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Rapid detection and quantification of milk adulteration using MALDI-MS protein profiling and multivariate calibration

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ABSTRACT

The adulteration of high-value milks with low-value milks is a common fraudulent practice in the dairy industry. This not only results in economic or quality prejudice, but also poses a potential threat to individuals with sensitivities or allergies. Profiling the major whey and casein milk proteins can be a crucial tool in combating this malpractice, given that each mammal species exhibits a unique protein fingerprint. In this study, we employed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) as a rapid and straightforward method for profiling proteins from bovine, caprine, and ovine milks. Subsequently, we analyzed binary mixtures of goat and sheep milks with bovine milk to establish multivariate calibration models using partial least-squares (PLS) to quantify the adulteration of goat and sheep milks. Proteins were identified according to their molecular mass (M_r) and their intensities were considered as multivariate data. To achieve satisfactory calibration and validation results, normalization through variable augmentation was necessary, enabling the quantification of bovine milk across the entire adulteration range. This uncomplicated approach based on MALDI-MS protein profiling and PLS shows significant potential for qualitative and quantitative assessments of milk adulteration.

1. Introduction

Milk adulteration encompasses a wide range of fraudulent practices involving the addition of certain substances to milk to dishonestly increase profits (Azad and Ahmed, 2016; Ionescu et al., 2023; Montgomery et al., 2020). Adulterants added to fresh milk may include water, various chemicals (e.g. urea, starch, or detergents), and low-value milks in different states (e.g. liquid, powdered, or frozen). This prevalent malpractice in the dairy industry, not only results in economic prejudice and compromises milk quality, but also poses potential health risks to consumers with sensitivities or allergies (Cubero-Leon et al., 2023). Regulatory bodies and food safety organizations implement stringent measures to detect and prevent milk adulteration (Montgomery et al., 2020), emphasizing the crucial need for developing accurate and reliable analytical methods to support these preventive efforts (Azad and Ahmed, 2016; Ionescu et al., 2023).

The adulteration of high-value milks with low-value milks stands as one of the most common fraudulent practices in the dairy industry (Azad and Ahmed, 2016; Ionescu et al., 2023). Profiling the major whey and casein milk proteins can be a crucial tool in combating this malpractice, considering that milk from each mammal species possesses a distinctive protein fingerprint, which can be analyzed through different methods (Dupont et al., 2018). Among the most efficient methods are those based on high performance separation techniques. Various capillary electrophoresis (CE) and liquid chromatography (LC) with ultraviolet absorption (UV) or mass spectrometry (MS) detection methods have been described for the analysis of milk proteins from different species (Fuerer et al., 2020; Ghafoori et al., 2022; Heck et al., 2008). However, these methods require time-consuming and labor-intensive separations compared to those based on direct MS for the identification of the components of the protein fingerprints, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) (Cozzolino et al., 2002; Cunsolo et al., 2013; Di Girolamo et al., 2014; Liland et al., 2009; Piras et al., 2021; Rau et al., 2020; Sassi et al., 2015). MALDI-MS has been used for milk protein profiling, both at the intact protein level (Cozzolino et al., 2002; Cunsolo et al., 2013; Di Girolamo et al., 2014; Ghafoori et al., 2022; Liland et al., 2009; Piras et al., 2021; Sassi et al., 2015) and the peptide level (Rau et al., 2020; Sassi et al.,

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2015), typically after releasing proteotypic peptides from proteins through enzymatic digestion (Rau et al., 2020). The resulting mass spectra have served as fingerprints for animal species identification, classification, and detection of adulteration. Generally, the analysis of intact proteins is more straightforward, and conventional MALDI mass spectrometers are sufficient to obtain characteristic protein profiles to differentiate milks from different species (Cozzolino et al., 2002; Cunsolo et al., 2013; Di Girolamo et al., 2014; Ghafoori et al., 2022; Liland et al., 2009; Piras et al., 2021; Sassi et al., 2015). However, despite the widely accepted potential of MALDI-MS for the identification of low-value milks in high-value milks at the intact protein level, only a few studies have moved beyond qualitative analysis to demonstrate its applicability in quantifying milk adulteration (Cunsolo et al., 2013; Liland et al., 2009; Nicolaou et al., 2011; Piras et al., 2021). Nevertheless, in most cases, these studies propose to work with the complete profile of whey and casein milk proteins for appropriate discrimination, and the proposed experimental and chemometrics procedures often remain unclear and complex for a widespread implementation (Liland et al., 2009; Nicolaou et al., 2011; Piras et al., 2021).

In this study, we present a rapid and straightforward MALDI-MS method for the identification of proteins from cow, goat, and sheep milks, and quantification of adulteration of goat and sheep milks with cow milk. The method relies on the accurate identification of the proteins from the different species in their binary mixtures, as a necessary step to use their intensities, measured in the mass spectra, as fingerprints. These fingerprints are then employed to establish multivariate calibration models using partial least squares (PLS). The proposed uncomplicated approach based on MALDI-MS protein profiling and PLS shows significant potential for both qualitative and quantitative assessments of milk adulteration.

2. Materials and methods

2.1. Chemicals and reagents

Analytical reagent grade chemicals and reagents were used in the preparation of buffers and solutions. Sinapinic acid (SA, ≥99.0%), acetone (99.8%), TFA (99.0%), sodium citrate dihydrate (≥99.0%), urea (99.0–100.5%), DL-dithiothreitol (DTT, 97%), and sodium hydroxide (≥99.0%, pellets) were provided by Merck (Darmstadt, Germany). ACN and water (both LC-MS grade) were supplied by PanReac Applichem (Barcelona, Spain).

2.2. Sample preparation

Ultra-high temperature (UHT) semi-skim cow, goat, and sheep milks from three different commercial brands produced in Spain were acquired at a local market of Barcelona. Pure semi-skim milks were defatted by centrifuging a sample aliquot of 1.5 mL at 14,500 × g and 4ºC for 30 min in a cooled Rotanta 460 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). After collecting a 500 μL volume from the middle of the tube, centrifugation was repeated for 15 min, and 300 μL of skim milk was collected from the bottom of the tube. Cow-goat and cow-sheep binary mixtures were prepared by volume, covering the entire concentration range, from 0% to 100% (v/v) of cow milk. The calibration and validation sets included ten and six cow milk concentration levels (i.e., 0%, 5%, 15%, 25%, 35%, 45%, 55%, 65%, 85%, and 100% and 2.5%, 10%, 30%, 50%, 70%, and 90% (v/v) cow milk, respectively).

Pure skim milk samples or the binary mixtures (1 mL) were diluted with reduction buffer (5 mL) and incubated for 1 h at room temperature. The reduction buffer was prepared as described in previous works (De Jong et al., 1993; Ghafoori et al., 2022). Briefly, sodium citrate dihydrate (73 mg) and DTT (38 mg) were mixed with 37.5 mL of 8 M urea in a 50 mL volumetric flask. Then, pH was adjusted to 8.0 with sodium hydroxide solution, and the volume was made up with water.

The resulting solution from protein extraction was filtered through 0.20 μm nylon filters (MSI, Westboro, MS, USA). The filtered solution was further desalted using MF-Millipore® membrane filters (Merck) before MALDI-MS analysis. For desalting, 10 μL of sample solution and 2 μL of ACN were deposited onto the membrane filter and dialyzed with water for 45 min at room temperature. Desalted samples were immediately analyzed by MALDI-MS.

2.3. MALDI-MS

MALDI-MS mass spectra were obtained using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Waltham, MA, USA) equipped with a nitrogen laser (355 nm) and a microchannel plate detector (MCP). The laser intensity was set to 7900 (100%). Mass spectra were acquired over a range of 5000–30,000 *m/z* using the mid mass positive mode. Data acquisition and data processing were conducted using the 4000 Series Explorer™ and Data Explorer® software (Applied Biosystems). For analysis, sample-matrix mixtures were freshly prepared by depositing onto the stainless-steel MALDI plate 1 μL of SA in 99:1 (v/v) acetone:water (final concentration 27 mg·mL^{-1}), 1 μL of sample solution (allow drying for 15 min), and 1 μL of SA in 50:50 (v/v) ACN:water with 0.1% (v/v) of TFA (final concentration 10 mg·mL^{-1}) (Ghafoori et al., 2022; Pont et al., 2020). Four replicate analyses were carried out at each concentration level.

2.4. Data preprocessing and PLS

After conducting the MALDI-MS analyses, proteins were identified based on their molecular mass (M_r) (Table 1), and their intensities were

Table 1
Mass accuracy in molecular mass (M_r) determination by MALDI-MS for the cow, goat, and sheep milk proteins considered for adulteration quantification (±standard deviation, SD, n=3).

Proteins[a]	Uniprot entry	Theoretical M_r[b]	Experimental M_r (±SD)[c]	ΔM_r
Cow milk				
αLA	P00711	14186.0	14180.1 (±0.4)	5.9
βLG-B	P02754	18281.1	18281.0 (±0.7)	0.1
βLG-A	P02754	18367.1	18359.0 (±0.2)	8.1
κCN-B (1 Pyr,1 P)	P02668	19005.2	19008.6 (±0.5)[d]	-3.4
κCN-A (1 Pyr, 1 P)	P02668	19037.2		28.6
βCN-A2 (5 P)	P02666	23983.0	23980.8 (±1.3)[d]	2.2
βCN-A1 (5 P)	P02666	24023.0		42.2
Goat milk				
αLA	P00712	14194.0	14187.5 (±1.5)	6.5
βLG	P02756	18191.1	18184.6 (±2.5)	6.5
κCN (1 Pyr, 2 P)	P02670	19289.1	19287.7 (±1.6)	1.4
βCN (5 P)	P33048	23740.7	23710.3 (±4.3)	30.4
Sheep milk				
αLA	P09462	14255.0	14181.9 (±3.1)	73.1
βLG	P67976	18150.9	18153.6 (±2.4)	-2.7
κCN (1 Pyr, 2 P)	P02669	19286.2	19269.2 (±3.1)	17.0
βCN (5 P)	P11839	23750.8	23733.5 (±1.1)	17.3

[a] The considered protein sequences and post-translational modifications were described in UniProtKB database (http://www.uniprot.org) and reported in previous papers species (Fuerer et al., 2020; Ghafoori et al., 2022). αLA, α-lactalbumin, βCN, β-casein, βLG-A, β-lactoglobulin A, βLG-B, β-lactoglobulin B, κCN, κ-casein.

[b] Theoretical M_r values were calculated with mMass open source software (version 5.5.0, http://www.mmass.org/).

[c] Experimental M_r values were calculated from the *m/z* values of the detected singly-charged molecular ions for the different proteins as an average of three replicate analyses of pure milk samples (standard deviation, SD).

[d] Mass accuracy and resolution was not enough to discriminate both proteins. α-s1-CN-B could not be detected in any of the milk samples, probably because it was codetected with βCNs. This was not considered for the calculations.

arranged as vectors for subsequent data preprocessing. To ensure the best performance in multivariate data analysis, the intensities from at least three of the four replicate analyses at each concentration level were averaged before proceeding with normalization. Under the optimized normalization conditions, each protein intensity was divided by the intensities of all other proteins (Lerma-García et al., 2007). This normalization approach resulted in variable augmentation. The total number of new variables was easily calculated considering that they could not be redundant (i.e., each intensity ratio was considered only once: (number of proteins)x(number of proteins-1)/2)). Additionally, the average % relative standard deviation (RSD) for the normalized protein intensity variables decreased from 13.8% to 5.7% in cow-goat mixtures and from 16.1% to 7.5% in cow-sheep mixtures. Finally, the normalized vectors were centered and employed for multivariate data analysis. First-order PLS was performed using MVC1 toolbox (Giménez et al., 2008; Olivieri et al., 2004), running in MATLAB R2016a (The Mathworks Inc., Natick, MA, USA).

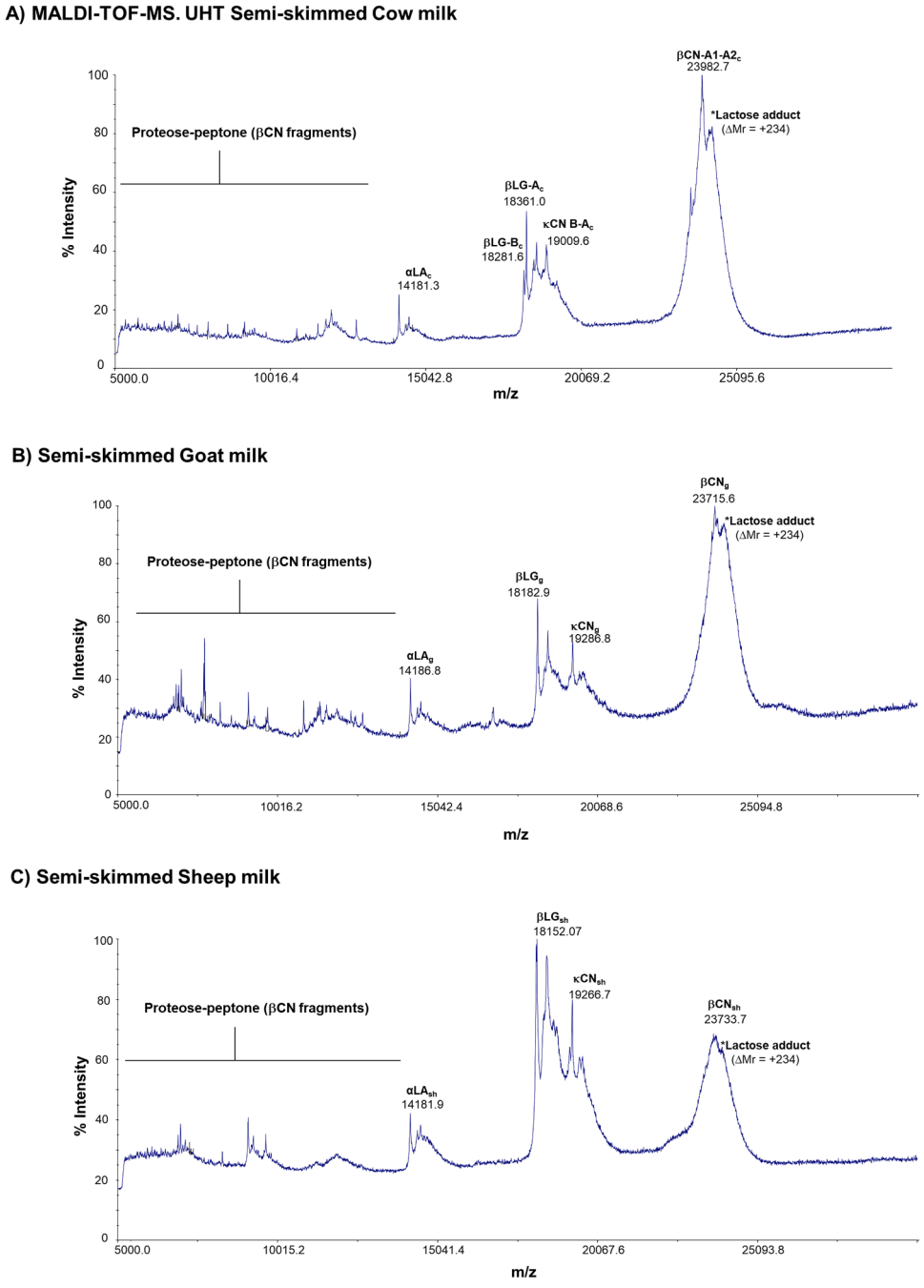

Fig. 1. MALDI-MS mass spectra of A) cow, B) goat, and C) sheep milks. The *m/z* values of the molecular ions of the major proteins unambiguously identified are labelled with their names, because they were relevant for multivariate data analysis. An example of the typical lactose adducts from Maillard reaction after UHT treatment ($\Delta M_r = +234$), as well as the *m/z* range of the proteose-peptone β-casein fragments, are indicated in each mass spectrum. αLA, α-lactalbumin, βCN, β-casein, βLG-A, β-lactoglobulin A, βLG-B, β-lactoglobulin B, κCN, κ-casein. Subindexes: c, g, and s stand for cow, goat, and sheep, respectively. Some of the protein biomarkers are a mixture of several proteoforms (e.g. βCN-A1-A2$_c$).

3. Results and discussion

3.1. MALDI-MS

MALDI-MS is a very useful technique for qualitative analysis, allowing the rapid and straightforward generation of protein fingerprints for milk characterization, as we and other authors have previously demonstrated (Cozzolino et al., 2002; Cunsolo et al., 2013; Di Girolamo et al., 2014; Ghafoori et al., 2022; Liland et al., 2009; Piras et al., 2021; Sassi et al., 2015). Proper sample preparation, MALDI matrix selection, matrix-sample preparation, and deposition onto the stainless-steel plates are extremely important to obtain optimal results (Giménez et al., 2007), especially in quantitative analysis (Vergara-Barberán et al., 2023). In this study, we followed the experimental procedures described in our prior work for cow milk analysis (Ghafoori et al., 2022). These procedures allowed obtaining good sensitivity and repeatability for the three milk types investigated. Fig. 1-A, -B, and -C depict the mass spectra for pure cow, goat, and sheep milks, respectively. Each milk exhibited a characteristic protein profile, including the major whey and casein proteins, along with their lactose adducts resulting from Maillard reactions during the UHT treatment ($\Delta M_r = +234$) and different proteose peptone β-casein (βCN) fragments (Ghafoori et al., 2022). Table 1 shows the theoretical and experimental M_r of the major proteins unambiguously identified in each milk type. As can be observed, the mass accuracy given as ΔM_r between the theoretical and experimental M_r values, ranged between 0.1 and 73.1 mass units, with the standard deviations (SD) of the experimental M_r values falling between 0.2 and 4.3 mass units. Unfortunately, the mass accuracy and peak resolution of our conventional MALDI-TOF mass spectrometer were not sufficient to differentiate closely related protein variants with similar M_r values. This was the case of κ-casein A and B (κCN-A and κCN-B, ΔM_r=32), or βCN-A1 and βCN-A2 in cow milk (ΔM_r=40). Consequently, a total of 5, 4, and 4 proteins were experimentally identified in cow, goat, and sheep milks. Additionally, this limited performance also impacted protein identification in cow-goat and cow-sheep milk mixtures. As shown in Table 1, the difference between the M_r values of α-lactalbumin (αLA) in the three milk types was not sufficient for an appropriate discrimination (ΔM_r=8 and 69). Similarly, due to the considerably lower concentration of βCN in sheep milk compared to cow milk (~60% vs 100% of the maximum mass spectrum intensity, respectively, Fig. 1), it was impossible to distinguish between sheep and cow βCNs in cow-sheep mixtures (Table 1). In contrast, this differentiation was possible in cow-goat mixtures (Table 1). As a result, a total of 8 and 7 protein variables were considered for the multivariate data analysis of the cow-goat and cow-sheep mixtures, respectively (Fig. 2). It is worth highlighting that, in contrast to previous studies (Liland et al., 2009; Nicolaou et al., 2011; Piras et al., 2021), the remaining protein components, including adducts and peptide fragments, were discarded as variables in subsequent analyses. This decision was made due to their potential variability depending on milk origin and processing. The aim was to establish the selected protein variables as a universal set of protein biomarkers for a reliable and robust quantitative multivariate data analysis.

3.2. PLS calibration

A subset of the typical mass spectra obtained for the cow-goat and cow-sheep mixtures used for PLS calibration is shown in Fig. 2. The annotation of the protein biomarkers considered as variables for multivariate data analysis was based on a careful and systematic comparison of experimental and theoretical M_r values, as explained in the previous section. Non-detected protein biomarkers are indicated in red. As can be observed, the annotation of the different protein peaks at 5% (v/v) cow milk concentration revealed the detection of cow proteins in goat and sheep milks, even at this low concentration, confirming the applicability of MALDI-MS for the qualitative analysis of milk adulteration.

The raw intensity data required preprocessing to optimize performance in PLS calibration, which correlated protein biomarker intensities with cow milk concentration levels in goat and sheep milks. Following the MALDI-MS analyses on the cow-goat and cow-sheep calibration sets and identifying the protein biomarkers, their raw intensities in the replicates of each concentration level underwent normalization. In a preliminary normalization approach, each protein intensity was divided by the sum of the intensities of all other proteins (Benavente et al., 2006; Lerma-García et al., 2007). Subsequently, these normalized values were averaged for the different replicates. However, poor results were obtained in PLS calibration (data not shown), likely due to the limited number of normalized variables employed to establish the PLS calibration models (i.e., 8 and 7 variables in cow-goat and cow-sheep mixtures, respectively). As an alternative normalization approach, each protein intensity was divided by the intensities of all other proteins (Lerma-García et al., 2007) before averaging and PLS calibration. This resulted in an increased number of normalized variables (i.e., 28 and 21 variables in cow-goat and cow-sheep mixtures, respectively) and demonstrated the best calibration performance. The number of PLS factors was selected using Haaland's criterion, which considers that the best calibration model explains a large percentage of the total variance of the data with the minimum number of factors (Olivieri et al., 2004). Three and two factors were selected for cow-goat and cow-sheep models, with correlation coefficients (R^2) of around 0.98 in both cases. Additionally, no outliers were detected (the F values were below one, when calculated as the ratio between the squared prediction error for a sample left out during cross-validation and the average squared prediction error (Olivieri et al., 2004). Overall, the selected strategy for PLS calibration resulted in an excellent performance.

3.3. PLS validation

In the validation step, the calibration models were used to predict the cow milk concentration in the cow-goat and cow-sheep validation set mixtures. Table 2 shows the nominal and predicted cow milk concentrations in the mixtures. The agreement between both concentrations was acceptable, with average absolute prediction error (RMSEP) values of 7.7% and 8.8% for cow-goat and cow-sheep mixtures, respectively. The accuracy of the estimation was especially lower at the lowest and highest cow milk concentrations, as indicated by the relative error values at 2.5% and 10% of cow milk in both milk mixtures and 90% of cow milk in cow-sheep mixtures. Attempts were made to improve the PLS calibration models for a more accurate quantification in the mixtures by excluding pure cow, goat, and sheep milk samples from the calibration models. However, the results did show significant improvement. Therefore, we recommend building the PLS calibration models covering the entire concentration range, but considering the quantification of adulterant milk concentrations quantified at the extremes of the calibration range as a very rough estimation. Anyway, in intentional adulteration of goat and sheep milks with cow milk, the addition of very low or very high amounts of cow milk is typically avoided to generate economic profit without evident changes in the milk properties. Compared to previous MALDI-MS methods described for quantifying milk adulteration, our approach maintains simplicity by employing a small yet reliably identified set of protein biomarkers, without relying on a single protein biomarker. The latter strategy performs reasonably well only in specific cases, such as the adulteration of donkey milk with cow and goat milks due to the M_r differences between αLA variants (Cunsolo et al., 2013). Additionally, our method provides an excellent alternative to using the complete mass spectra, which requires complex data processing procedures (Liland et al., 2009; Nicolaou et al., 2011; Piras et al., 2021). These procedures must be implemented with care to construct representative calibration models for a reliable quantification. Therefore, the described MALDI-MS PLS-based quantification method may be very useful for rapidly and simply estimating adulteration levels. This extends beyond the mere qualitative confirmation of milk

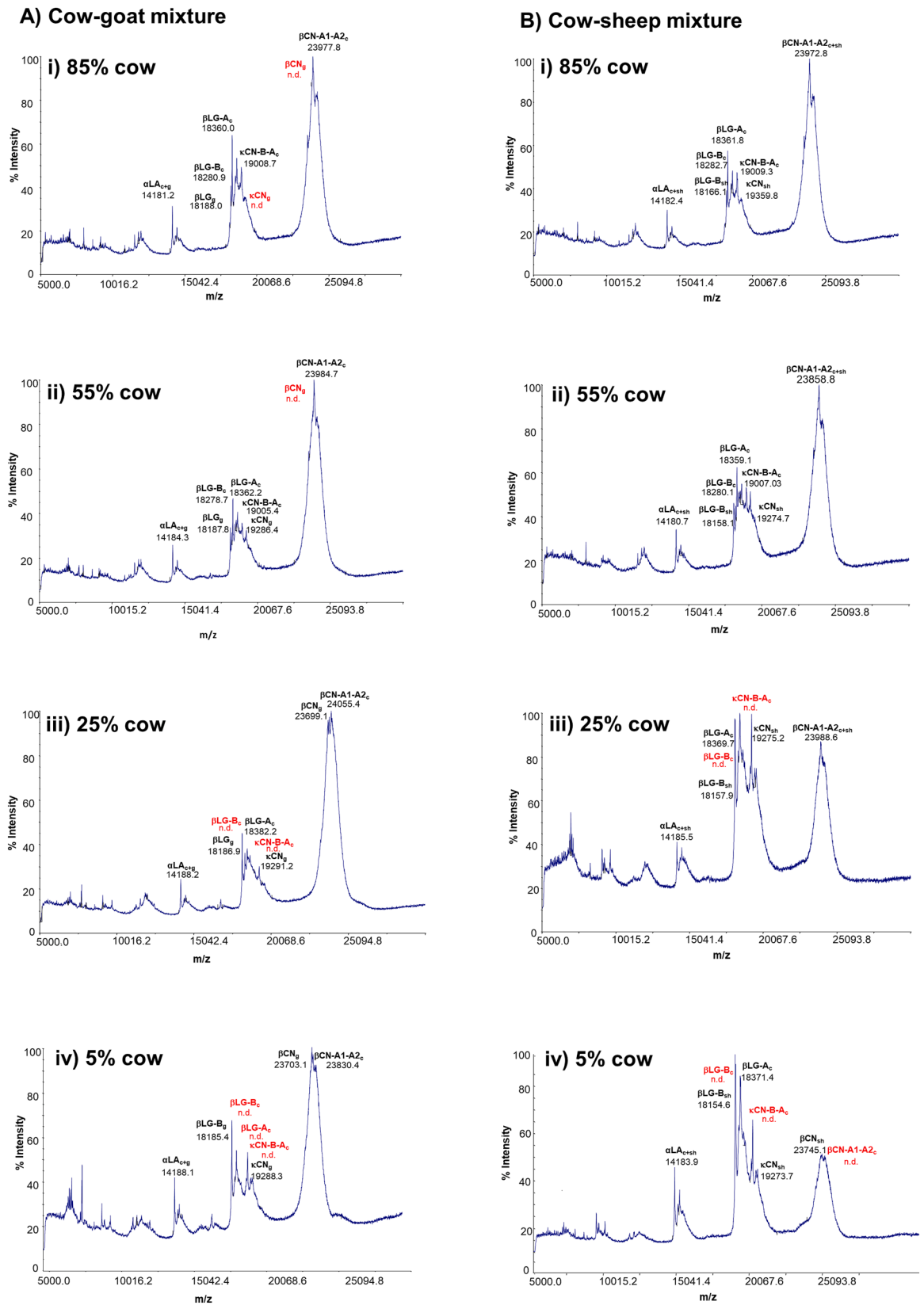

Fig. 2. MALDI-MS mass spectra of A) cow-goat and B) sheep-goat milk mixtures at different concentrations. i) 85%, ii) 55%, iii) 25%, and iv) 5% (v/v) of cow milks. Non-detected protein biomarkers considered as variables for multivariate data analysis are indicated in red color.

Table 2
Optimized number of factors and statistical parameters obtained by PLS for prediction of adulteration of goat or sheep milks with cow milk in the validation set.

	% Cow milk		
Cow-Goat mixtures	**Nominal (%v/v)**	**Predicted (%v/v, PLS)**	**Relative error (%)**
1	2.5	3.5	40.0
2	10	8.0	20.0
3	30	27.5	8.3
4	50	57.5	15.0
5	70	65.3	6.7
6	90	100.9	12.1
No. of factors	3		
RMSEP[a]	7.7%		
	% Cow milk		
Cow-Sheep mixtures	**Nominal (%v/v)**	**Predicted (%v/v, PLS)**	**Relative error (%)**
1	2.5	3.1	24.0
2	10	12.1	21.0
3	30	32.9	9.7
4	50	43.8	12.4
5	70	67.4	3.7
6	90	110.1	22.3
No. of factors	2		
RMSEP[a]	8.8%		

[a] RMSEP: Average absolute prediction error

adulteration by unequivocally identifying exogenous milk proteins.

4. Concluding remarks

In this study we introduced a rapid and straightforward MALDI-MS PLS method for profiling the major whey and casein milk proteins in bovine, caprine, and ovine milks, as well as for quantifying the adulteration of goat and sheep milks with cow milk. The method, using a small yet reliably identified set of protein biomarkers, demonstrated satisfactory performance in quantifying cow milk across the entire adulteration range. The proposed method expands the applicability of MALDI-MS beyond the qualitative screening of milk adulteration and may be considered an excellent complement to more accurate analytical methods for adulteration quantification (e.g. LC-MS and CE-MS). Its potential applications include rapid screening and quantitative estimation of adulteration in quality control and food safety programs related to milk and dairy products. More widely, a similar analytical strategy may be applied to other protein-rich foodstuffs.

CRediT authorship contribution statement

Fernando Benavente: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Tahereh Tehrani:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Laura Pont:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on reasonable request

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