the ratio between the peak intensities of ions at m/z 706.6 and m/z 703.6

Sample	Intensities ratio (706.6/703.6)	CV intra-assay (%)	CV inter-assay (%)
Raw cows' milk 1	1.44	6.95	12.8
Raw cows' milk 2	1.39	7.10	12.5
Raw cows' milk 3	1.46	7.34	13.1
Commercial cows' milk 1	1.65	9.78	15.8
Commercial cows' milk 2	1.67	10.03	18.9
Commercial cows' milk 3	1.76	9.89	17.8
Commercial cows' yoghurt	1.55	7.52	12.3
Raw goats' milk 1	0.605	7.62	16.8
Raw goats' milk 2	0.632	8.97	17.9
Raw goats' milk 3	0.665	9.23	18.3
Commercial goats' milk 1	0.323	5.87	7.18
Commercial goats' milk 2	0.303	6.98	8.59
Commercial goats' milk 3	0.345	7.21	9.67
Commercial goats' yoghurt	0.352	6.88	12.2
Raw sheep's milk 1	0.331	8.32	12.9
Raw sheep's milk 2	0.293	7.93	10.0
Raw sheep's milk 3	0.341	8.68	13.6

different origin or from different producers. The mean and standard deviation of the 706.6/703.6 intensity ratio were calculated for all the replicates of each type of milk and the two-sample t -test was performed to determine whether the populations were significantly different. By comparing the calculated t -value, with k degrees

of freedom, with the critical t -value from the t -distribution table at the chosen confidence level we could decide whether to accept or reject the null hypothesis (means are equal). We could reject the null hypothesis when the calculated t -value > critical t -value. For comparison of raw bovine milk with commercial bovine

Fig. 3 (A) MALDI mass spectra obtained from lipid extracts of raw goats' milk, raw goats' milk adulterated at 5 %, 20 %, and 50 % with raw cows' milk, and raw cows' milk. (B) MALDI mass spectra obtained from lipid extracts of commercial goats' milk, commercial goats' milk adulterated at 5 %, 20 %, and 50 % with commercial cows' milk, and commercial cows' milk



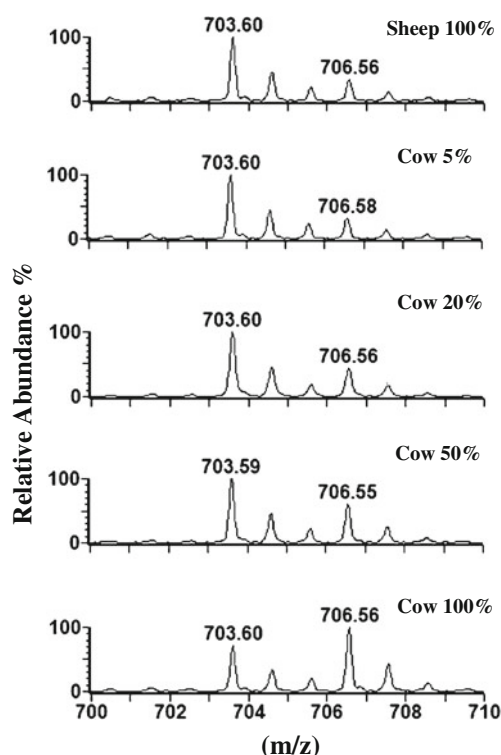


Fig. 4 (a) MALDI mass spectra obtained from lipid extracts of raw sheep's milk, raw sheep's milk adulterated at 5 %, 20 %, and 50 % with raw cows' milk, and raw cows' milk

milk the calculated t -value at the 95 % confidence level (two-tailed) was less than the critical t -value so the two kinds of milk were considered equal (the null hypothesis is accepted).

The same test was performed by comparing the mean value of the ratio for cows' milk with that for goats' and sheep's milk. In this case, the computed value for t was greater than the critical value of t and the null hypothesis was rejected (the mean values were significantly different). The test was then performed to compare goats' and sheep's milk adulterated at the 5 % and 20 % levels against pure goats' and sheep's milk, respectively. Here, the computed value for t was greater than the critical value of t and there was a significant difference between the means for milk samples adulterated at the 20 % level compared with those for the pure milk samples, whereas the differences between the intensity ratios for milk samples adulterated at the 5 % level were not significantly different from those of the pure samples. For the lower adulteration levels (10 or 5 %), however, the method can still be used to alert the analyst and can function as a screening method. Samples that exceed the level of attention can be analyzed further by conventional methods, for example gas chromatography.

These results suggest the method is not affected by natural variations, at least for the samples investigated, and that the marker intensities are not significantly affected by industrial treatment of the milk or by individual variability. In fact, for biological variability it is worth noting that we have also analysed different UHT milk samples. UHT milk samples come from cows of different breeds raised in several EU countries with different diets and different periods of lactation. These results were particularly significant, because MALDI data, as a result of lack of homogeneity of the matrix crystals, are usually affected by lack of analysis-to-analysis and sample-to-sample reproducibility. It is likely that the good reproducibility obtained here could be ascribed to the use of the new matrix CCICA that produces more homogeneous crystals than traditional matrices, for example DHB.

According to the results reported so far, a concentration-dependent relationship seems to exist between the relative intensities of the phospholipid markers and the level of adulteration. This observation was confirmed by plotting the average ratio of the relative intensities (six replicates) of the diagnostic peaks (706.6/703.6) against the percentage of adulterant cow milk. Results for raw milk sample mixtures, i.e. cows' milk in goats' milk (a) and cow's milk in sheep's milk (b) are reported in Fig. 5, as an example. The progressive increase of the ratio is clearly apparent, as also

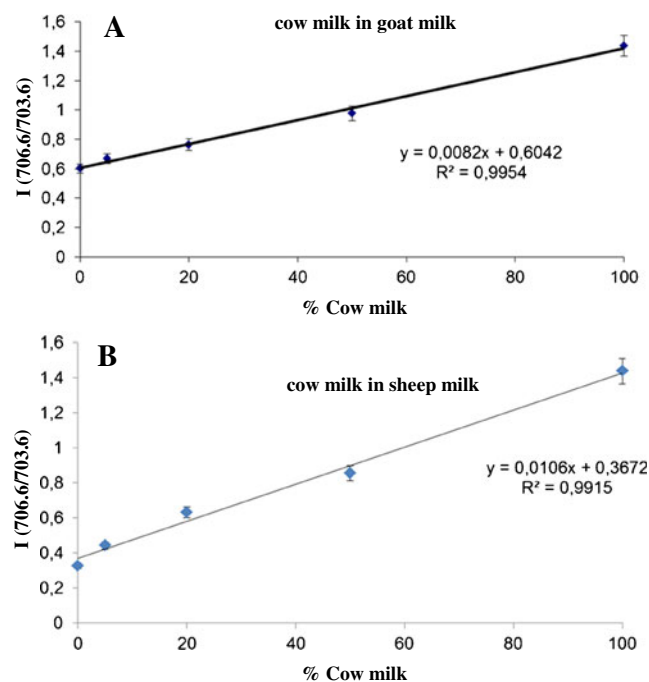
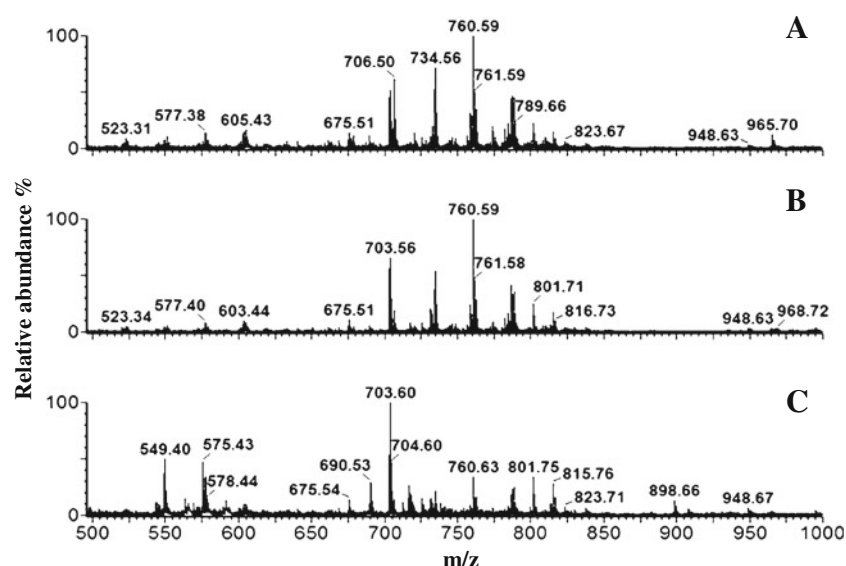


Fig. 5 Plot of the ratios of the abundance of the marker ions of m/z 706 and 703 for (A) cows' milk in goats' milk and (B) cows' milk in sheep's milk against the percentage of cows' milk. Error bars indicate technical inter-assay reproducibility

Fig. 6 MALDI mass spectra obtained from lipid extracts of Grana Padano DOP 100 % (A), Pecorino Romano DOP 100 % (B), and goat ricotta cheese (C)



is its linear dependence on adulteration level, which clearly indicates that this method could be used for the proposed purpose. To validate our results we also performed experiments on 24 samples of unknown identity; by calculating the marker ratio it was possible to identify adulteration with sensitivity of 88 % and specificity of 91 %.

Finally, cheese samples were analyzed by use of the proposed method to verify its applicability to processed dairy products also. MALDI-TOF mass spectra obtained from extracts of lipids from Grana Padano DOP 100 % (a), Pecorino Romano DOP 100 % (b), and goat ricotta cheese (c) are reported in Fig. 6. The spectra obtained were very similar to those reported in Fig. 1 for milk samples, indicating that the intensity ratio 706.6/703.6 is even informative for cheeses for which thermal and enzymatic treatment was used. Two-sample *t*-tests performed to compare Grana with Pecorino and Grana with goat ricotta confirmed the populations were significantly different. In fact, at the 95 % confidence level (two-tailed) the computed value for *t* was greater than the critical value of *t* and there was significant difference between the means. Thus, the method could potentially be capable of detecting cheese adulteration also.

Conclusions

We have demonstrated the usefulness of direct milk phospholipid profiles obtained by matrix-assisted laser desorption/ionization-time of flight mass spectrometry as a rapid and sensitive method for the detection of milk adulteration, by searching for species-specific markers. Several pure raw and commercial milk samples and binary mixtures containing cows' and goats' milk and cows' and sheep's milk were processed and the

phospholipids were analysed using α -cyano-4-chlorocinnamic acid as matrix. The peak intensity ratio (706.6/703.6) is proposed as new marker for the identification of the adulterant cows' milk in sheep's or goats' milk. Furthermore, very promising preliminary data were obtained when the approach was applied to cheese samples.

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