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ARTICLE

Cheese Lipid Profile using Direct Imprinting in Glass Surface Mass Spectrometry

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Cheeses have always been targeted for frauds. Among the most common is the adulteration of highly-priced milk (goat and sheep) with less valuable cow milk, which is sold as raw material for the cheese industry. The need for sensitive techniques to assess authenticity encourages the development and improvement of analytical methods. Herein we bring this approach employing Direct Imprinting in Glass Surface Mass Spectrometry (DIGS-MS) for qualitative cheese analysis in a MALDI instrument, featuring easy sample preparation and fast data acquisition/interpretation. It is possible to readily identify complex lipids in different types of cheese, associating them as potential quality markers. Controlling productive stages may also be feasible using this technology by integrating analytical and statistical data, resulting in a powerful combination for sample discrimination based on lipid profiles. This approach presented great signal cleanliness in mass spectra, with easy data workup and interpretation.

Either for economic, ethical or public health reasons it is very important to detect the existence of lower quality and fraudulently labeled products on the market. The adulteration of dairy products is relatively frequent and diverse [1]. It is not unusual to find adulterations in the mixture of valuable – and highly priced – milk (goat or sheep) with lower value milk (cow) [2, 3]. This malpractice is usually performed in products destined for direct consumption or as cheese industry raw material. Fluctuations in the availability of goat and sheep milk and their higher price when compared to cow milk are the main causes for this adulteration, in a very profitable strategy [4]. In this way, determining if and how the raw material has been adulterated has great relevance, not only to ensure the genuineness of cheese with denomination of origin and of cheese manufactured with pure milk, but also to avoid the threat for consumers. Cow milk is known for its allergenic potential to a large number of people from the most diverse populations, especially due to the presence of proteins such as α1-casein and β-lactoglobulin, causing numerous effects on people's health [5]. There is no doubt that the use of mass spectrometry in food science and adulteration findings is one of the most suitable and successful combinations, as described in some previous works [6-10]. Numerous analytical techniques have been developed over the years

to assess authenticity of dairy products made from different milk species. However, most of them show some difficulties, either in terms of sample preparation, price, time, or availability [11-18]. The relevant need for sensitive techniques to assess authenticity of these products has thus encouraged the development and improvement of many analytical methods; despite that, it is still necessary to develop new techniques for faster analysis and enhanced results visualization. This background brings us to understand that developing improved methodologies must be based on the ease of sample preparation and readiness for data acquisition/interpretation. Methodologies have been widely developed with this mindset; a great approach previously developed describes a method that uses desorption/ionization on silicon mass spectrometry (DIOS-MS), in which a porous silicon (PSi) surface is prepared in plates by electrochemical etching and is very suitable for direct analysis of samples [19]. Despite the analysis being similar to MALDI-MS in its concept for laser desorption/ionization, it requires no matrix application, and has been applied for a wide range of molecules, from proteins to low-molecular weight species. And although it shows much improvement, some specific plate preparations are still required [20].

Herein, we propose a new and even simpler variation of this already simple technique: laser desorption/ionization using direct imprinting in glass surface mass spectrometry (DIGS-MS) in a MALDI instrument. This methodology uses no matrix in the process and requires minimal sample preparation, following a recent trend in our group regarding direct analysis of complex samples [21]. As seen in the results, it ensures great signal cleanliness in mass spectra, as well as assertive and effective data processing, proving to be one very viable alternative for food analysis and determination of authenticity/adulterations.

Materials and Methods

Sample Preparation. Five different fresh cheese samples were elected for the experiments (n=10/group, 5 different batches for each): cow, sheep, goat, buffalo and mixed cheese (composed of cow, goat and sheep milk, in the proportions of 80, 10 and 10%, respectively), purchased from local grocery stores. To ensure that the cheeses were not adulterated, all purchased samples belonged to the

same manufacturer, which had certified raw materials, production and products by the competent regulatory agencies. The sample preparation was inspired by the DIOS method reported by Wei *et al.* [22]. Our approach has employed a glass plate, suitable for optical microscopy, with a porous surface (Corning Glass Works, Corning, NY, USA), which was previously sonicated in a 50:50 solution of acetonitrile:methanol (J. T. Baker, Xalostoc, Mexico) so that no potential residues were adsorbed onto the surface. Pieces of the central part of each cheese were pressed against the plate and then removed right away, just like a stamp, resulting in a very thinly imprinted layer of lipids, with suitable width and distribution for direct analysis. The glass plate was then embedded on a regular MALDI plate and inserted in the equipment, without any further steps.

Mass Spectrometry. A MALDI-LTQ-XL (Thermo Fisher, California, USA) was used to acquire mass spectrometric data. The operation conditions were: 20 μJ Nitrogen laser power, 100 μm raster step size, sample size of 2000×2000 μm, three laser shots per step and normalized collision energy set at 50-70 for collision-induced dissociation (CID) in MS/MS experiments. Survey scan analyses (n = 10 for each cheese class) were performed in m/z range of 600 to 950 (positive ion mode). The compound classes were proposed using the obtained MS/MS data and supported by software calculations with Mass Frontier (v. 6.0, Thermo Scientific, California, USA) as well as literature [23-25] and lipid database information (Lipid Maps – University of California, San Diego, CA – www.lipidmaps.org).

MS data. Principal Component Analysis (PCA) was performed on data using Statistica v.7 (Statsoft Inc., Oklahoma, USA). Information from the mass spectra was extracted in tables with *m/z* ratios and their respective relative intensities. Ions with relative signal-to-noise ratios lower than 3:1 were excluded. The data was preprocessed using auto scale and the PCA method was then run.

Results and Discussion

Preliminary tests were conducted analyzing the plate with no sample added to assess background level and efficiency of the cleaning procedure. Since no spectra or signals were obtained in this phase, the plates were considered to be clear enough and hence suitable for experiments and data collection. Full scan in positive mode of each cheese was performed to identify larger mass compounds, such as triacylglycerols (TAGs) and phospholipids. Some recent works have reported that laser desorption/ionization of phospholipids may happen at the expense of triacylglycerols and can be substantially affected, particularly by the MALDI matrix used [26]. Nonetheless, previous works have reported that lipid fingerprinting in bovine milk samples is feasible to achieve with MALDI-TOF approaches, especially for the TAG content in these samples [27, 28]. The present work was performed without matrix application, therefore eliminating potential interferences from the matrix in the onset and resolution of signals. Figure 1 shows the sample spectra of (A) buffalo cheese; (B) mixed cheese; (C) goat cheese; (D) sheep cheese; (E) cow cheese.

Figure 1. Sample spectra of (A) Buffalo's cheese; (B) Mixed cheese; (C) Goat's cheese; (D) Sheep's cheese; (E) Cow's cheese. Data were acquired in the positive ion mode at the m/z range of 600 – 950.

Multivariate data analysis (PCA) was performed using all cheese samples analyzed (n = 10 for each group), with results portrayed in Figure 2. Although these types of milk present a very

similar lipid profile, as indicated by spectra from Figure 1, PCA was capable of differentiating four cheese classes, which are clearly separated. Cow and sheep samples presented very close similarities, and were therefore not separated with PCA.

Figure 2. Score plots of PCA for cheese samples: (■) Buffalo's cheese; (●) Mixed cheese; (♦) Goat's cheese; (▲) Sheep's cheese; (+) Cow's cheese.

Table 1 reports the assignments for some of the characteristic ions of each sample class. These results are based on the MS/MS spectra obtained through CID reactions and their comparison to characteristic fragmentations predicted by calculations performed with Mass Frontier software. The proposed structures, however, cannot be assigned to a specific molecule, since these *m/z* ratios cannot be assigned to a single structure, as most of these markers show position isomers within the same lipid class. It was possible to observe that goat and mixed cheeses have presented phospholipids as the characteristic lipid markers that helped differentiating from the other classes. On the other hand, TAGs were the main observed markers for cow, sheep and buffalo samples.

Table 1. Specific lipid markers for each cheese as indicated by PCA and MS/MS data.

The main feature of DIGS-MS is to use a treated porous glass plate as the main support for lipid trapping and extraction. By applying ("stamping") samples directly onto the surface of the glass plate with no matrix application, we were able to obtain high quality spectra, with clean signal. This work has demonstrated to readily identify compounds of interest by integrating both full scan (Figure 1) and MS/MS data, with no further actions regarding sample preparation neither matrix application. This approach can have its use further expanded for fast fingerprint of complex lipids, such as triacylglycerols and phosphoglycerols, for rapid lipid evaluation in dairy products, characterization of their compounds and adulteration findings.

Conclusions

We were able not only to identify complex lipids in cheese with direct mass spectrometric analysis, but also to differentiate all samples based on their lipid profiles. Cheese samples, namely cow, goat, sheep, buffalo and mixed cheese presented characteristic molecules within their lipid profiles, proving that our approach can be an important tool for industry to launch products of higher quality and for laboratories and governmental agencies to meet the needs for safety of consumers. Moreover, the absence of matrix may result in signal cleanliness in all dynamic mass ranges, including for free fatty acids and other low-mass molecules that would suffer signal suppression caused by the matrix effect.

Notes

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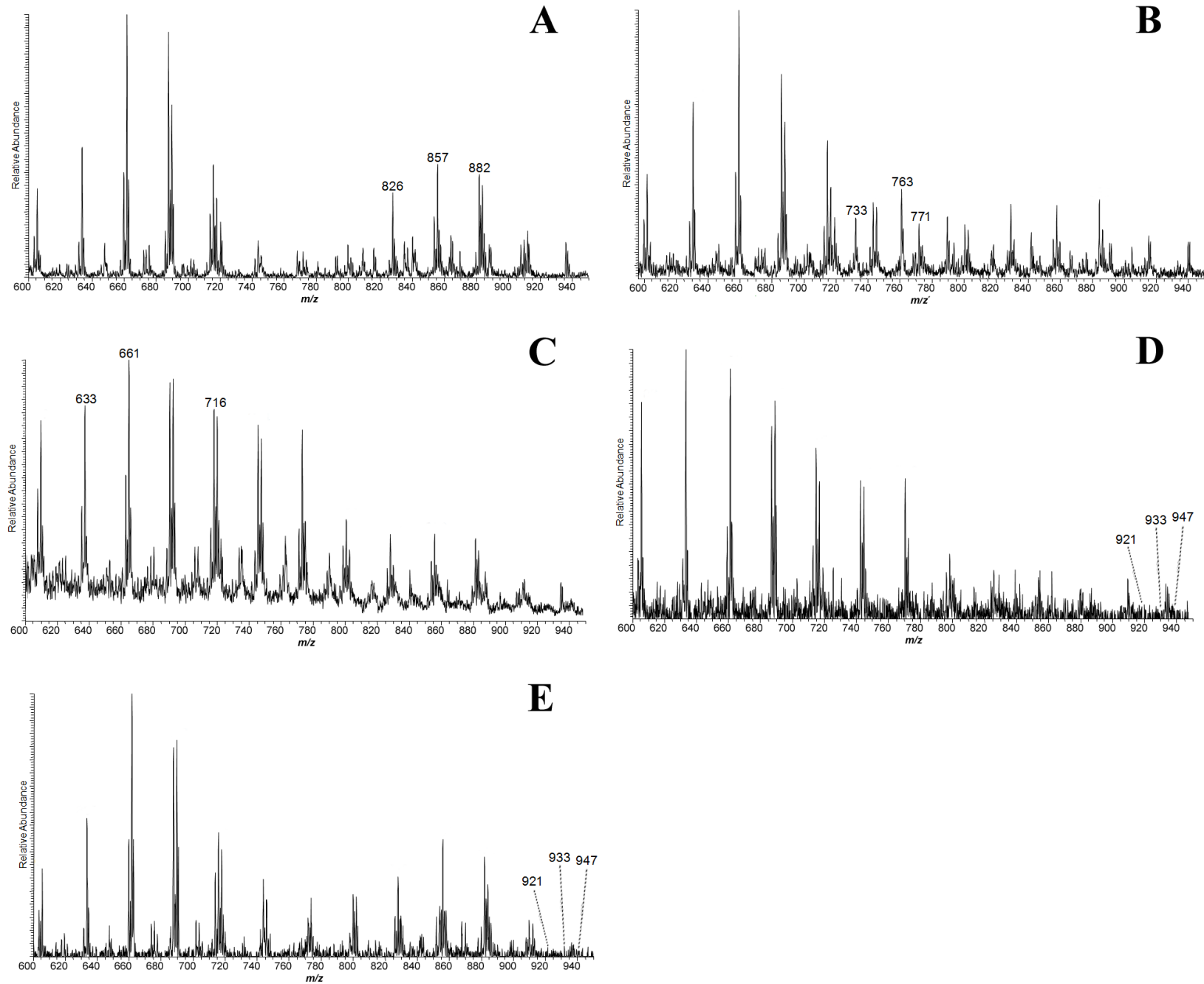28. G. Picariello, R. Sachhi, F. Addeo, Anal Chem, 2010, 82, 5783-5791.

Figure 1. Sample spectra of (A) Buffalo's cheese; (B) Mixed cheese; (C) Goat's cheese; (D) Sheep's cheese; (E) Cow's cheese. Data were acquired in the positive ion mode at the m/z range of 600 – 950.
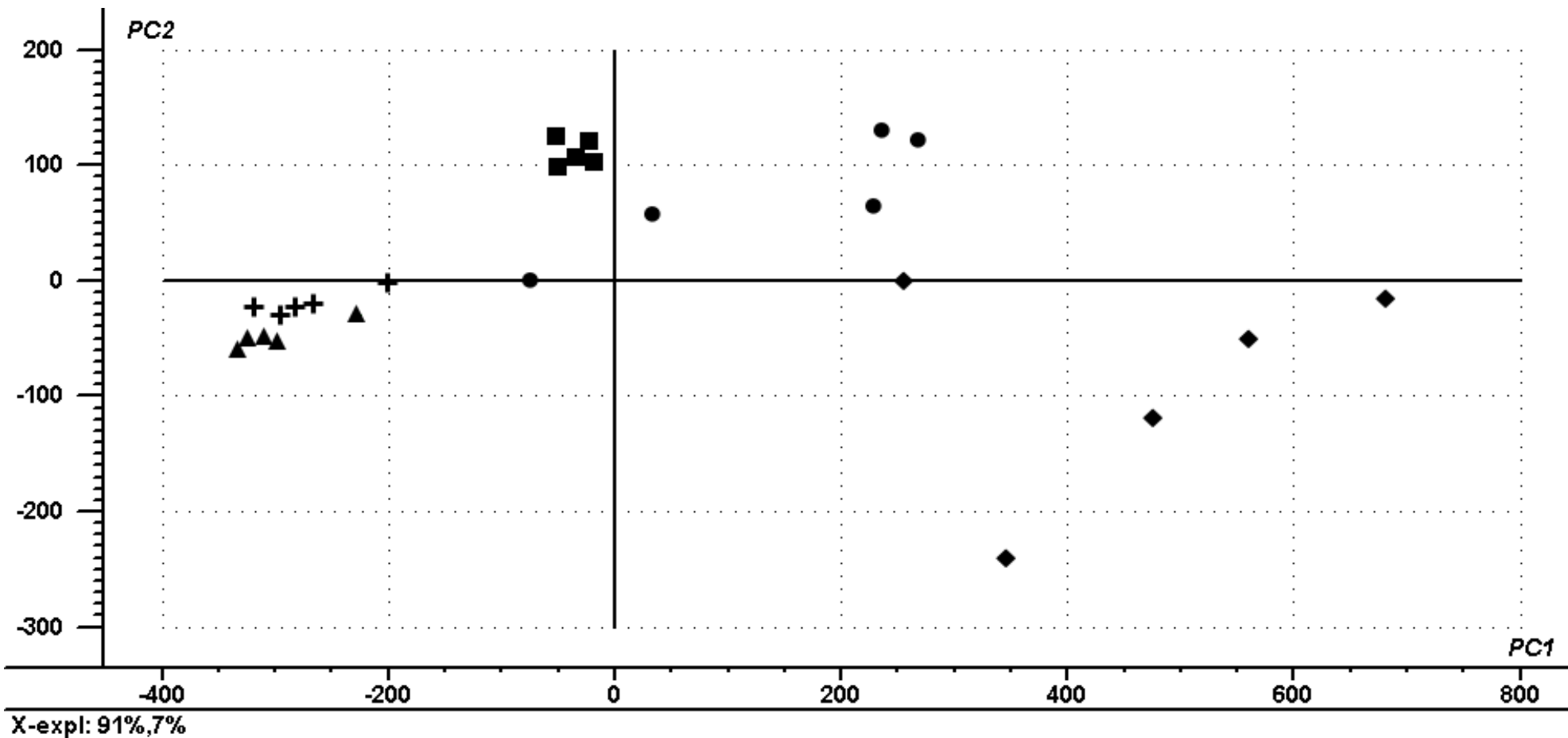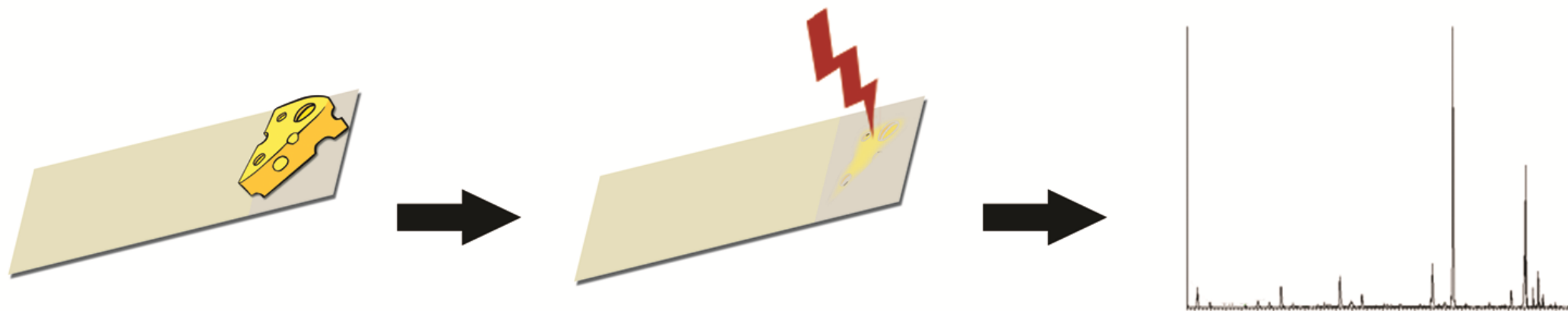
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Figure 2. Score plots of PCA for cheese samples: (■) Buffalo's cheese; (●) Mixed cheese; (♦) Goat's cheese; (▲) Sheep's cheese; (+) Cow's cheese.

Table 1 - Specific lipid markers for each cheese as indicated by PCA and MS/MS data.

Sample	[M+H$^+$] m/z	Lipid Class	Structural Formula	CN:DB [a]	CID Fragmentation
Cow /	921	Triacylglycerol	$C_{60}H_{104}O_6$	57:6	897, 702, 657, 511
Sheep	933	Triacylglycerol	$C_{61}H_{104}O_6$	58:7	913, 855, 782, 623
	947	Triacylglycerol	$C_{61}H_{118}O_6$	58:0	901, 800, 372, 887
	633	Diacylglycerophosphate	$C_{35}H_{69}O_7P$	32:1	437, 323
Goat	661	Phosphatidylethanolamine	$C_{35}H_{68}NO_8P$	30:1	434, 388, 643, 275
	716	Phosphatidylethanolamine	$C_{40}H_{81}N_2O_6P$	38:1	434, 351, 320, 515
	733	Glycerophosphoglycerol	$C_{39}H_{73}O_{10}P$	33:2	599, 716, 508, 480
Mixed	763	Phosphatidylcholine	$C_{42}H_{84}NO_8P$	34:0	720, 537, 509, 381
	771	Glycerophosphoglycerol	$C_{42}H_{75}O_{10}P$	36:4	755, 555, 432, 386
	826	Phosphatidylcholine	$C_{48}H_{92}NO_7P$	40:3	647, 543, 310
Buffalo	857	Triacylglycerol	$C_{55}H_{100}O_6$	52:3	790, 599, 549
	881	Triacylglycerol	$C_{57}H_{100}O_6$	54:5	824, 792, 625

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Direct sample imprinting in porous glass plate allows fast mass spectrometric profiling of lipids.