A MALDI-TOF platform for rapid detection of illegal adulterations of milk products.

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Abstract – Seasonality and economic factors are the cause of fraudulent action in diary industry. One of the most frequent fraud is the adulteration of ovine, caprine and buffalo milks with bovine material and the use of powdered milk instead of declared, fresh material. In this context, we present a fast analytical approach suitable both for speciation and thermal treatment analysis to guarantee the origin of raw milk.

Keywords: MALDI-TOF-MS; protein profiling, peptide profiling, milk analysis, speciation, thermal treatment.

1. INTRODUCTION

Raw milk is one of the main constituent of the human diet, unique source of nutrients for the newborns and an important food for adult individuals. It is also essential part of several dairy products that, depending on producing animals and transformation procedures, are characterized by different organoleptic/nutritional properties and economic values [1]. In this context, the exclusive use of a specific, pure raw milk is mandatory in the manufacturing of traditional high-grade European Protected Designation of Origin cheeses. However, it has been estimated that buffalo, goat and sheep milk only represent 11, 2 and 1.4% of the raw material produced worldwide and used for dairy productions, compared with the bovine counterpart (about 85%) [2]. The adulteration of ovine, caprine and buffalo milk with common bovine material occurs often because of the higher prices of the first ones, compared to the latter, and the fluctuation in their availability due to the corresponding seasonal shortages. Fraudulent activities are also associated with the addition of powdered/frozen material to fresh milk in the period of low lactation [3].

Adulteration practices mentioned above determined an ultimate need of rapid, sensitive, and reliable methods to determine the nature of raw milk (and of the corresponding dairy products).

In this study, an integrated platform for the combined peptidomic and proteomic profiling of milk samples has been developed to rapidly reveal illegal adulterations due to the addition of either non-declared bovine material to water buffalo, goat and ovine milks, or of powdered bovine material to the fresh counterpart.

2. METHODS

2.1. Sample preparation

Fresh raw bovine milk (BM), water buffalo milk (WM), ovine milk (OM) and goat milk (GM) samples were collected from local farms after morning milking, stored at 4 °C, and assayed within 5 h. Bovine pasteurized milk (PM), UHT milk (UM) and infant formula (IM) samples from different (20 in number) commercial brands were purchased from supermarkets and used before their expiration date. In all cases, 25 different samples were analyzed for each type of milk. For adulteration studies, binary mixtures of BM in WM, OM and GM counterparts, or IM in BM were prepared at 1, 2, 5, 10, 15, 20, 30, 40 and 50% v/v values. Three independent samples

were prepared for each specific binary mixture. Before MALDI-TOF-MS protein profiling (PR-P), skimmed milk samples were diluted 1:100 with water. Each sample (0.5 µl) was mixed with 0.5 µl of a solution of sinapinic acid (10 mg/ml) (Bruker Daltonics, Bremen, Germany) in 0.1% v/v trifluoroacetic acid, 30% v/v acetonitrile, placed onto a Ground Steel Target (Bruker Daltonics) and dried at room temperature. Before MALDI-TOF-MS peptide profiling (PE-P), peptide samples (0.5 µl) were mixed with 0.5 µl of a solution of α-cyano-4-hydroxycinnamic acid (25 mg/ml) (Bruker Daltonics) in 0.1% v/v trifluoroacetic acid, 30% v/v acetonitrile, placed onto the instrument target reported above and dried at room temperature.

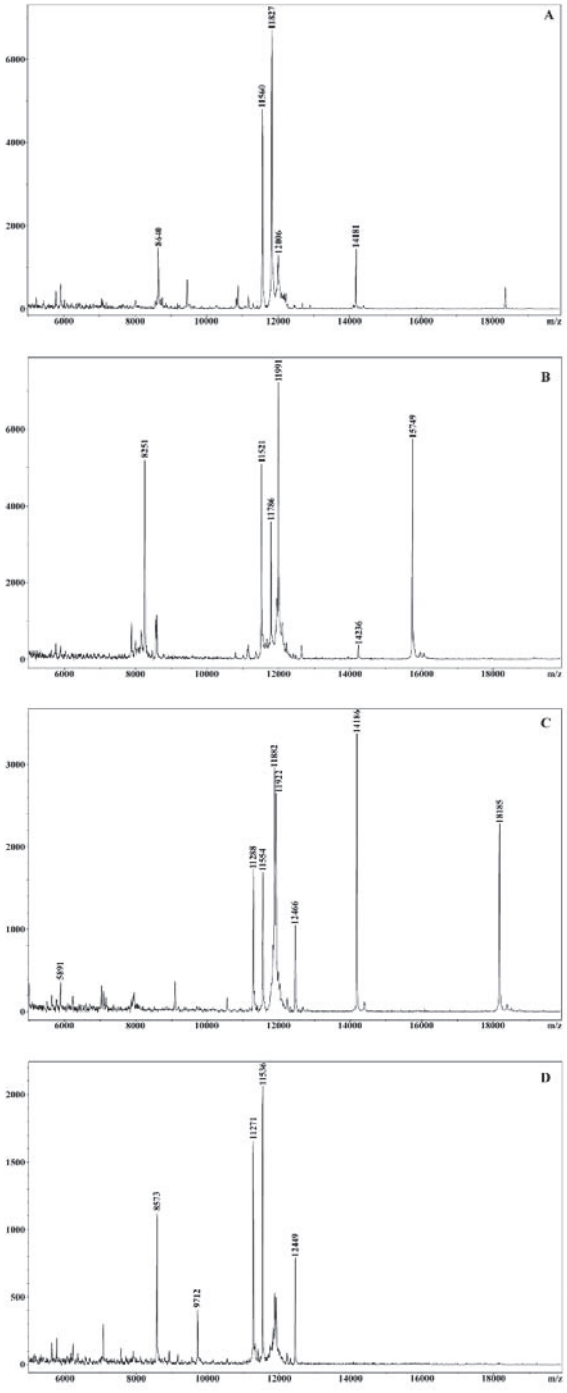

Fig. 1 MALDI-TOF-MS PR-P in LM of diluted milk samples. Spectra refer to BM (A), WM (B), GM (C) and OM (D).

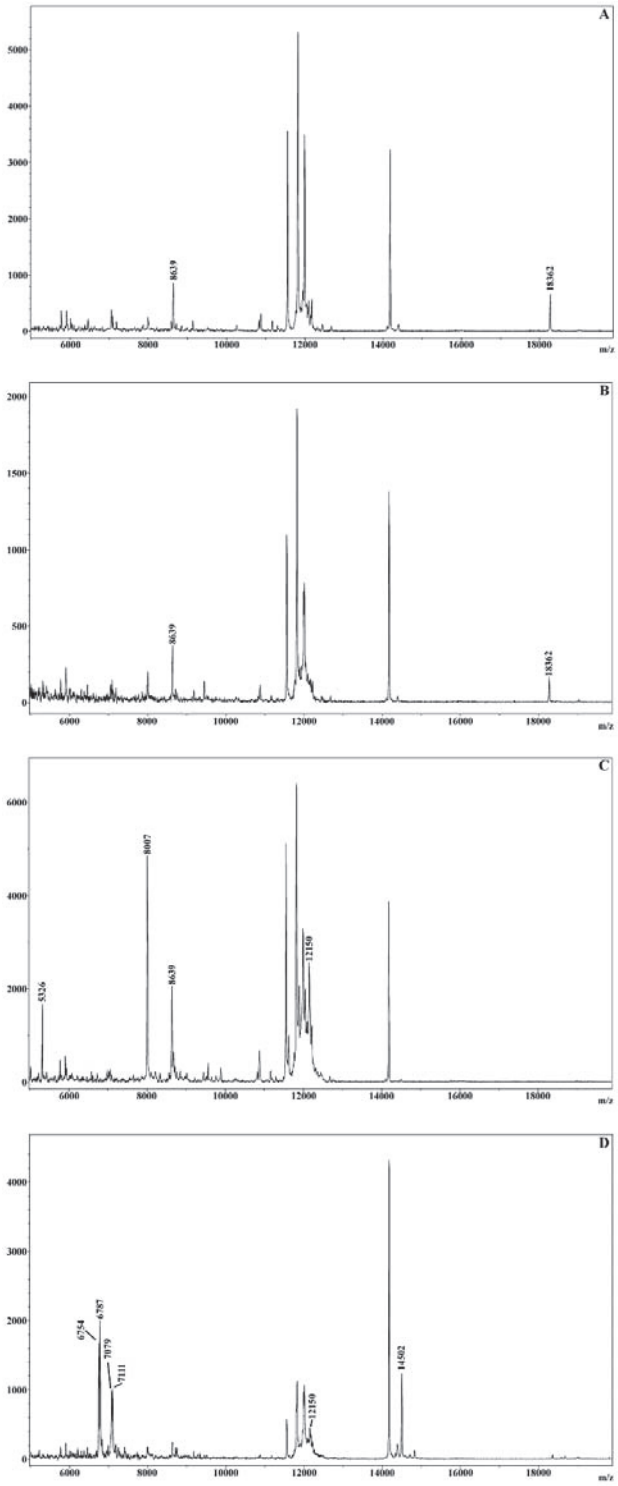

Fig. 2 MALDI-TOF-MS PR-P in LM of diluted milk samples. Spectra refer to fresh BM (A), PM (B), UM (C) and IM (D).

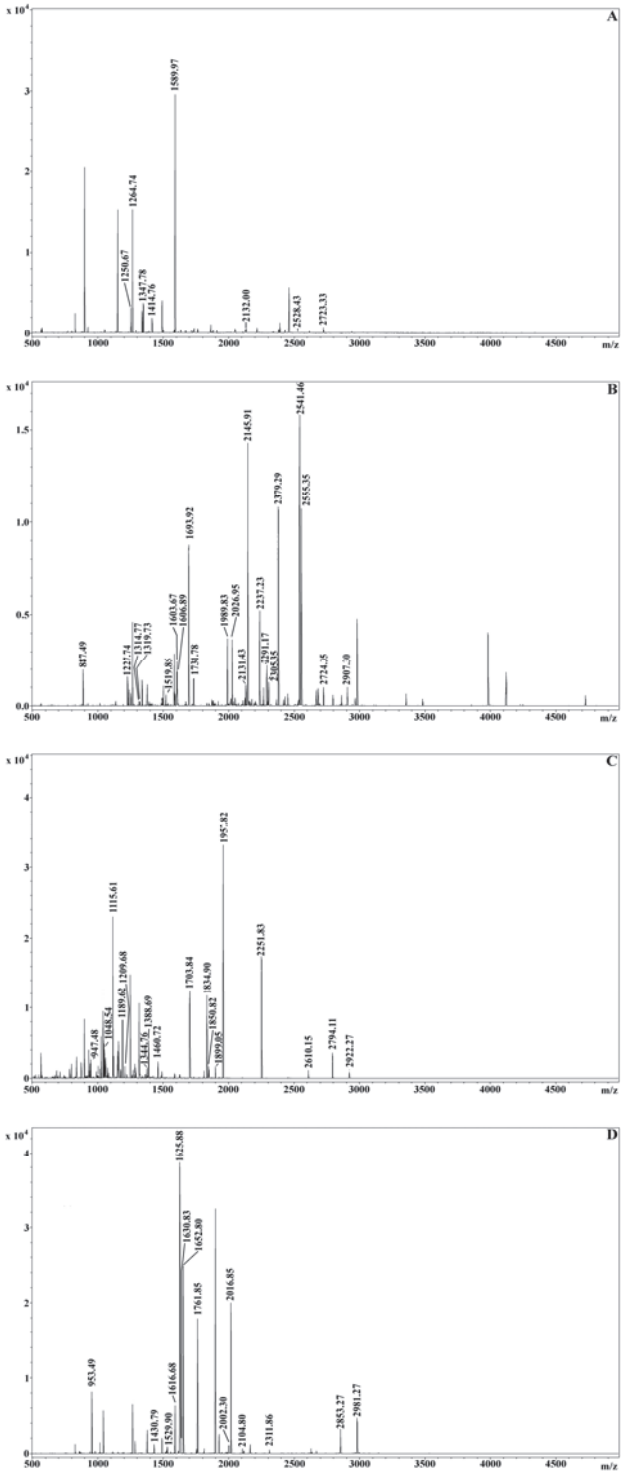

Fig. 1 MALDI-TOF-MS PE-P in RM of diluted milk filtrates subjected to C18ZipTip enrichment as resulting from BM (A), WM (B), GM (C) and OM (D).

2.2 Mass spectrometry and statistical analysis

In order to define an optimal method for the suitable discrimination of milk samples by MALDI-TOF-MS profiling, dedicated procedures were set up for the combined analysis of the corresponding protein and peptide components. Due to the composition complexity of each milk type, various experimental protocols were developed to this purpose, which were compared a posteriori for their discrimination performances. Protein Profiling (PR-P) assays were performed directly on diluted milk samples. On the other hand, Peptide Profiling (PE-P) measurements were realized on diluted low-molecular mass milk filtrates that were then straightly analyzed or extracted with concentration devices before MS

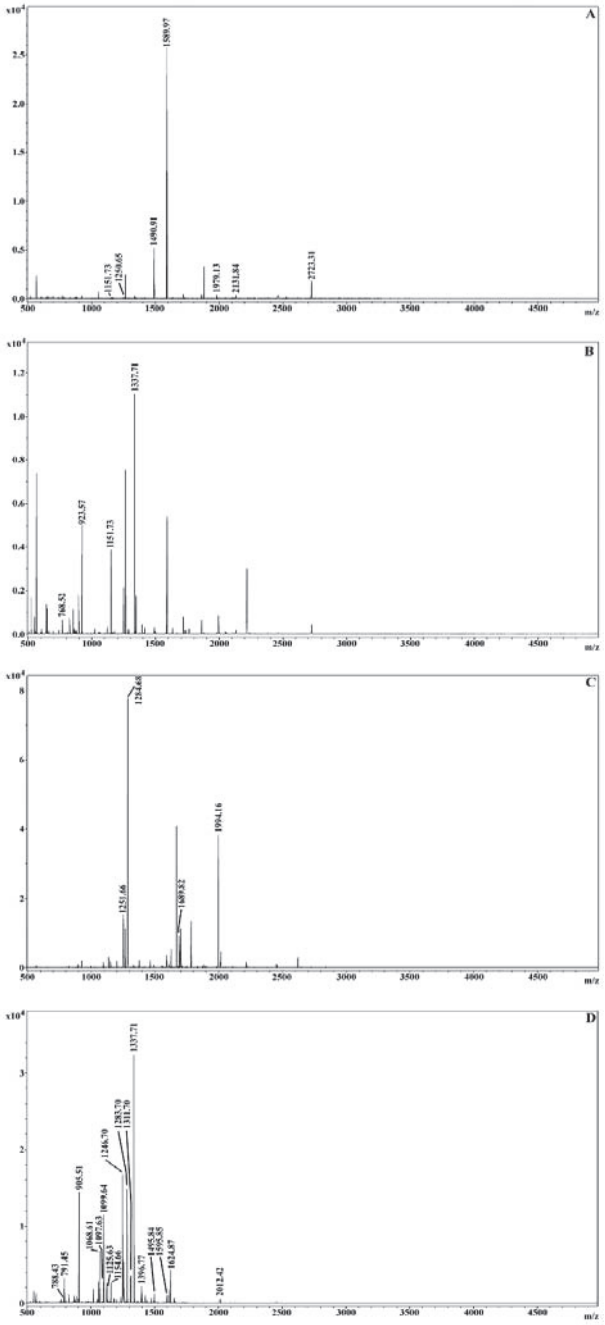

Fig. 4 MALDI-TOF-MS PE-P in RM of milk filtrates subjected to C18ZipTip enrichment from thermally-treated commercial milk samples. Spectra refer to fresh BM (A), PM (B), UM (C) and IM (D).

analysis by MALDI-TOF-MS using an UltraflexExtreme mass spectrometer (Bruker Daltonics). Optimized procedures were used either for the speciation of milk samples, for the characterization of commercial bovine milk samples subjected to various thermal treatment (TTs) and finally for the detection of adulterated milk test samples as result of the addition of non-declared material.

FlexAnalysis (version 3.4) and ClinProt Tools (version 2.2) software packages (Bruker Daltonics) were used for the analysis of all MS data visualization and statistical analysis. Peptides or proteins showing a statistically significant difference in signal intensity or mass value were determined by means of Wilcoxon- (PWKW), Anderson-Darling- (PAD) and t- (PTTA) test. Class prediction model was set up by Genetic Alghorithms (GA). Discriminant peaks were considered those presenting at least a p-value < 0.000001 among the PWKW, PAD and PTTA ones. Finally, it was performed a principal component analysis (PCA) of the spectra, which was carried out by an external MATLAB software tool integrated into ClinPro Tools software.

Regarding adulterated milk, corresponding principal components (PCs) scores were subjected to Partial Least Squares Regression (PLS) by XLSTAT software (Microsoft). Adulteration prediction at two points (5 and 15% v/v) was obtained by the forecast function of Excel software (Microsoft), using PLS results as matrix reference data.

In order to identify the corresponding nature, peptide species already present on the MALDI-TOF mass spectrometer sampling plate were also analyzed by MALDI-TOF/TOF procedures and/or nanoLC-ESI-linear ion trap (LIT)-MS/MS procedures. A LTQ XL mass spectrometer (ThermoFinnigan, USA) equipped with Proxeon nanospray source and connected to an Easy-nanoLC (Proxeon, Denmark) was used to this purpose.

3. RESULTS

Peptide and protein markers of each animal milk were identified after direct analysis of a large number of diluted skimmed and/or enriched diluted skimmed filtrate samples. In parallel, markers of thermal treatment were characterized in different types of commercial milks. Marker protein signals recognized for milk speciation and for TTs recognition were associated with specific components based on literature data and mass calculations and showed in Fig. 1 and Fig. 2 respectively. Peptide markers subsequently characterized by MALDI-TOF/TOF and/or nanoLC-ESI-linear ion trap (LIT)-MS/MS procedures are highlighted in Fig. 3 and Fig. 4.

For milk speciation were identified 23 markers (Protein Profiling (PP)), 65 markers (Peptide Profiling (PE-P) in Linear Mode (LM)), 106 markers (Peptide Profiling in Reflectron Mode (RM)), 63 markers (Peptide Profiling in Linear Mode after C18ZipTip enrichment) and 95 markers (Peptide Profiling in reflectron mode after C18ZipTip enrichment), together with additional statistical parameters. Corresponding data for the characterization of the TT of commercial bovine milk are: 10 markers (PR-P), 27 markers (PE-P in LM), 47 markers (PE-P in RM), 46 markers (PE-P in LM after C18ZipTip enrichment) and 53 markers (PE-P in RM after C18ZipTip enrichment), together with additional statistical parameters. As expected, a number of common peptide markers occurred between analyses performed in LM and RM. In particular, 43, 39, 18 and 23 common peptide markers were observed during PE-P on milk filtrates for speciation, on C18ZipTip-enriched milk samples for speciation, on milk filtrates for TT characterization, on C18ZipTip-enriched milk samples for TT characterization, respectively. Importantly, data from PWKW, PAD and PTTA analysis defined the recognition capability of each specific assay. They demonstrate that milk speciation (100% recognition capability) was guaranteed in the case of MALDI-TOF-MS profiling measurements performed either on proteins from milk filtrates, on peptides from milk filtrates analyzed in RM, and on peptides from C18ZipTip-enriched milk filtrates analyzed both in LM and RM. Conversely, the characterization of the TT of commercial bovine milks was ensured only in the case of peptides from C18ZipTip-enriched milk filtrates analyzed in RM. The latter result is probably dependent on the complexity of the protein and peptide mixtures present in commercial milk samples; it also underlines that the method used for spectral acquisition can highly affect the recognition capability of the whole platform. On this basis, particular care is necessary for the initial set up of the PR-P and PE-P experimental conditions to guarantee the maximum number of signals present in the mass spectra and of the corresponding signal to noise ratios. These instrumental setting have to be

maintained during the whole analysis of different milk samples with the aim of not compromising the recognition capability of the whole platform.

As expected, PCA of the data were in line with the recognition capability values. For example, a good separation of the colored spots was evident in the PCA 3D scatter plots of results from BM, WM, GM and OM proteins analyzed in LM and from BM, WM, GM and OM C18ZipTip-enriched peptides in RM, respectively, which were used for milk speciation (Fig. 5.). Similarly, the PCA 3D scatter plots of results from BM, PM, UM and IM proteins analyzed in LM, and from BM, PM, UM and IM C18ZipTip-enriched peptides in RM, respectively, were used for the

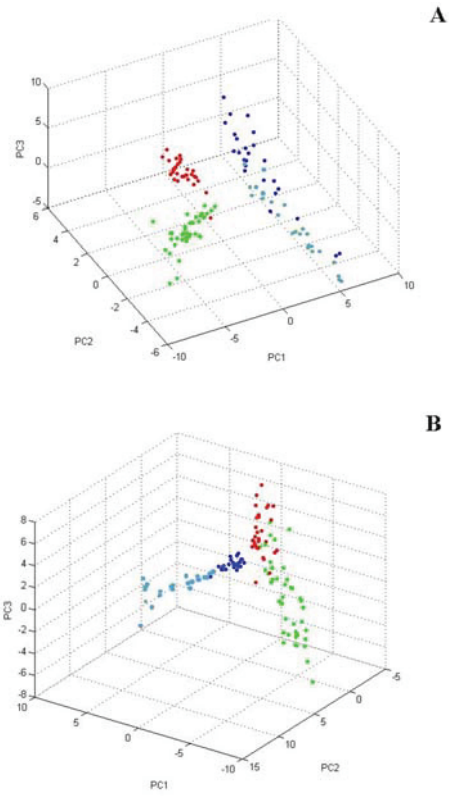

Fig. 5 PCA scatter plot of proteins (A) and peptides (B) MALDI spectra of Bovine (red), Buffalo (green), Goat (blue) and Ewe (light blue) milk.

recognition of the material thermal treatment (Fig. 6). Well-resolved colored spots were evident also in these cases, although a better dot separation occurred for peptides. Less resolved PCA 3D scatter plots were obtained in the case of the remaining methods used for milk speciation and for the recognition of milk TT.

On the basis of the data and the capability of this MS platform of performing independent analyses on

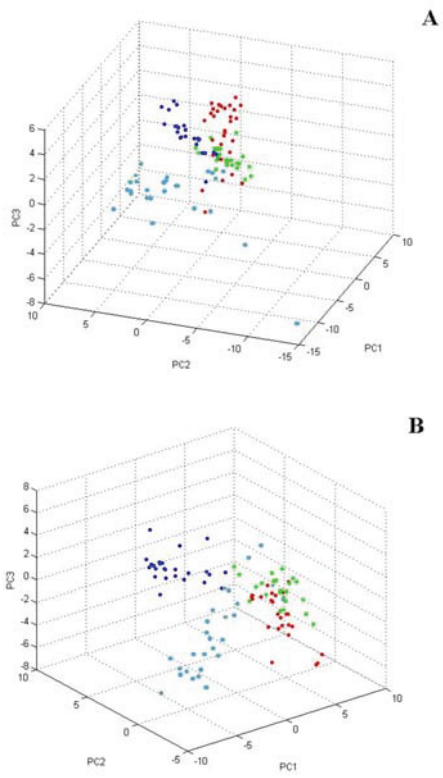

Fig. 6 PCA scatter plot of proteins (A) and peptides (B) MALDI spectra of Raw (red), Pasteurized (green), UHT (blue) and Powder (light blue) milk.

different molecules (proteins and peptides), we conclude that complementary information associated with the highest recognition capability values for milk speciation and for thermal treatment characterization are obtained when diluted skimmed milk samples and C18ZipTip-enriched diluted skimmed milk filtrates are analyzed for the respective protein and peptide content in LM and RM, respectively. Principal components scores of ad hoc prepared species- or thermal treatment-associated adulterated milk samples were subjected to partial least squares regression, permitting a fast accurate estimate of the fraud extents in test samples either at protein and peptide level (Fig. 8).

Plots of the predicted adulteration levels, as estimated from the above-mentioned PLS model based on the corresponding PR-P and PE-P data, are reported in Fig. 8. Data from PR-P and PE-P analyses matched each other and were in good agreement with real adulteration percentages (5 and 15%) within the experimental error. These results demonstrated the good capability of this platform to predict illegal adulteration levels of unknown milk

samples, when they are processed together with proper adulteration standards. They also demonstrated the importance of having analytical system that provides simultaneously two independent adulteration measurements realized on diluted skimmed milk samples and C18ZipTip-enriched diluted skimmed milk filtrates, which are analyzed for the respective protein and peptide content, respectively.

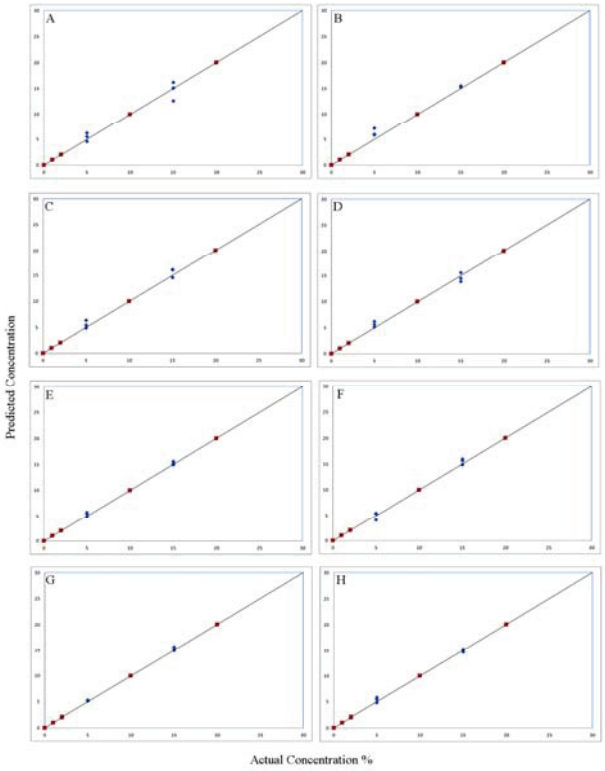

Fig. 7 Plots showing the predicted levels of milk adulteration in protein and peptide profiling (left and right panels respectively). The blue squares represent the training data set and the red rhombus, the test set. Different panel represent cows' milk when added to Buffalo (A, B), Goat (C, D), Ewe (E, F) and Powder milk (G, H).

4. CONCLUSIONS

The development of a versatile and fast method for the detection of fraudulent adulterations in milk is a very important issue, due to the economic impact that these frauds have in dairy productions. A MALDI-TOF-MS platform for combined proteomic and peptidomic profiling of milk samples has been developed in this study, which recognizes markers either for speciation and thermal treatment in BM, WM, GM and OM, as well as in BM, PM, UM and IM.

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