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Untargeted lipidomics by LC-QTOF-MS for biomarker selection and discrimination of plant- and animal-derived rennet in cooked-curd ovine cheese at different ripening stages

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ABSTRACT

In this study, the lipidomic alterations in cooked-curd ovine cheese were comprehensively assessed by comparing samples produced using either vegetal (*Cynara cardunculus* L.) or animal (calf) rennet. Lipid extracts were analyzed using high-resolution liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), and the resulting data were processed through multivariate statistical analyses to explore compositional changes across 18 months of ripening. The use of cardoon rennet, led to distinct modifications in the lipid profile compared to conventional calf rennet. A broad range of lipid classes, including phosphatidylserines, triacylglycerols, free fatty acids, phosphatidylcholines, ceramides, sphingomyelins, phosphatidylethanolamines, and monoacylglycerols, were significantly affected by the type of rennet employed. In contrast, the ripening process mainly influenced the abundance and composition of free fatty acids, reflecting ongoing lipolysis and lipid remodeling.

To enhance classification and biomarker discovery, a forward stepwise interval partial least squares discriminant analysis (PLS-DA) was applied, enabling the identification of two optimal feature subsets for discriminating samples based on rennet type and ripening stage. The resulting models achieved high classification accuracies of 88 % for rennet type (cardoon vs. calf) and 91 % for ripening time (24 h, 12 months, 18 months), underscoring the strong lipidomic signature linked to both technological variables.

These results allowed to better understand how the type of rennet and the ripening process can change the composition of lipids in cheese. This knowledge can be useful for improving product quality, ensuring authenticity, and developing new types of traditional cheeses with specific features.

1. Introduction

Cheese is a fermented dairy product with a complex food matrix that reflects the nature of its production recipe (Turri et al., 2021). Cheese final quality is known to be highly sensitive to renneting and ripening conditions, as they strongly affect the cheese yield, texture and flavor (Khattab et al., 2019; Liu et al., 2021). After the milk pretreatment steps, renneting is the first stage of cheesemaking process (Dalgleish, 1993). In this step, the enzymes added through the rennet promote the cleavage of phenylalanine-methionine bond of κ-casein, followed by the destabilization of milk colloid and the subsequent aggregation of casein micelles, which results in milk gelation (Cheryan et al., 1975). After undergoing additional steps, such as pressing and salting, the cheesemaking process is then completed with ripening, which contributes to the achievement of the product's desired properties through the evolution of a complex set of physical, chemical and microbiological phenomena (Sanjuán et al., 2002).

Historically, the most used rennet enzyme is calf rennet, derived from the extract of the suckling-calf abomasa (Andrén, 2021). However, the cheesemaking industry is interested in exploring alternatives to

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animal-derived milk coagulants such as microbial and plant-based rennet, owing to their limited availability, escalating price and concerns related to dietary preferences, including vegetarianism, and religious beliefs (Liu et al., 2021; Shah et al., 2014). Each of these alternatives confer unique sensory properties to the cheese, enhancing both the diversity and quality of products that can be introduced into the emerging markets. To this concern, in the last decade, extracts of *Cynara Cardunculus* plant have achieved an increasing interest in several industrial frameworks, such as cheesemaking, due to its wide availability and multifunctional properties (Gominho et al., 2011). *Cynara Cardunculus* is a herbaceous perennial diploid plant from which artichoke and cardoon are cultivated (Conceição et al., 2018; Timón et al., 2019).

To date, several works studied the different effects induced by vegetable rennet (such as *Cynara Cardunculus*) and animal rennet on the final product's quality, and elucidated their intricate behavior within different cheese ripening durations (Pacifico et al., 2024; Prieto et al., 2004; Rampanti et al., 2023; Sanjuán et al., 2002; Şengül et al., 2014). The cited studies contributed to defining the physical-chemical, proteolytic and proteomic characteristics of vegetable rennet-based cheese. This focus is attributed to the pivotal role of casein proteins, which are the main substrates in renneting processes. While rennet enzymes are primarily recognized for their proteolytic activity in cleaving milk caseins during cheesemaking, they can also include lipolytic enzymes, such as triacylglycerol lipase, capable of releasing free fatty acids throughout ripening (Lambré et al., 2021). Consequently, measurable lipolytic activity may be observed even in the absence of additional lipases.

As an example, one study examined the fluctuations in the concentrations of short, medium, and long-chain fatty acids across three different lamb rennets employed in the production of Pecorino Romano Cheese (Addis et al., 2005). Similarly, Santillo et al. examined the probiotic lipolytic activity contained in the same rennet paste for quantifying fatty acids increasing during ripening, within the same type cheese (Santillo et al., 2009).

Consequently, there is a significant gap in a broader characterization of the lipidic profiles of cheeses produced using vegetable rennet, with particular regard to higher molecular weight compounds such as sphingolipids, phospholipids and triacylglycerols. This lack extends to understanding the differences between vegetable-rennet cheeses and animal-rennet ones. Moreover, it is widely recognized that extracts of *Cynara Cardunculus* are affected by high variability of the biochemical profile (Conceição et al., 2018; Gomes et al., 2019), which can significantly slow down its introduction in the dairy industry. A broader knowledge of vegetable rennet-based cheese metabolomics can provide a powerful benchmark for enhancing and standardizing the final product's quality. Such metabolomic extension opens up the possibility to apply chemometric analysis tools for developing more reliable classification models for detecting different renneting technologies that can be employed in the industrial dairy panorama.

The present work proposes a lipidomic study with the aim of extending the current set of biomarkers for the recognition of different types of rennet during cheese ripening. Specifically, the content of fatty acids, triglycerides, sphingolipids and phospholipids obtained through LC-QTOF-MS measurements was coupled with a chemometric approach to study the lipidic profile of cooked-curd ovine cheeses coagulated through a vegetable enzyme extracted from the *Cynara Cardunculus* plant and the one obtained from calf abomasum. Different cheese ripening times were investigated for both the formulations. The application of a robust classification model for discriminating samples belonging to different classes facilitated the identification of lipidic features that exhibited significant variations across the investigated treatments.

2. Methodology

2.1. Dataset description

The metabolic dataset includes 18 cheese samples and 1018 LC-QTOF-MS features, combining 738 from the positive ion mode and 280 from the negative ion mode. The data matrix included two classes based on the rennet type used in cheese production: animal rennet and vegetal-rennet coagulated cheeses, each comprising nine samples. Within each rennet type category, the samples are further subdivided based on ripening time, with three samples belonging to each of the following ripening periods: 24 h, 12 months, and 18 months.

2.2. Cheese production

The cheese samples were obtained from three experimental cheesemaking trials carried out on three different days in a dairy farm located in Sardinia (Italy) according to the cheesemaking technology for hard, cooked-curd sheep's milk cheese. Every cheesemaking was performed using bulk sheep's milk, which was first divided into two aliquots, thermized (68 °C x 120 s) and then cooled to 37–38 °C. Subsequently, a thermophilic starter culture (CHOOZIT® SU CASU, Danisco, Copenaghen, Denmark) and a secondary support culture (CHOOZIT® FLAV 54, Danisco, Copenaghen, Denmark) were added to the milk. One aliquot of milk was coagulated using a commercial liquid calf rennet (Caglificio Manca, Thiesi, Italy; chymosin 75 %, pepsin 25 %; 150 IMCU (International Milk Clotting Unit) cm^{-3}) at a dosage of 35 cm^3 100 L–1 milk while the other one was coagulated with the commercial liquid vegetable rennet Gallium® containing plant enzymes extracted from *Cynara cardunculus* (Laboratorio Prodor, Bobbio, Italy; 55 IMCU cm^{-3}) at a dosage of 100 cm^3 100 L–1 milk. The dosage of each rennet was selected so as to standardize the clotting time to approximately 10 min. After an additional 15-min period necessary to allow curd firming, the curd was subsequently subjected to further mechanical and physical processing according to the diagram reported in Fig. 1. The cheeses wheels obtained for each cheesemaking process were sampled one day from production and after a ripening period of 12 months and 18 months under controlled conditions (10–12 °C and 78–85 % relative humidity); in particular, two opposite wedges weighing a total of approximately 1.5 kg (10 % of the entire wheel) were taken from each wheel of cheese weighing 15–16 kg. The wedges were then ground, homogenised and stored at −20 °C until further analysis. Further details about the production process are reported in a previous work (Pes et al., 2014).

2.3. Sample preparation and lipidomic analysis

Lipid fractions from animal-rennet and vegetal-rennet coagulated cheese samples were extracted following a modified Folch method (Folch et al., 1957). Briefly, 10 mg of each sample were mixed with 250 μL of methanol and 125 μL of chloroform, followed by vortexing at 15-min intervals for 1 h. An additional 350 μL of chloroform and 150 μL of methanol were then added, and the mixture was incubated for a further hour. After centrifugation at 17,700×*g* for 10 min, the resulting organic (400 μL) layer was carefully collected into separate glass vials and evaporated under a gentle nitrogen stream.

The dried lipid extract was reconstituted in 20 μL of a methanol: chloroform solution (1:1, *v/v*) and diluted with 800 μL of isopropanol: acetonitrile:water (2:1:1, *v/v*) for analysis by UHPLC-QTOF-MS in positive and negative ionization mode. The analysis of the lipid extract was carried out using a 6560 LC-QTOF-MS system coupled to an Agilent 1290 Infinity II UHPLC platform. For positive ionization mode, 2.0 μL of each sample was injected onto a Kinetex C18 column (1.6 μm, 100 mm × 2.1 mm; Phenomenex, Bologna, Italy), while 10 μL were injected for negative ionization mode. Chromatographic separation was performed at 50 °C, with a constant flow rate of 0.5 cm^3/min.

The mobile phase composition differs by ionization mode. In positive

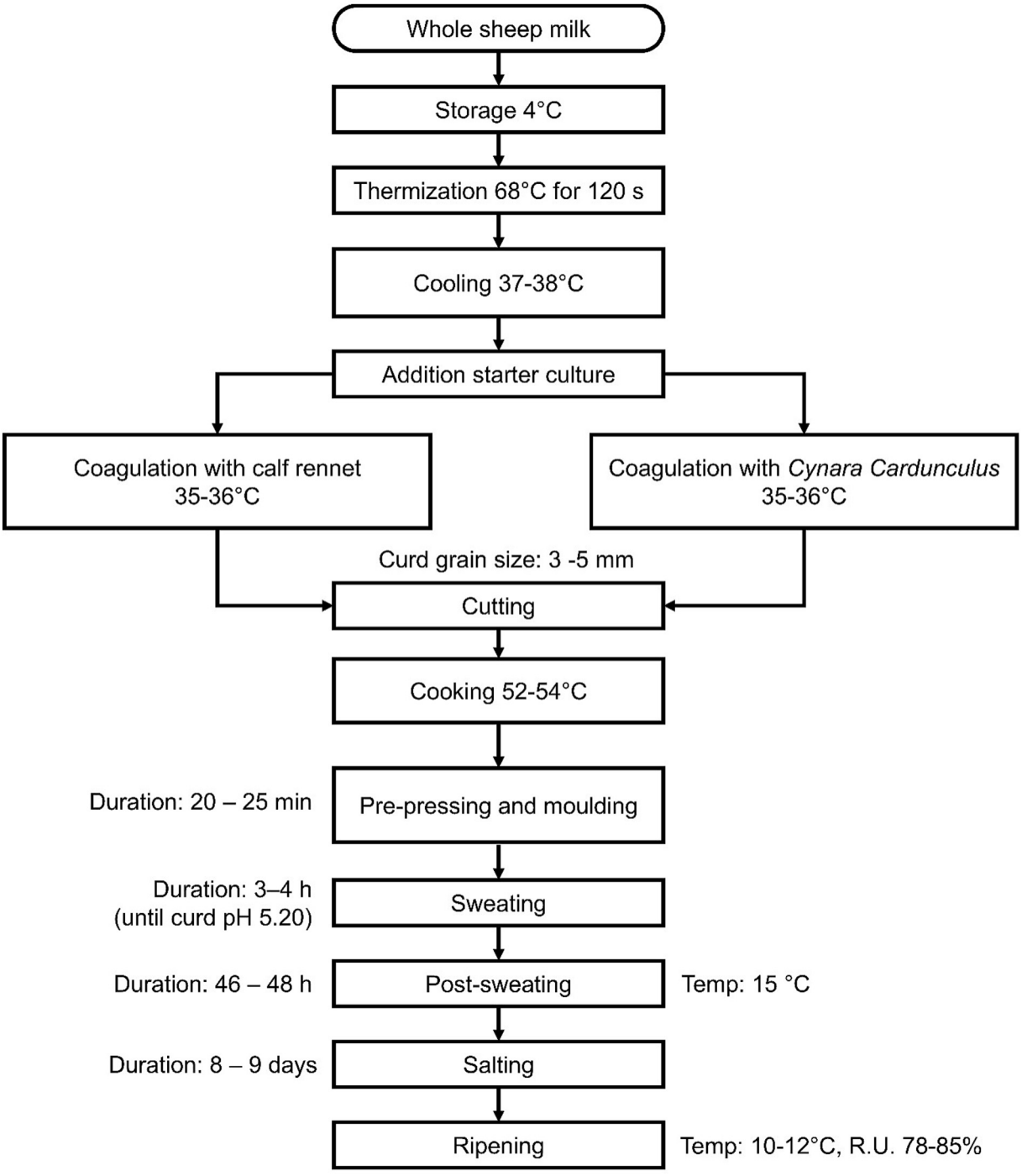

Fig. 1. Flowchart of the Cheesemaking process. There is an error in the humidity unit (78–85 %).

mode, eluent A consisted of 10 mM ammonium formate in 60 % Milli-Q water/40 % acetonitrile, while eluent B was 90 % isopropanol/10 % acetonitrile (9:1) containing 10 mM ammonium formate. For negative ionization mode, 10 mM ammonium acetate replaced ammonium formate in both phases. The elution gradient began with 60 % A, decreased to 50 % at 2.1 min, then to 30 % by 10 min, and further reduced to 1 % A, maintained for 1.9 min, before re-equilibrating to initial conditions within 1 min.

Mass spectrometric detection employed an Agilent Jet Stream ESI source, operating in both ionization modes. Source conditions were as follows: gas temperature at 250 °C, drying gas flow at 5 L/min, nebulizer pressure at 20 psig, sheath gas temperature at 275 °C, and sheath gas flow at 12 L/min. The capillary voltage was set to +4000 V (positive mode) and − 3000 V (negative mode); nozzle voltage was 500 V, fragmentor voltage 400 V, skimmer 65 V, and octopole RF 750 V. The mass range acquired was m/z 50–1700, with a collision energy of 20 eV in positive mode and 25 eV in negative mode, allowing for up to 3 precursors per MS/MS cycle.

Instrument calibration was performed prior to analysis using a standard Agilent tuning mix (m/z 50–1700). A reference mass solution was continuously infused throughout the run for real-time mass axis correction. Data acquisition was performed using the Agilent MassHunter LC/MS Acquisition software, and the results were processed with the Agilent Lipid Annotator software (Agilent Technologies, Santa Clara,

USA) as part of a comprehensive lipidomics workflow.

Data acquired with LC-QTOF-MS in both, positive and negative ionization mode, were pre-processed with the software MassHunter Workstation suite (Agilent Technologies, Santa Clara, USA). This software (Mass Profinder 10.0) allowed to perform Time Alignment and deconvolution of signals removing the background noise and unrelated ions through the tool called Recursive Feature Extraction (RFE), yielding a matrix containing all the features present across all samples. With the aim to minimize the observed instrumental variation that can affect the detection of the variation between the different class of samples and to eliminate non-specific information, the quality assurance was performed on the matrix. Quality assurance of the LC–MS dataset was ensured through calculation of relative standard deviation (RSD%) across technical replicates, confirming analytical reproducibility. Moreover, blank samples were analyzed, and background signals were subtracted to minimize matrix interferences. Quality control injections were included throughout the run to monitor instrument stability. Only features meeting these criteria were retained for downstream data analysis, obtaining a data matrix of 738 features for the positive ion mode and 280 features for the negative ion mode. These filtered matrices were combined and then subjected to statistical analysis. Discriminant metabolites were annotated using iterative MS/MS acquisition, which provided characteristic fragmentation spectra at a collision energy (CE) of 20 eV. When feasible, annotations were further supported by comparison with analytical standards. Representative LC-QTOF-MS results and feature distribution for cooked-curd ovine cheese samples are reported.

2.4. Statistical analysis

A Two-way ANalysis Of VAriance (ANOVA) test was conducted on raw LC-QTOF-MS data to assess whether the investigated factors and their interaction have a significant effect on each variable. After conducting the univariate test, two sets of *p*-values (one set for each main factor) were arranged in ascending order. Each p-value denotes the significance of the factor's effect on every individual feature. Univariate tests such as two-way ANOVA, though useful for preliminary feature screening, may yield high false negative rates after multiple testing correction. To account for the multivariate structure of the data, an iterative forward variable selection approach was implemented, initialized with the top-ranked ANOVA features and followed by PLS-DA. Briefly, the algorithm is initialized by collecting a subset of variables from the original matrix, comprising the first two variables with the lowest p-values determined by two-way ANOVA. PLS-DA was executed on unity variance preprocessed data subset. This iterative procedure involved a progressive addition of each variable and validation of the subset-based model through a Montecarlo cross-validation with 1000 simulations (Xu & Liang, 2001). For each subset, the optimal number of Latent Variables (LV) was determined based on achieving the highest accuracy. After examining the entire feature space, the model with the highest accuracy was chosen as the best-performing one. In each Monte Carlo simulation, 20 % of the dataset for validation and the remaining for calibration. This procedure refers to a forward selection interval-PLS-DA algorithm, wherein variables are sequentially added to an initially low-feature subset candidate until the assessment of the overall feature space (Nørgaard et al., 2000). The variables subset which corresponded to the best discrimination accuracy was selected as the biomarkers set for the specific factor (i.e. rennet type and ripening time). Data analyses were carried out in the MATLAB® environment. PLS-DA was performed using the Classification Toolbox 6.0 from Milano Chemometrics (Ballabio & Consonni, 2013). Fig. 2 shows a scheme of the implemented algorithm.

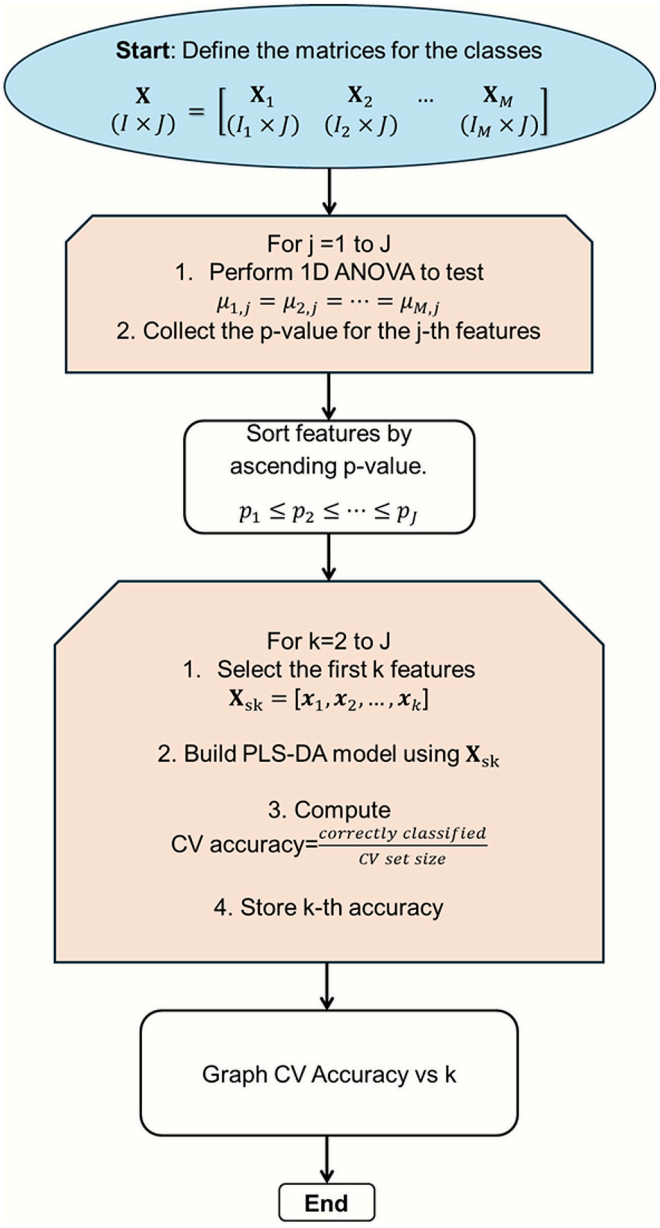

Fig. 2. Schematic representation of forward-selection algorithm for biomarkers determination. In the presented algorithm, I_1, I_2 and I_M respectively denote the number of samples corresponding to class 1,2 and M for the data matrix **X** with I samples and J features, while μ_{1j}, μ_{2j} and μ_{Mj} are the relevant class mean values associated with the feature j. *p*-values rank is then used to sort feature vectors used to generate the subset Data Matrix $\mathbf{X}_{sk}$.

3. Results and discussion

After conducting ANOVA on LC-QTOF-MS data, a set of 1018 *p*-values corresponding to each metabolite was sorted in ascending order. Due to the high dimension of the data matrix, part of such variables is expected to be redundant or uninformative for the assessment of the effect of the *stimuli* under investigation. The employment of a local modeling procedure involving a variable window selection can significantly improve both the model's predictive ability and computational time (Nørgaard et al., 2000). Indeed, the overall computational complexity of PLS algorithm is proportional to the square of the number of variables (Qin et al., 2022).

In the following discussion, a lipidomic investigation of the impact of different rennet sources (vegetable vs animal) on the content of a broad set of complex lipids in cheese is presented for the first time. The subsequent analysis of ripening time provides an additional means of validation of the applied chemometric method and the overall structure of

the collected data, while providing a deeper understanding of both triglycerides and phospholipids lipolysis mechanisms.

3.1. Rennet type analysis

The discrimination potential of LC-QTOF-MS data was assessed through forward stepwise interval PLS-DA, which selects the subset of metabolites associated with the maximum accuracy of classification. Fig. 3 depicts the classification accuracy in cross validation for 1017 variables subsets and their associated optimal number of LVs. For the case of vegetable vs animal rennet, a 40 metabolites subset was selected as the optimal window, giving rise to classification accuracy in cross-validation of 88 %. Conversely, only 56 % of accuracy was achieved when the entire dataset was considered for the analysis. It is worth noting that the employment of two LVs guaranteed the achievement of the optimal performance when the first 40 variables with the smallest *p*-value were included in the window, meaning that the essential discriminatory information was captured in a parsimonious bidimensional latent space. On the other hand, when data dimensionality was high (i.e., number of features >450), the optimal number of latent variables (LVs) increased sharply. This was likely due to the increased system complexity, which in turn reduced the model's ability to reliably discriminate between samples.

Table 1 indicates the metabolites included in the optimal subset and their associated *p*-value from two-way ANOVA test, from which *p*-values between 0.006 and 0.041 were obtained. The rennet analysis identified a broad range of complex lipid families. In the context of this study, significant differences were observed between the two rennet types in the content of triacylglycerols (TG), monoacylglycerols (MG), free fatty acids (FFA), phosphatidylserines (PS), phosphatidylcholines (PC), phosphatidylethanolamine (PE), ceramides (Cer), and sphingomyelins (SM). Such compounds were identified in other cheese lipidomic studies with diverse biomarker selection purposes using an unspecified rennet type (Haddad et al., 2022; Tomaiuolo et al., 2023). It is worth noting that among the presented compounds, several triacylglycerol-derived compounds such as MGs and FAs resulted to significantly vary between cardoon rennet-based cheeses and calf rennet-based ones. This outcome can be due to the relatively high abundancy of triacylglycerols in milk substrates, including sheep's milk, as they account for 95 % of total fat (Zhao et al., 2022), supporting their main involvement in lipolytic phenomena. The remaining fraction (5 %) is mainly constituted

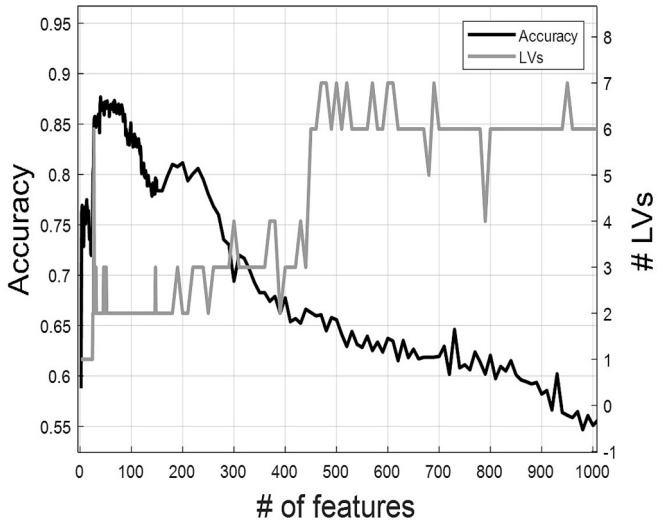

Fig. 3. Classification accuracy in cross-validation as a function of the number of features considered in the PLS-DA classifier for the rennet type (black line), and the corresponding optimal number of LVs obtained from each subset (grey line).

Table 1
Two-way ANOVA *p*-values for the subset of metabolites demonstrating optimal performance of the PLS-DA classifier for rennet type.

Metabolite	p-value	Metabolite	p-value
U1	0.0062	PE (18:1_18:1)	0.0269
PS (18:1_18:1)	0.0077	Cer (d18:1_23:0)	0.0272
TG (51:5)	0.0085	PE (P-18:1_18:1)	0.0274
FA (21:3)	0.0162	dihydroxy FA (20:0)	0.0301
FA (22:5)	0.0166	U4	0.0303
14:0 Cholesteryl ester	0.0166	MG(21:0)	0.0304
SM (23:0;O5)	0.0167	MG(16:0)	0.0315
PC (14:0)	0.0180	U5	0.03263
PS (18:0_22:0)	0.0198	Octadecanedioic acid	0.0328
U3	0.0198	PI (18:0_18:2)	0.0328
Cer (d18:0_23:0)	0.0203	Cholesterol glucuronide	0.0330
SM (d33:1)	0.0227	PS (18:1_20:0)	0.0335
FA (18:2)	0.0232	FA (19:1)	0.0343
FA (24:2)	0.0252	FA (13:0)	0.0346
PC (P-16:0_18:4)	0.0254	FA (22:1)	0.0382
FA (21:1)	0.0258	FAHFA(24:5–17:0)	0.0382
SM (d36:1)	0.0261	N-(4-benzenesulfonamide) arachidonoyl amide	0.0396
PE (18:0_18:1)	0.0261	U6	0.0400
FA (24:1)	0.0266	U7	0.0404
SM (d37:1)	0.0267	Cer (m18:0_24:0)	0.0405

U: Unknown; PS: PhosphatidylSerines; TG: TriacylGlycerols; FA: Fatty Acids; PC: PhosphatidylCholines; Cer: Ceramides, SM: SphingoMyelins; PE: Phosphatidyl-Ethanolamine; MG: MonoacylGlycerols.

of phospholipids. The five major phospholipids in dairy products are PC, PE, SM, PI and PS and are located in the milk fat globule membrane (Manis et al., 2023; Verardo et al., 2013). Their considerable contribution to the discrimination of cheese samples obtained from different rennet can be attributed to a possible high content of phospholipases enzymes in the calf rennet. These enzymes are known to facilitate the degradation of the fat globule membrane, which promotes the releasing of its constituents in the dairy matrix (Patton & Keenan, 1975).

To this regard, differences between calf and cardoon rennet lipidomics are depicted in Fig. 4, which shows the biplot obtained from the PLS-DA model including the optimal lipids subset. As can be seen, the LV space displays a clear separation between the scores belonging to the different groups. From a visual inspection of loading values, it can be concluded that only 3 metabolites were clearly upregulated in cardoon-based cheese samples, while the other 37 features resulted to show either a strong or a weak upregulation in the calf rennet-based samples. This pattern is consistent with the mean values of LC-QTOF-MS peaks observed in each class comparison across all metabolites.

It has been demonstrated that several animal rennets contain pregastric lipolytic enzymes (PGLs) and esterases that contribute significantly to triacylglycerols (TG) lipolysis during cheese production, releasing free fatty acids (FFA) and monoacylglycerols (MG), and modifying phospholipid classes such as phosphatidylcholines (PC), phosphatidylethanolamines (PE), sphingolipids, and ceramides (Moschopoulou, 2011; Santillo et al., 2009). This can explain the pronounced upregulation of these lipid species in cheeses prepared with animal rennet.

Interestingly, similar loading values were identified for ceramides (Cer) and sphingomyelins (SM). This overlap likely reflects their close structural relationship (Liu et al., 2020): SM are phosphocholine-derivatives of ceramide, which can be hydrolyzed by sphingomyelinases into ceramide and phosphatidylcholine (Hannun & Obeid, 2008). The higher abundance of sphingomyelins (SM) observed in cheeses made with animal rennet compared to those made with vegetable rennet may be linked to both enzymatic activity and compositional factors. Animal rennets often contain pregastric lipases, pepsin, and other enzymes capable of disrupting the milk fat globule membrane (MFGM), thereby releasing and remodeling sphingolipids (Moschopoulou, 2011).

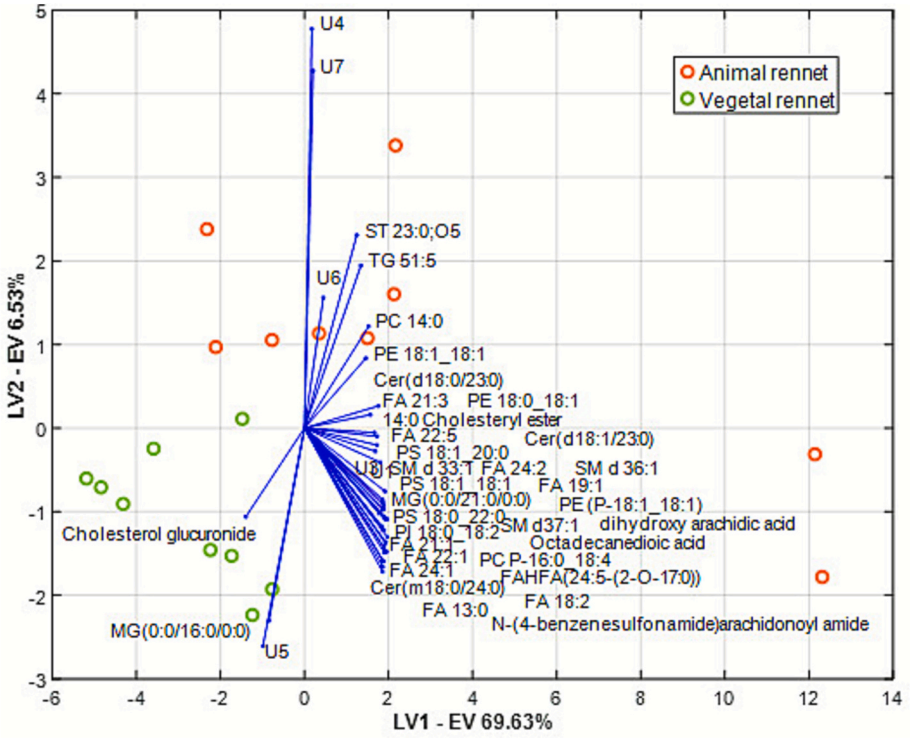

Fig. 4. PLS biplot showing scores for different rennet types and the lipids loading values obtained from the optimal features subset of rennet analysis. For a clear visual analysis, loading values were multiplied 10 times.

Since SM are major constituents of the MFGM, their relative enrichment could result from selective hydrolysis or structural reorganization processes. Indeed, baseline levels of sphingomyelin in milk are influenced by species, lactation stage, diet, and animal breed (Lopez et al., 2008). Growth of cheese-ripening lactic acid bacteria on MFGM components further suggests that SM serve as substrates in microbial remodeling during ripening (Moe et al., 2013). These factors suggest that the up-regulation of SM in animal-rennet cheeses reflects both a greater release of SM from membrane structures and distinct remodeling dynamics compared to cheeses made with vegetable rennet (Liu et al., 2020; Moschopoulou, 2011). Noteworthy, the positive correlation between cholesterol-glucuronide levels and cheeses coagulated with *Cynara cardunculus* rennet may reflect a direct contribution of plant-derived lipophilic compounds and/or specific enzymatic activities introduced by the vegetable coagulant. Lipophilic profiling of *C. cardunculus* shows a significant content of sterols and fatty acids, which can be present in crude pistil extracts used as rennet (Ramos et al., 2013). Particularly, Ramos et al. indicated that sterols account for 1 to 11 % of the total detected compounds in all morphological parts of the plant (Ramos et al., 2013). Moreover, cardoon rennet are known to contain highly active proteases and have been reported to exhibit esterase/lipase activities in crude preparations, potentially producing monoacylglycerols (MG) from plant triacylglycerols and influencing cheese lipid profiles (Pimentel et al., 2007).

Overall, these findings suggest that the distinctive lipid profile of cheeses made with cardoon rennet, including the elevated levels of MG (16:0) and sterol derivatives, reflects both the intrinsic composition of the plant-based coagulant and its contribution to enzymatic lipid remodeling during cheese production.

3.2. Ripening time analysis

Ripening stage duration is a dominant factor for FAs profile of cheese due to lipolysis of TGs (Haddad et al., 2022). The present chemometric analysis of the effect of ripening time on the lipidic profile of cheese allows to acquire a deeper understanding of cheese lipolytic processes.

Fig. 5 depicts the accuracy of ripening time classification in cross validation for 1017 subsets (1018 variables) and their associated optimal number of LVs concerning the analysis of cheese ripening time. A 15-feature subset was selected as it achieved an optimal CV classification accuracy of 91 %. On the other hand, only 74 % accuracy was achieved when the entire dataset was considered for the analysis. In this case, the optimal performance was achieved from a three LVs-model

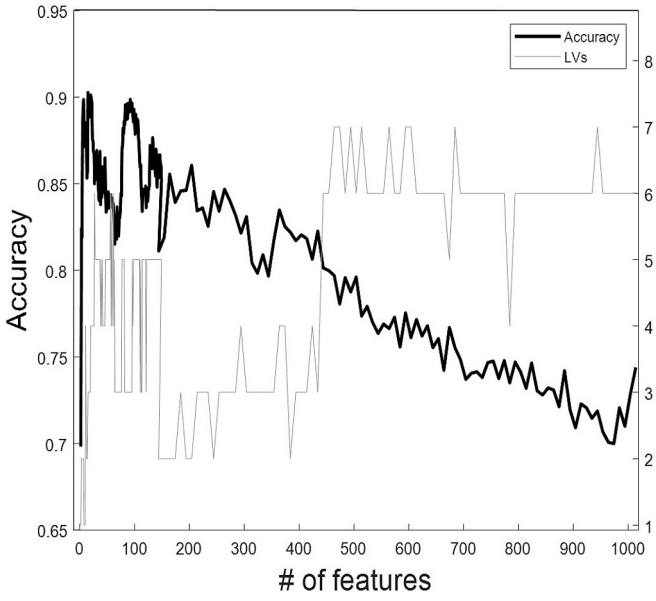

Fig. 5. Classification accuracy in cross-validation as a function of the number of features considered in the PLS-DA classifier for the ripening time (black line), and the corresponding optimal number of LVs obtained from each subset (grey line).

when the first 15 lipids were included in the window, meaning that the essential discriminatory information was explained in a tridimensional latent space. Also in this scenario, when dealing with high data dimensionality (#features>450), the optimal number of LVs showed a sudden increase along with a remarkable decreasing of model's accuracy.

In the examination of cheese ripening time, as delineated in Table 2, the first lipid group found to exhibit significant variation across different ripening durations is that of FAs. Notably, the associated *p*-values for ripening time are considerably lower compared to those linked with the type of rennet used. In fact, cheese ripening is one of the most influential factor for determining the metabolic composition (Scano et al., 2019).

It is important to specify that none of the compounds reported in Table 2 resulted to significantly vary between the different rennet types, meaning that the following rationale for ripening times is statistically consistent for both calf and cardoon rennet-based cheeses.

Fig. 6 reports the PLS-DA biplot obtained from the optimal subset for ripening time cheese classification. A clear separation of samples ripened for different times can be observed from the scores' positions. The 24-h ripened cheese samples exhibit prominently negative score values along the LV1 axis, contrasting with the predominantly positive or marginally negative scores observed for the 12 and 18-month clusters. Furthermore, LV2 delineates a notable difference between the 12-month and 18-month clusters, with the former displaying high values compared to the latter, indicative of discernible compositional differences between these ripening durations. The analysis of loading values provides a positive correlation of medium to long chain FFAs content with cheese samples obtained from 18 months of ripening. Such an outcome can be attributed to the occurrence of multiple phenomena:

1) Following an initial phase of the lipolysis process, during which the outer and sterically less hindered position of the triglyceride (the sn-3) is preferentially hydrolyzed, the lipolytic enzymes are able to hydrolyze, in a later ripening time, even the less accessible sites corresponding to the stereochemical number positions 1 and 2, where medium and long-chain fatty acids are preferentially esterified (Caboni et al., 2019). Indeed, the loadings position of TG (18:0_18:4_18:0), placed in the middle of 12 and 18 months-ripened cheese clusters, as well as the subsequent upregulation of C18 FAs in 18 months-ripened cheese is probably related to a delay of ester bonds cleavage due to steric hindrance.
2) A higher content of LysoPE (18:1) is detected in 12 months-ripened cheese. Probably, the hydrolysis of this phospholipid through the action of lysophospholipase enzymes contributed to the remarkable increasing of FA 18:1 concentration in 18 months-old cheeses (García-Cano et al., 2020).

Interestingly, two hydroxy-fatty acids (OH-FAs) resulted to be upregulated in 18 months-ripened samples. Traces of OH-FAs were detected in other works, which recognized this group of lipids as an important marker for characterizing foodstuffs (Jenske & Vetter, 2009). The different regulation of hydroxy-palmitic acid and hydroxy-octadecanoic acid in cheese with different ripening time, as reported in this study, is probably related to the hydroxylation of fatty acids resulting from their microbial catabolism (Collins et al., 2003).

Ultimately, due to the characteristic times of lipolysis reactions, it can be deduced that none of the here studied lipids are apparent to be upregulated in 24-h cheeses. This can be ascribed to the short residence time of lipolysis enzymes in the cheese substrate.

Further details about the models' outcomes are reported in the supplementary materials (Section and Fig. S2).

3.3. Joint analysis

After identifying the significant lipids, a new matrix was constructed by concatenating the two datasets obtained from the different analyses, and the Pearson correlation matrix was assessed. Fig. 7 presents a correlation heatmap of the measured metabolites. Each cell represents the pairwise correlation coefficient between two metabolites, with the intensity of the color indicating the strength and direction of the relationship. Positive correlations are depicted in shades of red, while negative correlations appear in shades of blue, with the scale ranging from −1 to +1. The strong clustering of red blocks highlights groups of metabolites that exhibit highly concordant behavior, as a result of the overall lipid upregulation in long stage ripening samples and viceversa. On the other hand, isolated blue lines reflect negative associations between specific metabolites. In particular, MG(16:0) and Cholesterol glucuronide resulted to be negatively correlated with other metabolites, thus highlighting their upregulation in plant-based rennet. It is worth noting that strong positive correlations was observed among fatty acids and hydroxy-fatty acids with a high degree of saturation. Similar conclusions can be drawn from the correlation clustergram, reported in the supplementary material (Fig. S3).

The matrix obtained by concatenating the selected feature matrices was used to calibrate a PLS-DA model, enabling the assessment of class separability when considering both ripening time and rennet type. In this analysis, samples were divided into six distinct classes, each reflecting their membership to the corresponding combination of the two factors. The PLS-DA score plot (Fig. 8) displays the distribution of samples across the first two LVs, explaining 68.72 % and 8.85 % of data variance, respectively. Samples are grouped according to the different experimental conditions. Distinct clustering patterns are observed, thus confirming the substantial differences in the lipidic profile among different rennet recipies and ripening times. Specifically, samples obtained from *Cynara cardunculus* renneting can be seen in the upper-left portion of the score plot, whereas those derived from animal rennet appeared in the opposite region. With respect to ripening time, samples at 24 h were associated with positive values of the second latent variable, while those at 18 months corresponded to negative values. No clear positioning trend was observed for samples ripened for 12 months, although they still exhibited evident clustering.

4. Conclusions

Cynara cardunculus from which rennet is extracted has great potential for different applications within pharmaceuticals, energy as well as in the food industry, thanks to its multifunctional biochemical profile. This study presented for the first time a lipidomic investigation of cheese

Table 2
Two-way ANOVA p-values for the subset of metabolites demonstrating optimal performance of the PLS-DA classifier for ripening time.

Metabolite	p-value	Metabolite	p-value	Metabolite	p-value
LysoPE 18:1	2.2610e-07	FA 11:0	0.0002	hydroxy-octadecadienoic acid	0.0003
Cer(d18:1/26:0)	5.7332e-07	hydroxy palmitic acid	0.0002	DG 15:0_22:2	0.0004
FA 22:2	0.0001	PS 18:1_22:0	0.0002	FA 18:2	0.0004
U2	0.0001	FA 18:1	0.0002	FA 15:1	0.0004
TG 18:0_18:4_18:0)	0.0001	FA 12:0	0.0003	U8	0.0004

U: Unknown; PS: PhosphatidylSerines; TG: TriacylGlycerols; FA: Fatty Acids; PhosphatidylCholines; Cer: Ceramides, SM: SphingoMyelins; PhosphatidylEthanolamine; MG: MonoacylGlycerols.

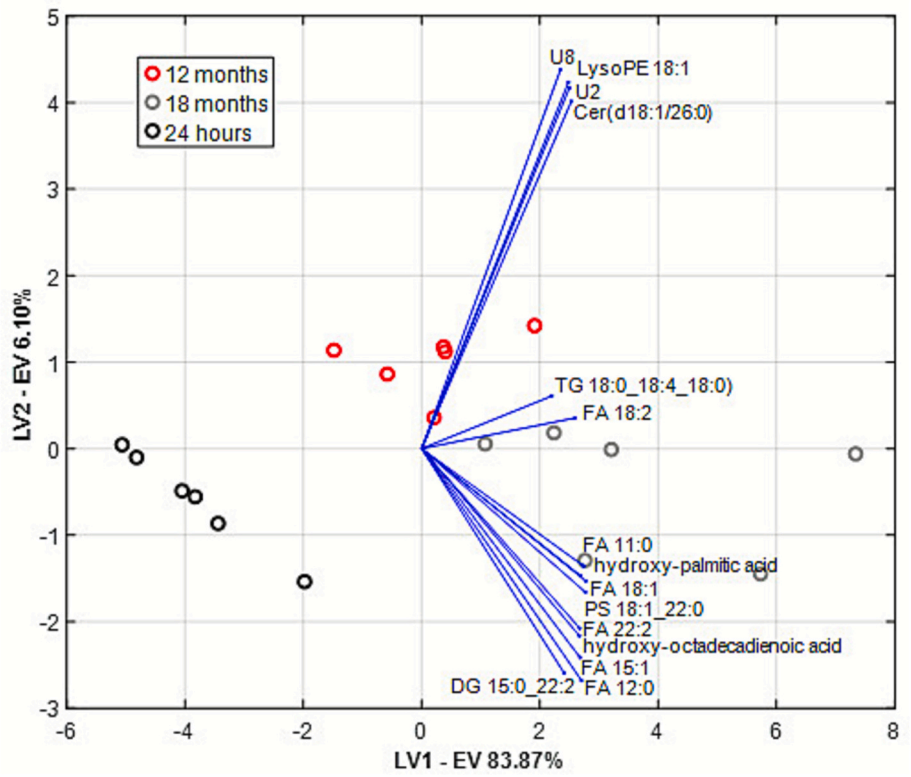

Fig. 6. PLS biplot showing scores for different ripening times and the lipids loading values obtained from the optimal features subset of ripening time analysis.

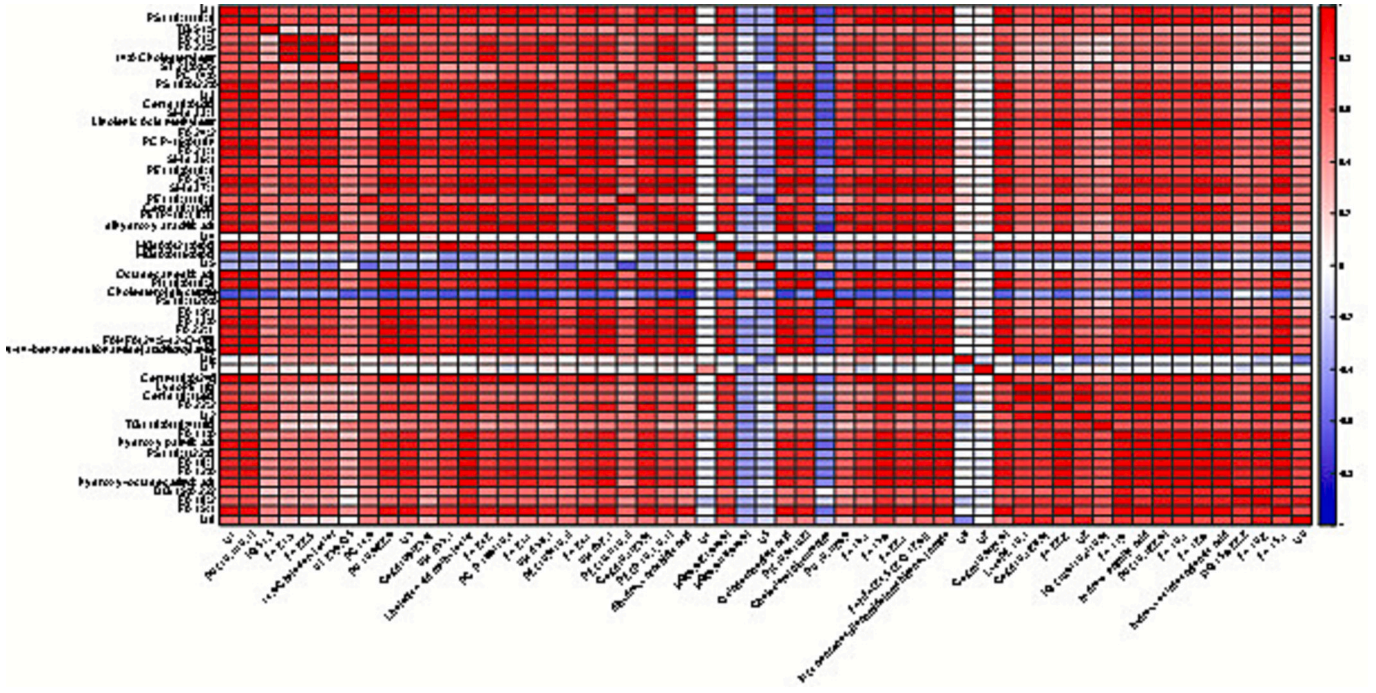

Fig. 7. Results of the correlation analysis, depicted in the Pearson correlation coefficient heatmap.

deriving from vegetable and animal coagulant enzymes, proving the cardoon-derived cheese to be significantly different in its fat's composition to the traditional calf rennet. The utilization of chemometrics coupled with LC-QTOF-MS data provides a powerful tool to detect the variability of specific biomarkers within complex biomatrices such as cheese.

Several multivariate phenomena, such as lipolytic processes, may exhibit diverse extent of variations under different factors, leading to disparities in performance when employing a classifier model like PLS-DA for the investigation of more than one stimulus. A proper selection of metabolites subset allowed the achievement of high performances of the classification model for both factors. Regarding rennet type

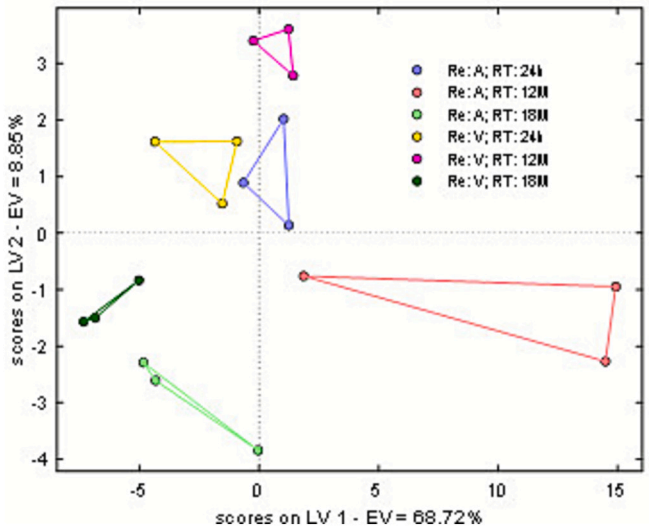

Fig. 8. PLS-DA score plot of the lipid data matrix obtained by concatenating LC-QTOF-MS signals of the selected features from rennet type analysis with those from ripening time analysis.
Abbreviations. Re: Rennet; A: Animal; V: Vegetal; RT: Ripening Time.

investigation, a broad picture of biomarkers was identified, denoting various lipid classes such as triglycerides and various phospholipids to be upregulated in animal rennet-based cheeses. This diversity reflects the significant dissimilarities in the lytic behavior of two coagulants from different sources (i.e. vegetable and animal). Conversely, biomarkers associated with ripening time were predominantly found within fatty acid molecules, probably due to the high lipolysis extent of triacylglycerols. However, one limitation of the present study lies in evaluating cheeses only as groups produced with either calf rennet or cardoon rennet, without assessing the effects of individual enzyme preparations. Cardoon rennet, in particular, can vary naturally depending on the plant source and extraction method, potentially influencing cheese characteristics. Future research should examine individual rennet preparations under controlled conditions to provide more precise insights into their specific impact on cheese quality.

These findings emphasize the importance of metabolite selection in achieving robust predictive outcomes for diverse factors influencing cheese quality. Moving forward, the use of such lipidomic knowledge could enhance control over technological characteristics of cheese, thus supporting cheesemaking research in establishing efficient formulations of plant rennet aimed at reducing enzyme's profile variability and optimizing cheese production processes for product quality enhancement. In conclusion, this study demonstrates that untargeted LC-QTOF-MS lipidomics, coupled with chemometric analysis, can identify markers that differentiate plant- and animal-derived rennets across ripening stages, supporting both cheese authentication and the optimization of technological processes.

CRediT authorship contribution statement

Leonardo Sibono: Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Conceptualization. **Massimiliano Grosso:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Massimiliano Errico:** Writing – review & editing, Validation, Conceptualization. **Stefania Tronci:** Writing – review & editing, Validation, Conceptualization. **Giacomo Lai:** Writing – review & editing, Investigation, Conceptualization. **Massimo Pes:** Writing – review & editing, Investigation, Conceptualization. **Margherita Addis:** Writing – review & editing, Investigation, Conceptualization. **Pierluigi Caboni:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Cristina Manis:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.117987.

Data availability

Data will be made available on request.

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