Rapid Detection of Feta Cheese Adulteration via MALDI-TOF MS

Easy and efficient protein profiling coupled with chemometrics to protect product integrity and support consumer safety

Abstract

"Feta" is a Protected Designation of Origin (PDO) Greek cheese, produced exclusively from pasteurized sheep milk or a mixture of sheep and goat milk. The worldwide recognition of feta is attributed to its unique sensory characteristics and high nutritional profile, rendering it one of Greece's most important exports in the dairy industry. Due to its major economic impact, fraud control of these highest-grade PDO feta cheeses is vital. In this study, an integrated, untargeted protein-based workflow has been developed for the rapid detection of the adulteration of feta cheese with cow milk using MALDI-TOF MS profiling and chemometrics. Feta cheese was completely discriminated from similar white cheeses (prepared only from cow milk), and adulteration was detected to 1% via analysis of protein profiles. These results clearly demonstrate that MALDI-TOF MS can be applied as a reliable and rapid screening tool to detect adulteration of dairy products.

Keywords: Feta cheese, microflex LRF, food adulteration, flexAnalysis, profile analysis, cow milk addition, PDO products, prediction models

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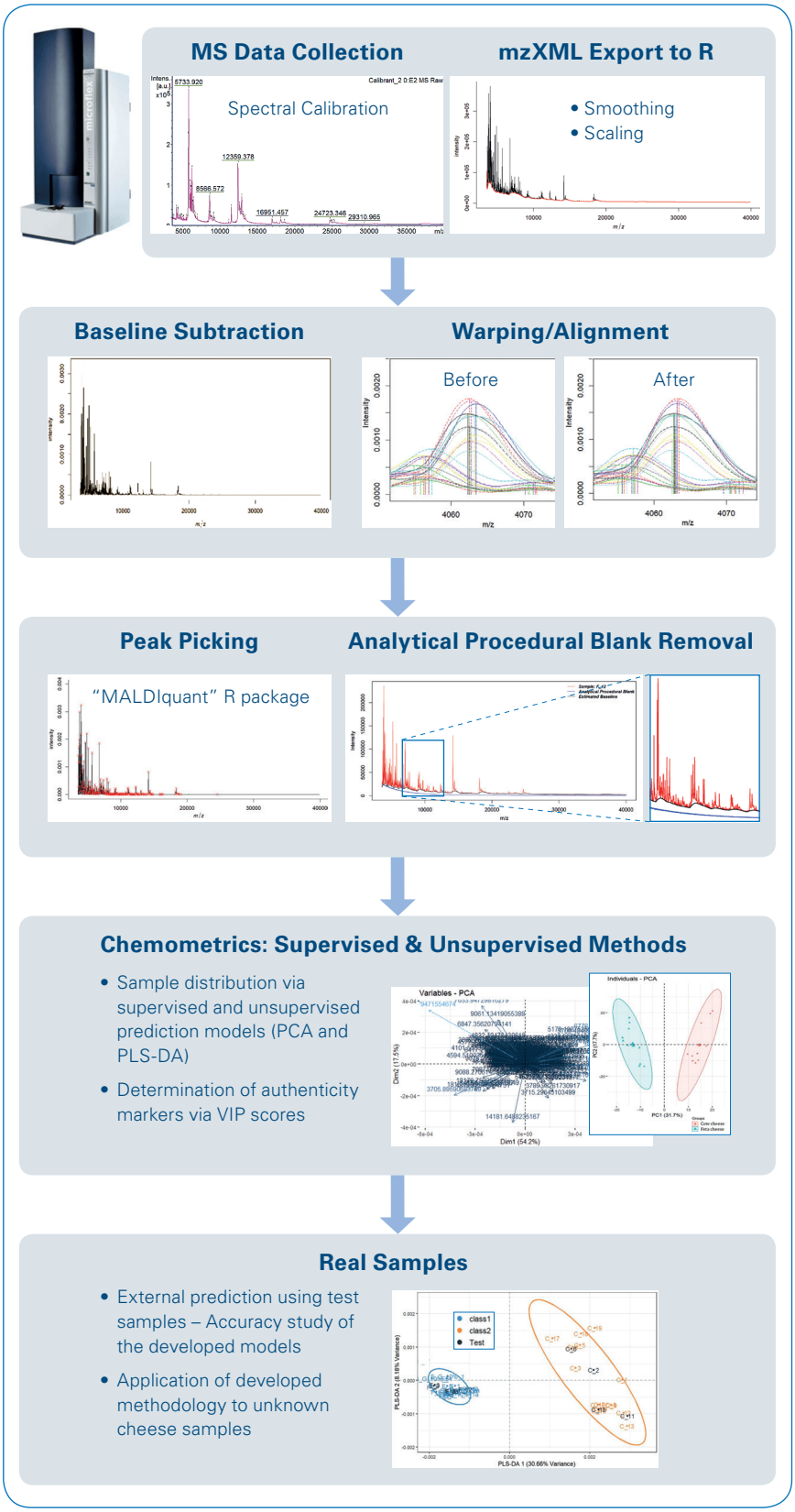

Figure 1: Schematic workflow for MALDI spectral collection, data treatment, and discriminatory analyses. MS data were collected on the microflex LRF. Raw MS spectra were calibrated and evaluated using flexAnalysis software. Data were exported as mzXML files and were imported to the R environment for data pre-processing (normalization, baseline subtraction, analytical procedural blank removal, etc.). The peak peaking process was performed using the MALDIquant R package.

Introduction

"Feta" is the most important semi-soft white Greek cheese, ripened in brine and traditionally made from sheep milk or a mixture of sheep and goat milk (the latter not to exceed 30%). Its unique sensory characteristics arise from several important factors, including the regional flora of the animals' diet and its traditional methods of production and maturation. Feta cheese has been produced since the 8th century B.C., and its integrity has been protected within the EU as a Protected Designation of Origin (PDO) product since 2002 [1].

In the dairy industry, using milk blends is not uncommon for the development of specific flavor, texture, or dietary profiles, or as a means to reduce raw material costs. Similar white brined cheeses, made in part or entirely from cow milk, are produced in many countries of the eastern Mediterranean Sea, and, under law, these products cannot be classified as feta cheese. The lower relative milk yield of ewes contributes to the premium cost of authentic feta cheese, and the adulteration of sheep milk with more abundant (lower cost) cow milk in cheeses labeled as feta is food fraud. Further, the presence of hidden milk allergens can risk consumer safety. Therefore, analytical methodologies aimed at the characterization of milk species and related dairy products are significant for authenticity control.

The continual growth and development of novel MS-based technologies and analytical tools are a valuable first line of defense for both detecting and deterring food adulteration, as well as supporting food safety. MALDI-TOF MS is a powerful analytical technique for food authenticity studies as it combines speed, reliability, and substrate flexibility with straightforward and user-friendly operation. The aim

of this study was to develop a rapid and reliable high-throughput MALDI-TOF MS strategy to discriminate between feta cheese and (similarly produced) white cheeses prepared only from cow milk. Moreover, a method based on derived protein content to identify feta cheese adulteration with low levels of cow milk was developed.

Materials and Methods

Reagents/Standards

Sinapinic acid (SA, # 1869451) and protein calibration standard (# 8206355) were obtained from Bruker and were prepared according to product information guidelines. All solvents were LC-MS grade: water (Millipore), ethanol (Fluka), acetonitrile (Sigma-Aldrich) and trifluoroacetic acid (TFA, Merck).

Cheese samples

48 cheese samples were collected from two cheese types, authentic feta (n=25) and similar white cheese prepared only from cow milk (n=23). Adulterated "feta" cheese samples (n=43), produced by the same manufacturers on request, contained seven different adulteration levels of cow milk (1%, 2%, 3%, 5%, 10%, 20%, 30%, 40% and 50%). Apart from the milk types and ratios used, standard commercial feta cheese production protocols were followed. Commercially available samples were also purchased and used for the applicability study of the method.

Preparation of cheese samples for MALDI-TOF analysis

All cheeses were prepared according to a simple methodology, based on an optimization of the method reported by Cozzolino et al. [2]. Briefly, cheese samples were diluted 10-fold with water containing 0.1% TFA. Following satisfactory homogenization using an overhead shaker, the samples were subjected to ultrasonic-assisted extraction for

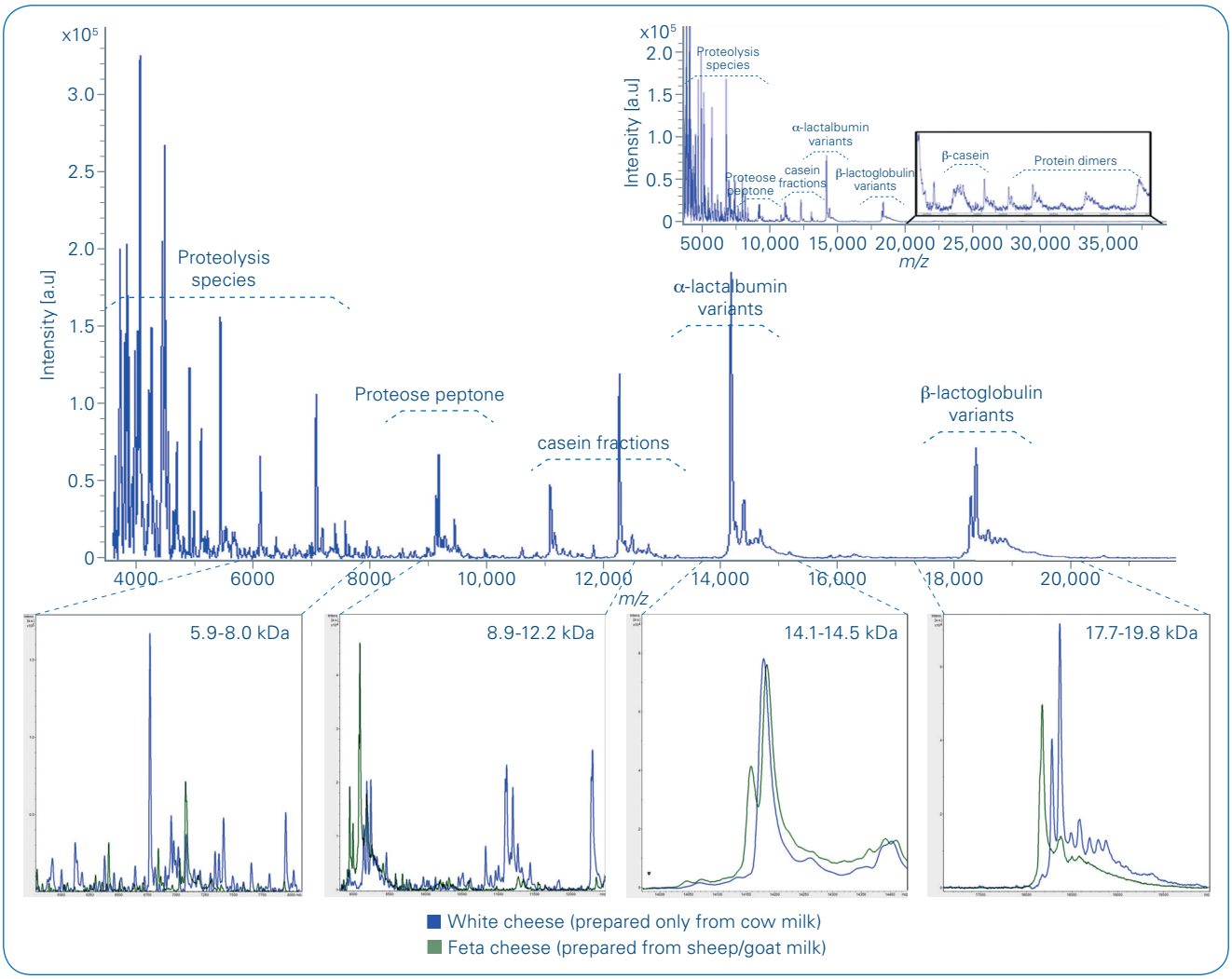

Figure 2: Typical MALDI mass spectra of white cheese prepared from cow milk. In the lower magnified figures, profile differences between white cheese (blue spectra) and feta cheese (green spectra) are shown as examples.

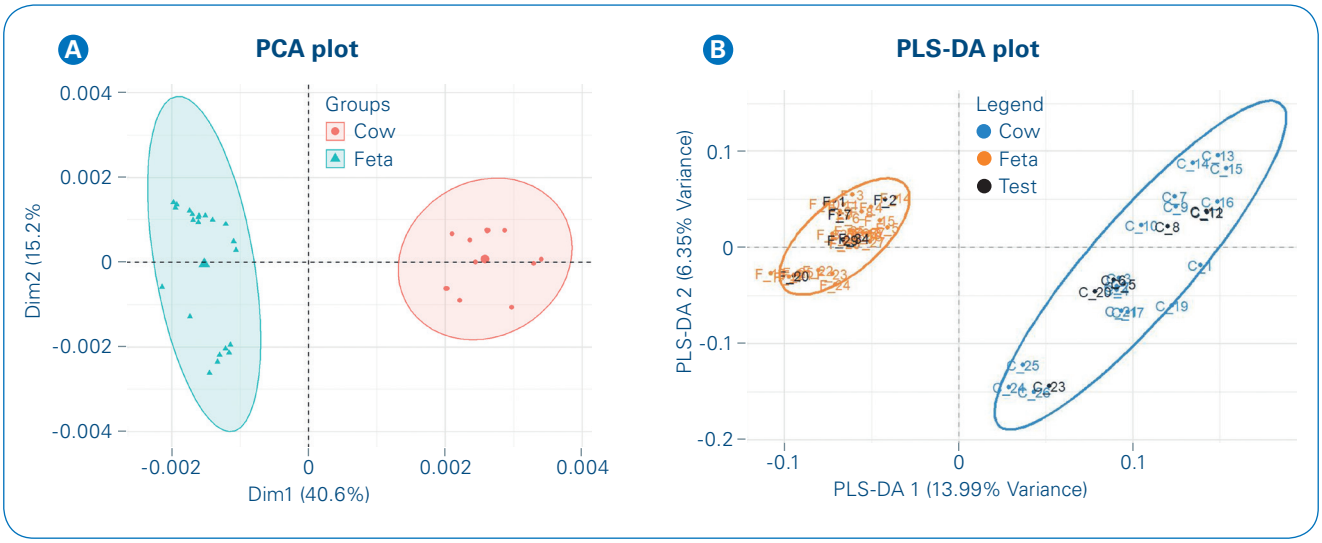

Figure 3: Discrimination of feta cheese and white (cow) cheese using PCA (Panel (A)) and PLS-DA prediction models (Panel (B)). Training and test samples in the PLS-DA prediction model are identified as feta (F) or cow (C). Optimization of latent variables and permutation tests for each model have also been studied [6, 7].

15 minutes at room temperature, followed by a five-fold dilution in the same solvent. All samples included in the study were prepared (extracted) in triplicate.

MALDI target preparation

A "double layer" approach was used for the spotting of prepared samples. Individual target positions on a ground steel target plate were covered with a thin layer of sinapinic acid prepared in ethanol and dried at room temperature. Cheese extracts were mixed in a 1:1 ratio with a saturated sinapinic acid solution (prepared in 30% acetonitrile, 0.1% TFA) and applied to the previously prepared sample positions. Each extracted sample was spotted in triplicate to confirm the reproducibility of the MALDI target preparation.

MALDI-TOF MS analysis

Proteomic profiles from all samples were collected using a benchtop microflex LRF model MALDI-TOF mass spectrometer (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) operated in linear detection mode with positive ion acquisition, with data collection from *m/z* 3.5-40 kDa. Mass spectra were automatically collected, summing 700 shots for each sample. Random walk mode (partial spot) was used, with 50 shots collected at each raster position. MALDI spectra were collected in triplicate for all tested cheese samples (white cheese, feta cheese, and adulterated cheese). Bruker "Protein Calibration Standard I" (including insulin, ubiquitin, myoglobin, and cytochrome c $[M+H]^+$ and $[M+2H]^{2+}$ ions, as well as the protein dimers) was used for external quadratic calibration.

Data treatment

Spectral data from MALDI-TOF MS were processed as shown in Figure 1. In brief, the collected MS data were calibrated using flexAnalysis (Bruker Daltonics). Raw data were exported as mzXML files and imported to the R environment. Spectral quality control was implemented, including evaluation of mass accuracy, homogeneity of the sample and the effect on the spectra, as well as a baseline comparison against the procedural blank. Sample spectra with acceptable profiles were subsequently normalized using unit variance and total ion chromatogram. This step was helpful to exclude experimental error, as was the calculation of the Signal-to-Noise Ratio. Peak smoothing was made using the Savitzky-Golay-Filter (half width size of 15 points, order of 3). The baseline of each spectrum was estimated locally and removed using the Sensitive Nonlinear Iterative Peak (SNIP) algorithm. A "grouping & alignment" step was performed to (re) calibrate, combine, and align similar *m/z* values. The "Non-linear Lowess" method was used with a half-width size of 15 points, as for the smoothing step. A mass accuracy tolerance of 0.03 Da and S/N ratio of 3 were used. Median Absolute Deviation "MAD" method and "detectPeaks" method from the MALDIquant R package [3] were used for peak detection. A custom wrapper function was then used for peak binning (mass accuracy tolerance of 0.03 Da and minFrequency=0.25) prior to peak list extraction.

Chemometric analyses

Using the extracted peak lists from authentic feta and white (cow) cheeses, an unsupervised PCA was built for these cheese types using *m/z* values with significant fold changes (between the two groups) to initially evaluate their distribution in PCs space. A pairwise PLS-DA model was further built between white and feta cheese samples to discriminate the two types of cheeses and study the class-regulated markers via variable importance in projection (VIP) values. A cut-off value of (greater than) 0.83 was used for the VIP score [5]. The PLS-DA model was validated internally and externally using a misclassification error rate (error rate in leave-one-out cross validation), k-fold-cross-validation (k=5), and a test set of 21 (unique) samples. In addition, Receiver Operating Characteristics (ROC) curves were plotted to evaluate specificity, sensitivity, and total accuracy. Statistical parameters were also calculated for the developed PLS-DA model, including R2X (measure of the accumulative variance explained by the selected number of latent variables), R2Y (measure of the goodness of fitted class), Q2 (k-fold-cross-validated, predictive ability of the PLS-DA model) as well as the square root of the mean error between the actual and the predicted classes for the training (RMSEE) and test (RMSEP) sets, respectively. A single PLS-DA model was likewise built for the detection of adulteration in feta cheese. The extracted peak lists from authentic feta, white (cow), and adulterated feta cheese samples were used as the data set. As with the pure feta and white cheese analyses, mass values were evaluated for their relative contribution to discriminate between sample types.

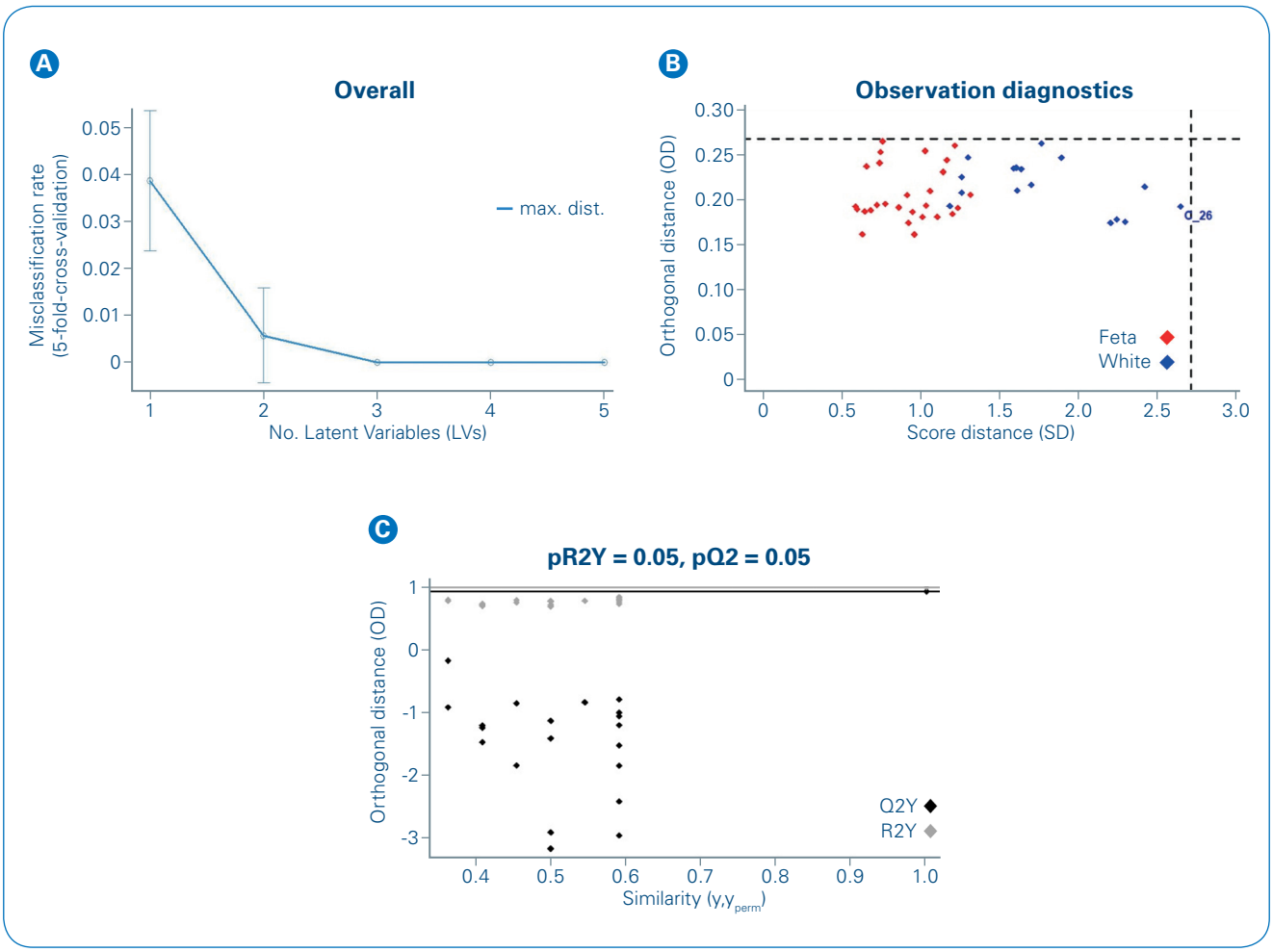

Figure 4: (A) Optimization of the number of latent variables in the PLS-DA model for white and feta cheeses; (B) outlier detection plot; (C) permutation test.

Results

Discrimination of feta cheese and white cheese

Protein components of feta cheese and other white cheeses (prepared only from cow milk) were detected by MALDI-TOF MS and putatively identified based on public protein molecular mass databases and previously published studies [5]. A typical MALDI mass spectrum of white cheese produced exclusively from cow milk is shown in Figure 2. Similar protein profiles are observed in the two types of cheeses, however, significant differences between feta and white cheese peak patterns are illustrated in example mass ranges (lower panel). More than 800 peaks were detected in the majority of samples. Many protein-related peaks were detected, including the major whey proteins (α-lactalbumin and β-lactoglobulin), casein fractions, and proteose peptone. The peaks detected at higher *m/z* values (*m/z* > 30,000) primarily represent protein multimers. Many peaks below *m/z* 9,000 are believed to be proteolysis species (of higher mass proteins) resulting from thermal, enzymatic, or temporal cheese processing factors.

In the PCA model constructed, two PCs (Principal Components) explain more than 40% variance, suggesting that any supervised methods based on the principal component concept are likely to be successful. Furthermore, using the generated MALDI-TOF MS profiles, feta cheese samples and white cheese samples (prepared only from cow milk) were fully discriminated with 100% classification accuracy using PLS-DA models (Figure 3). Test samples were successfully predicted and fully grouped with the training set. The PLS-DA model developed showed a very low misclassification error rate (RMSEE=0.053) and high discriminating power (Q2=0.920). Feta and cow cheeses could be discriminated from each other within a confidence interval of 95%. No substantial outliers were detected in either model (Figure 4). Only one cow cheese sample had a high(er) robust score distance, and its removal did not dramatically affect the PLS-DA model constructed.

Detection of adulteration

The PLS-DA model constructed using the MALDI-TOF MS peak list permitted the clear discrimination between white, feta, and adulterated feta cheese samples, with a low misclassification error rate (RMSEE = 0.121) and acceptable discriminating power (Q2 = 0.835) (Figure 5). All three groups were discriminated from each other within a 95% confidence interval. Details regarding the optimization of latent variables, outlier analysis, and permutation test have been previously described [6, 7]. All shuffled cases in the permutation test showed lower Q2 and R2Y values in comparison to the actual PLS-DA model. Significant discriminatory *m/z* values according to VIP values were noted (Figure 6). Each *m/z* value with a VIP score higher than 0.83 was evaluated for its contribution to the discrimination. Additionally, the marker candidates (for a specific type of cheese) were required to be present in at least 80% of the raw mass spectra belonging to the same group. The majority of the *m/z* values meeting these criteria were in the mass range of 4 kDa – 18 kDa. An example marker unique to authentic feta is shown in Figure 7.

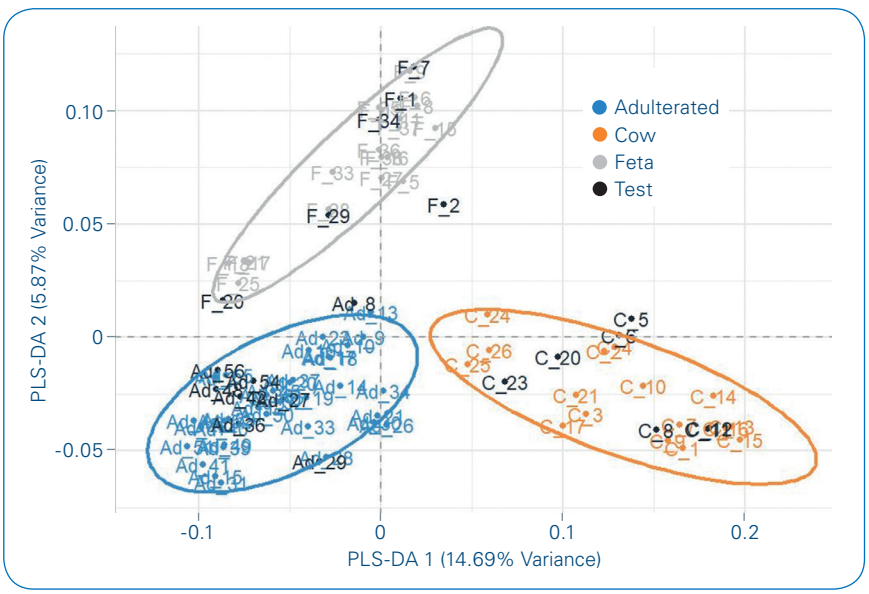

Figure 5: PLS-DA model for discriminating feta, white (cow), and adulterated feta cheese. As all samples shown, the test samples are identified as feta (F), cow (C), or adulterated (Ad).

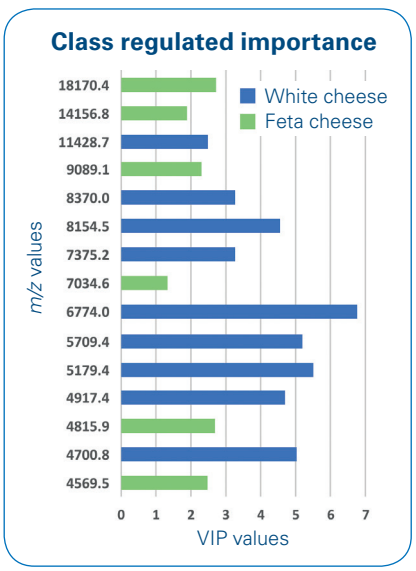

Figure 6: Significant class-regulated m/z *values, as exported from the PLS-DA model and VIP scores, for white and feta cheese discrimination.*

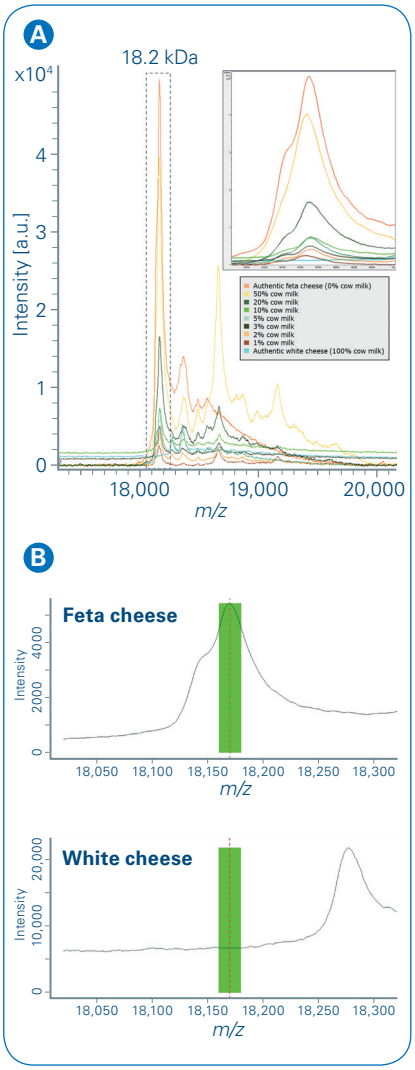

Figure 7: (A) Example discriminating peak (marker) distribution in feta and adulterated cheese samples, as seen within flexAnalysis. (B) Marker review following data processing. The red dotted line indicates the target m/z *value, and the green box the* m/z *tolerance for peak detection. The feta marker at* m/z *18170.4 (VIP 2.7532) is clearly absent in the white cheese sample.*

Discussion

Current EU regulations [8] for the detection of cow milk within cheese samples are based on the detection of bovine casein protein fragments. The method of reference is iso-electric focusing (IEF), where band patterns for target cheese samples are compared to standards containing 0 and 1% cow's milk. This method is considerably more time-consuming, more expensive (per sample), and more difficult to interpret than mass spectrometry-based methods. Specific bovine peptides have been targeted in tryptic-digest based MS/MS sample screenings [9-11], and MALDI-TOF MS profiling of phospholipids for detection of milk adulteration has also been reported [12]. The success of these experimental workflows supports the use of MALDI-TOF MS for analytical characterization of dairy products.

Our approach, with simple, straightforward sample preparation and automated analysis of the MALDI-TOF profiles, is rapid and robust. The reproducible detection of unique mass fingerprints and subsequent data treatment enables clear identification of adulterated feta cheese, even at very low adulteration levels, and in spite of potential profile complexities in commercial cheese samples due to the raw milk sources (e.g., different breeds of the same animal species, dietary differences based on local geography) and variations in processing and maturation conditions (e.g., temperature controls and the animal or vegetable rennet used). The developed workflow was also successfully applied in the detection of sheep yogurt adulteration with cow milk, paving the way to unravel new authenticity challenges. Further, the compact instrument footprint and ease of operation support its use as a routine methodology in the food and dairy industry. The integration of multiple protein and peptide signals supports high confidence detection of feta cheese adulteration and can serve as a valuable analytical tool to protect the integrity of this important national food product.

Conclusion

- MALDI-TOF technology and chemometrics have been successfully combined for the complete discrimination of feta cheese from similar white cheeses prepared from cow milk. Reliable detection of feta cheese adulteration was achieved down to 1% cow milk.
- Supervised statistical data analysis was used to detect potential species-specific markers to differentiate different milk species from the complex spectral features detected within each sample, as in non-targeted "omics" studies.
- The simplicity of sample preparation, the speed of data collection and analysis, and low per-sample costs support the use of MALDI-TOF MS for the detection of dairy product adulteration.

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