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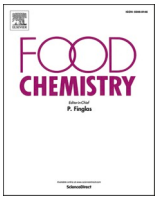

Metabolomic profiling of Fiore Sardo cheese: Investigation of the influence of thermal treatment and ripening time using univariate and multivariate classification techniques

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ABSTRACT

The effect of different sub-pasteurization heat treatments and different ripening times was investigated in this work. The metabolite profiles of 95 cheese samples were analyzed using GC–MS in order to determine the effects of thermal treatment (raw milk, 57 °C and 68 °C milk thermization) and ripening time (105 and 180 days). ANOVA test on GC–MS peaks complemented with false discovery rate correction was employed to identify the compounds whose levels significantly varied over different ripening times and thermal treatments. The univariate *t*-test classifier and Partial Least Square Discriminant Analysis (PLS-DA) provided acceptable classification results, with an overall accuracy in cross-validation of 76% for the univariate model and 72% from the PLS-DA. The metabolites that mostly changed with ripening time were amino acids and one endocannabinoid (i.e., arachidonoyl amide), while compounds belonging to the classes of biogenic amines and saccharides resulted in being strongly affected by the thermization process.

1. Introduction

The Food and Agriculture Organization of the United Nations (FAO) estimated that 6 billion people worldwide consume milk and milk products (FAO, 2023; OECD/FAO, 2021). Milk and milk products are an important source of protein, calcium, and other essential nutrients (Silva et al., 2017). They are also a major source of calories and energy for many people. There is an undeniable relationship between cheese and territory in terms of culture and recognized typical products. Therefore, in order to produce high-quality food products that meet the legal requirements and consumer demands, it is imperative to implement advanced technological and monitoring methods within the cheese-making industry (Khattab, Guirguis, Tawfik, & Farag, 2019) while preserving regional traditions associated with the product. The analysis of cheese flavour and safety embraces hundreds of different chemical compounds produced by food microbiota metabolisms such as biogenic amines (BA), fatty acids, amino acids (AA) and sugars, to name a few (Hassan, Abd El-Gawad, & Enab, 2012; Khattab et al., 2019; Sibono et al., 2023). Among the several variables that influence the concentration of such metabolites, cheese ripening time and milk heat treatment are considered to be the primary factors in the development of cheese characteristics (Atasoy & Türkoğlu, 2008; Combarros-Fuertes et al., 2016; Santiago-López et al., 2018). It has been broadly reported that ripening time has a strong effect on cheese microbial metabolic reactions such as amino acid decarboxylase (Benkerroum, 2016; P. Caboni et al., 2019; Kandasamy et al., 2021) due to the advancement of the proteolysis effect (Santiago-López et al., 2018), and fat degradation phenomena associated with lipolytic enzymes (Collins, McSweeney, & Wilkinson, 2003). Consequently, cheese ripening is responsible for determining the extent of accumulation of amino acids, some of which serve as precursors for the synthesis of BA, such as tyramine, histamine, putrescine, and cadaverine (Kandasamy et al., 2021; Moniente et al.,

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2023). Excessive intake of these compounds is thought detrimental to human health. (Combarros-Fuertes et al., 2016; del Rio, Fernandez, Redruello, Ladero, & Alvarez, 2024; Ferrante & Mercogliano, 2023). Concerning milk heat treatment, only a few works studied the effect of the thermization process, identifying the key features that discriminate heat-treated milk cheese from raw milk cheese (Anedda, Melis, & Curti, 2021; Caboni et al., 2019; Caboni, Murgia, et al., 2019; Dedola et al., 2020; Natrella, Gambacorta, Squeo, & Faccia, 2023). In particular, (Caboni, Maxia, et al., 2019; Caboni, Murgia, et al., 2019) studied the polar low-molecular-weight metabolites profile to differentiate raw milk cheese from cheese whose milk was previously treated at 68 °C for 30 s. However, milk thermization can be carried out within a relatively broad operative window that ranges from the mildest process of 57 °C for 15 s to the most severe thermal treatment of 68 °C for 30 s.

Due to the effect of different variables on the distribution of chemical compounds in a biological system, metabolomics was introduced as the comprehensive identification and quantification of metabolites (Idle & Gonzalez, 2007). In this discipline, the metabolome represents the complete set of these compounds that entirely depicts an organism's or tissue's biochemical state (Oliver, Winson, Kell, & Baganz, 1998). Metabolomics has emerged as a powerful tool in the field of dairy product characterization, as it enables the identification of metabolic signatures associated with different physiological states and treatments (P. Caboni, Maxia, et al., 2019; Rocchetti et al., 2018). The application of gas chromatography–mass spectrometry (GC–MS) to metabolomics has proven to be highly effective in analyzing dairy food quality, allowing for a widespread identification of biomarkers associated with a specific product attribute (Putri et al., 2022). Moreover, it is recognized that statistical analysis of metabolomic data is very challenging due to the nature of the data and the complexity of metabolomics as a research field.

In this work, the effect of heat treatment and ripening time on the metabolite profile of Fiore Sardo cheese was studied by means of GC–MS analysis. The ultimate goal was to develop classification tools capable of discriminating between cheeses subjected to different ripening times (105 and 180 days) and different thermization processes (non-thermized milk, milk thermized under mild conditions, and milk thermized under severe conditions). This investigation was approached from a comparative perspective, as it evaluates the performance of univariate classifiers compared to the widely employed Partial Least Squares Discriminant Analysis (PLS-DA).

2. Materials and methods

2.1. Chemicals

Analytical LC grade (99.9% of purity) methanol, chloroform, and hexane as well as pyridine, methoxamine hydrochloride, potassium chloride, *N*-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma Aldrich (Milan, Italy). Bi-distilled water (18.2 MΩ x cm at 25 °C) was obtained with a MilliQ purification system (Millipore, Milan, Italy). Analytical standards with a purity >98% were used to prepare a QA samples. Succinic acid, 2,2,3,3-D4-succinic acid, malic acid, citric acid, aspartic acid, pyruvic acid, ketoglutaric acid, oxalacetic acid, fumaric acid, lactic acid, butyric acid, beta-hydroxybutyric acid, glycolic acid, myoinositol, scylloinositol, ribitol, alanine, glycine, glutamine, leucine, isoleucine, serine, homoserine, ornithine, phenylalanine threonine, proline, valine, glucose, fructose, xylitol, ribitol and phosphate were purchased from Sigma Aldrich (Milan, Italy).

2.2. Cheese production and sampling

Cheese samples of this study were produced from bulk milk from mature Sarda sheep collected from February 2019 to June 2019, leading to a total of 45 cheese-making trials from the experimental farm of Agris Sardegna and transformed into the Fiore Sardo type cheese (three levels of milk temperature treatment × three cheese-making per month × six months). For each cheese-making trial, 130 kg of bulk raw sheep's milk were divided into 3 aliquots: 31 kg raw milk (RM), 32 kg low thermized milk (LTM) and 32 kg of high thermized milk (HTM). The heat treatment occurred in a batch process using a tubular infrared exchanger (model Stoutz-Actinator, ACTINI Group, Evian-les-Bains, France), where the milk was heated to 57 °C for LTM and 68 °C for HTM for 30 s, respectively, and then rapidly cooled to 38 °C. The processing diagram is reported in Fig. 1. The process was repeated under the same conditions, to obtain 3 replicates for each milk thermal treatment, within three separate days each month, in the Agris Sardegna pilot plant. In each cheese-making session day, three (3) vats were used in batch, 1 with RM and 1 with LTM and 1 with the HTM alternately, and the cheesemaker was the same for all cheese making. By following these preventative measures, it was possible to eliminate the effects associated with the vat and the cheesemaker. In each process, nine cheese samples of approximately 1.5–1.7 kg were produced (3 cheese for vat), for a total of 31 raw milk cheeses (RC), 32 cheeses at 57 °C (HCA) and 32 cheeses at 68 °C (HCB), that were analyzed at 105 (16 RC cheeses, 16 HCA cheeses and 16 HCB cheeses) and 180 days (15 RC cheeses, 16 HCA cheeses and 16 HCB cheeses), as reported in Table S1.

2.3. Sample preparation and GC–MS analysis

Fine shredded cheese samples were stored in sterile plastic Falcon tubes at −20 °C before analysis. After thawing on ice, 50 mg of cheese were extracted with 250 μL of methanol, and 125 μL of chloroform (Caboni et al., 2016). Samples were sonicated at 4 °C for 15 min at 25 kHz, through three cycles of 5 min each using ExstractorOne (GM solution, Cagliari, Italy). Subsequently, 380 μL of chloroform and 90 μL of aqueous 0.2 M KCl containing 5 mg/L of 2,2,3,3-D4-succinic acid as the internal standard, were added and finally vortexed for 10 s. The solution obtained was centrifuged at 17700 rcf at 4 °C for 10 min, and 0.200 mL of the aqueous layer were extracted from each sample and dried under a gentle nitrogen stream. The dried samples were derivatized first using 50 μL of methoxyamine hydrochloride dissolved in pyridine at 10 mg/mL, homogenized for 20 s, and kept at room temperature for 17 h. Then, 100 μL of *N*-methyl-N-(trimethylsilyl)trifluoroacetamide was added to the mixture and vortexed. Following a 1-h incubation period, 600 μL of hexane was added, and the samples were subjected to GC–MS analysis.

One microliter of derivatized samples was injected splitless in a 6850-gas chromatograph coupled with a 5973 mass spectrometer (Agilent Technologies Inc., Santa Clara, CA). The injector temperature was set to 200 °C. The gas flow rate through the column was 1 mL/min. The fused silica capillary column was a DB5-MS 0.25 μm column (30 m × 0.25 mm i.d.; J&W Scientific Inc., Folsom, CA). The initial temperature was 50 °C for 3 min in isothermal mode, which was then increased to 250 at 3 °C/min and maintained at 250 °C for 25 min. The transfer line and the ion source temperatures were 280 and 180 °C, respectively. Ions were generated at 70 eV with electron ionization and were recorded at 1.6 scans/s in the mass range m/z 50 to 550. GC–MS data analysis was conducted by integrating each peak of the resolved chromatogram. The identification of metabolites was performed using the NIST08 standard mass spectra library (NIST, 2005), and, when available, compared to analytical standards. Confirmation of metabolites was performed by comparison of their relative retention times and mass fragmentation as well as retention indices as calculated according to Kováts for alkanes C9 − C36 (Kováts, 1958).

In our untargeted metabolomics pipeline, we used authentic standards submitted to the same silylation procedure of cheese samples. Furthermore, we used QA and QC samples to improve the integrity and robustness of our datasets while obviating the need for repeated analysis. Spectral peaks associated with potentially formed adducts (M + TMS, M + 2TMS, M + 3TMS, M + 4TMS, M + 5TMS) were examined for each metabolite using Chromeleon software 7.2.8 (Themofisher,

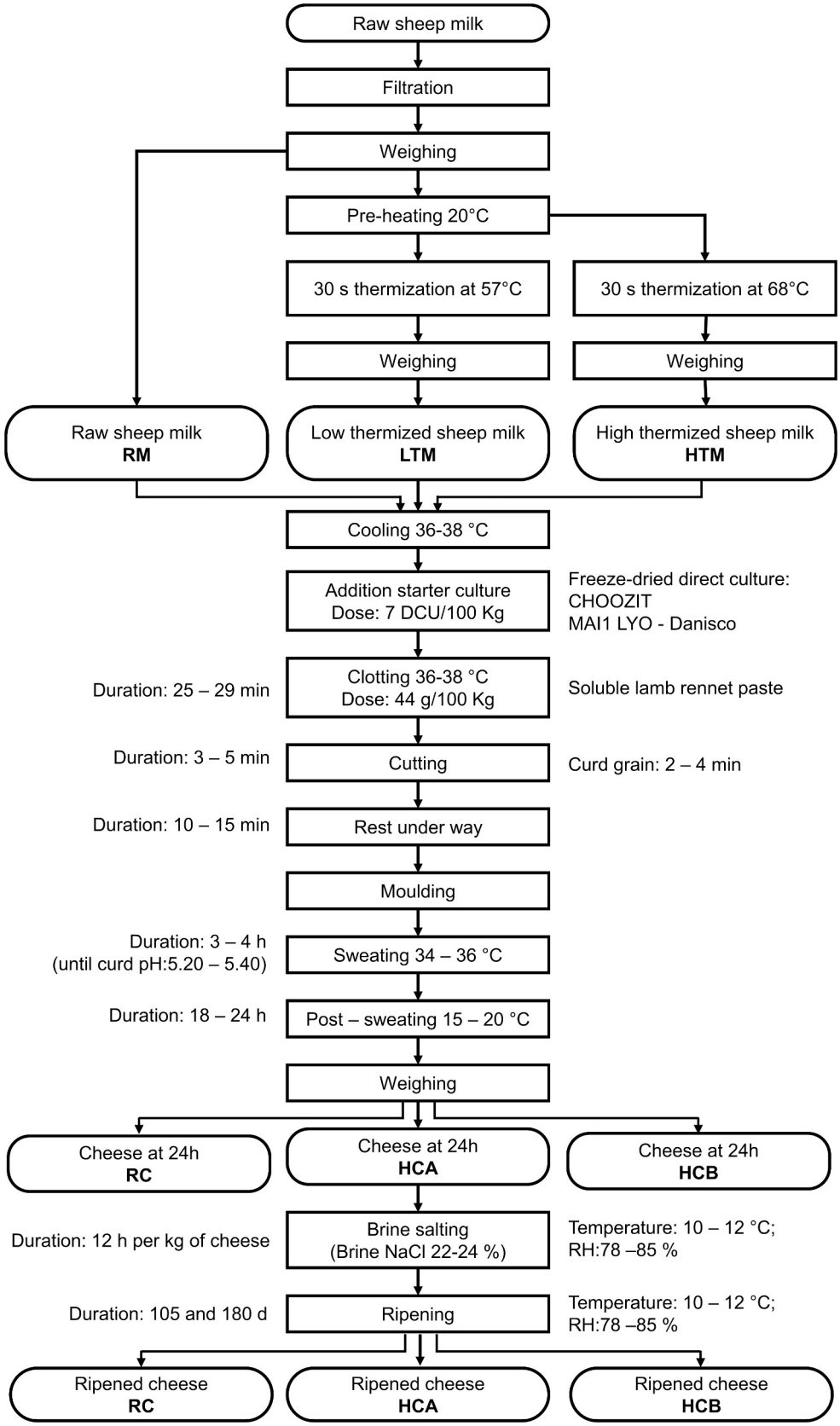

Fig. 1. Fiore Sardo cheese production flowchart. RC = cheese from raw milk; HCA = cheese from thermized milk at 57 °C; HCB = cheese from thermized milk at 68 °C.

Massachusetts, USA). When multiple adducts were detected, the most reproducible (technical replicate CV) MS1 adduct was selected for annotation and peak area integration. Metabolite reproducibility was then checked by evaluating consistency across replicate experiments; metabolites which are not consistent across replicates were labelled as irreproducible.

A representative chromatogram for RC, HCA and HCB cheese samples were reported in Fig. 2.

2.4. Dataset description

The metabolic dataset consists of 95 cheese samples and 745 GC–MS features. Cheese samples were classified based on different heat treatments and different ripening times. Among these samples, 31 were obtained from Raw Cheese (RC) milk samples (i.e. not thermally treated), 32 were obtained from 57 °C heat-treated milk (HCA), and 32 were subjected to a 68 °C thermal treatment (HCB).

2.5. Statistical methods

2.5.1. Data pre-analysis

Principal Component Analysis (PCA) was carried out on unity variance preprocessed data to reduce the complexity of the high-dimensional dataset, assess the trends among samples and eventually identify outliers (Varmuza & Filzmoser, 2016). The captured variance V_C, explained by a specific Principal Component (PC), was computed using components' eigenvalues expressed as percentages relative to the total variance (Brereton, 2009).

Before the classification step, a two-way ANOVA test was used on raw data to identify the metabolites contributing to sample discrimination based on heat treatment and ripening time. The following independent variables were considered in the analysis: ripening time, denoted by letter A, and milk thermal treatment, indicated by letter B, with a number of levels equal to a and b, respectively. The GC–MS experimental results obtained for each combination of these variables were denoted as x_{ijk}, where "i" represents the i-th level of the A factor, "j" represents the j-th level of the B factor, and "k" represents the k-th replication of the experiment. The effect model can be thus expressed as (Montgomery, 2013):

$$x_{ijk} = \mu + \tau_i + \gamma_j + (\tau\gamma)_{ij} + \varepsilon_{ijk} \quad \begin{cases} i = 1, 2 \ldots a \\ j = 1, 2 \ldots b \\ k = 1, 2 \ldots n \end{cases} \tag{1}$$

In Eq. 1, μ is the overall mean, τ_i is the effect of the i-th level of the independent variable A, γ_j is the effect of the j-th level of the independent variable B, $(\tau\gamma)_{ij}$ is the interaction effect of the i-th level of A and the j-th level of B, ε_{ijk} is eventually the residual for the ijk observation. To be rigorous, the two-way ANOVA tests the null hypothesis, which concerns the equality to zero of treatment effects with respect to the factors investigated. The level of significance α was set equal to 0.01. The procedure was performed for each metabolite, meaning that the effect of ripening time, thermal treatment and the interaction of both factors was tested on all the 745 spectral peaks collected. The ANOVA test revealed a set of metabolites found to be statistically significant in distinguishing between the ripening time and the thermal treatment of cheese samples. It is essential to note that, in situations involving multiple comparisons, the risk of committing Type I errors drastically increases (Lee & Lee, 2018). A more stringent approach was thus used to address this concern and control the inflation of false positives. More in detail, to identify the metabolites that are mostly responsible for variations in ripening, Benjamini-Hochberg method, also known as the False Discovery Rate (FDR; (Benjamini & Hochberg, 1995)) correction was employed for a FDR value equal to 0.01. This technique provided the opportunity to undertake a further elimination process with the objective of identifying the most significant metabolites that distinguish cheese samples based on their ripening times and thermal treatments.

2.5.2. Classification tools

For the sake of comparative analysis, data were analyzed by means of a univariate and a multivariate approach, and the performances were assessed in terms of accuracy and False Positive Rate (FPR). Firstly, a code employing univariate statistics was developed as a two-class classifier that identifies cheese samples' maturation time and thermal treatment. Specifically, the implemented code uses a *t*-test-based classification method to classify samples (Liao & Akritas, 2007) with a pairwise approach. This means that samples subjected to the same thermal treatment were binarily classified depending on the two ripening times. In contrast, for samples aged for the same ripening time, all possible pairs of thermal treatments among the three investigated were analogously tested. A detailed explanation of such a technique is reported in the Supplementary materials section. Besides the development of a univariate classifier, PLS-DA is the multivariate counterpart proposed for comparative purposes (Wold, Sjöström, & Eriksson, 2001). A pairwise classification approach is also adopted in PLS-DA for an unbiased comparison between the two models. Regarding the thermal treatment, each pair of classes is tested separately, meaning samples subjected to pairwise discrimination are labelled 1 for one class and 2 for the other. Regarding ripening time, the value 1 was assigned to cheese ripened for 105 days, whereas a value of 2 was assigned to cheese ripened for 180 days. The Bayesian assignation criterion collocated samples in a specific class (Ballabio & Consonni, 2013). Models' goodness of classification was assessed through 3-fold cross-validation and averaging performance indices (i.e. Accuracy and FR). Concerning the univariate classifier, in order to reduce the variance of the estimated performance measure, cross-validation was repeated 100 times with different 3-fold subsets at each time (Berrar, 2018), while 3-fold Venetian blind cross-validation was employed when assessing the multivariate classifier's performances (Ballabio & Consonni, 2013). The optimal number of PLS-DA latent variables was defined as the number that satisfies the following criteria:

(1) The error rate in cross-validation was minimized (i.e. the accuracy was maximized);
(2) The proportion of samples not assigned by the Bayesian criteria was <20% to ensure an unbiased assessment of the model's accuracy.

Univariate analysis and PCA were performed through Matlab®2023a (Mathworks Inc. Natick, MA, USA). The univariate procedure was carried out by means of codes developed by the authors, and "Milano Chemometrics" toolbox (version 6.0) was involved in performing PLS-DA (Ballabio & Consonni, 2013).

3. Results and discussion

3.1. GC–MS data pre-analysis

Principal Component Analysis was initially performed to explore dissimilarities and similarities among 95 cheese samples. A 99.99% confidence ellipsoid was employed in the 3D score plot (Fig. S.1 in supplementary materials), which illustrates the first three principal components, in order to identify any potential outliers within the data. The resulting dataset was a 94 × 745 matrix.

A two-way ANOVA test was performed on 745 GC–MS features to assess the impact of a specific factor, comprising the interaction term, on the individual metabolite. The hypothesis tests showed that 60 of these features underwent a statistically significant variation due to the effect of at least one of the two investigated factors. Specifically, 28 features were found to be influenced by the cheese ripening time. At the same time, 16 features were affected by the heat treatment. The contribution of the interaction term was remarkably lower since only 1 metabolite

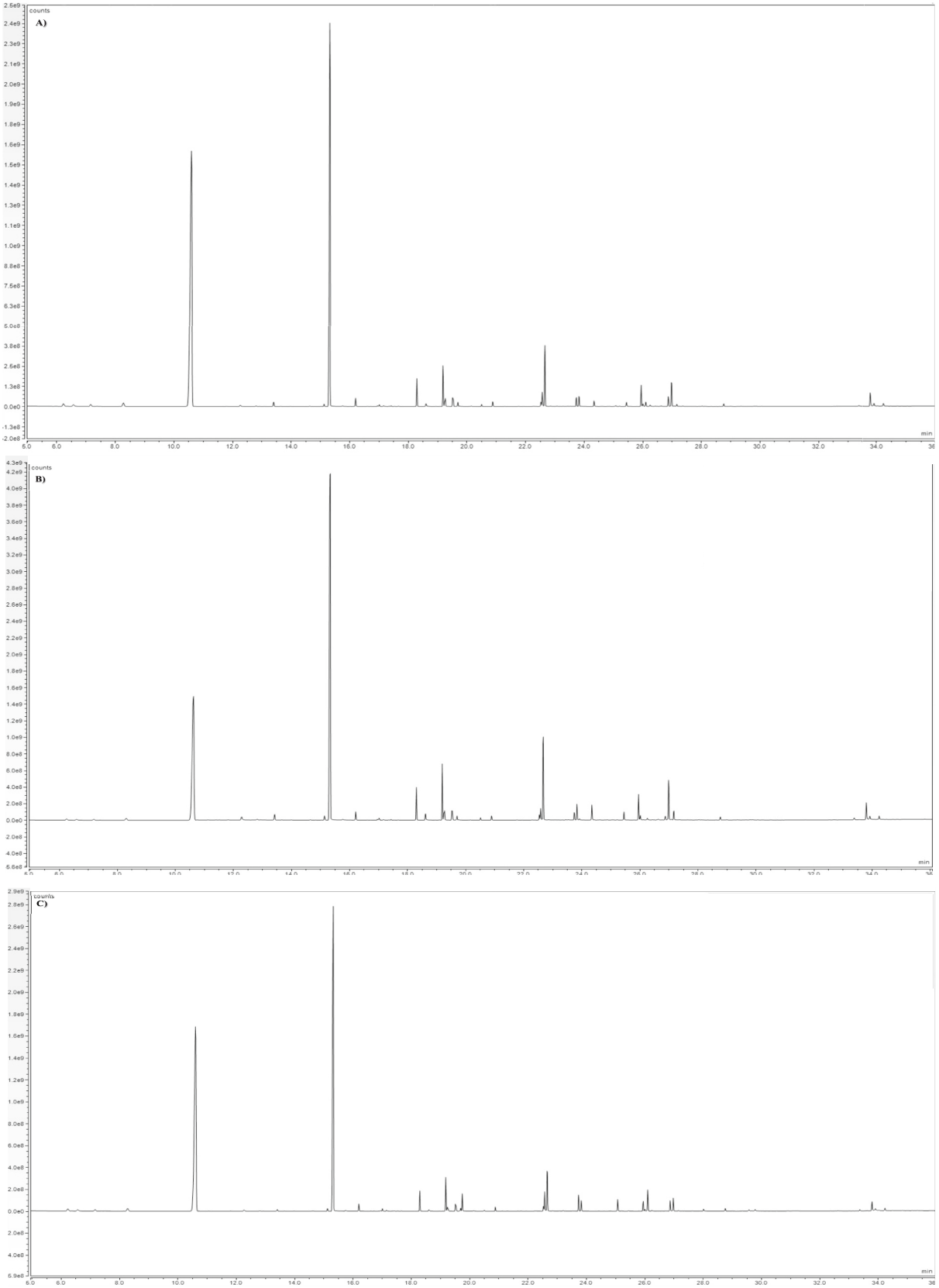

Fig. 2. Representative GC–MS chromatograms for A) cheese from thermized milk at 57 °C (HCA), B) cheese from thermized milk at 68 °C (HCB), and C) raw milk cheese (RC) at 105 days of ripening.

manifested a significant effect of the interaction term, which showed *p*-values smaller than 0.01. A Venn diagram is provided in Fig. 3, displaying all the metabolites that have shown a statistically significant effect ($p < 0.01$) from both factors, including the interaction effect. Metabolite 301 (arachidonoyl amide) resulted as the most important metabolite from the ripening time analysis, although it was also found to change significantly over the interaction term. Analogously, metabolite 1 (tyramine) resulted as the one that mostly changed over the thermal treatments, although the effect of ripening time was also found to be statistically significant. Metabolite identification numbers are listed in Table 1.

A Benjamini-Hochberg method was used to select the significant metabolites while simultaneously maintaining small type I errors. The false discovery rate was set equal to 0.01. Table 2 presents the metabolites and their p-values derived from the two-way ANOVA whose null hypothesis was rejected using the Benjamini-Hochberg method. Concerning maturation time, 8 metabolites were not discarded by the FDR correction, while only one metabolite was retained when the correction method assessed the effect of heat treatment. Finally, the interaction term was discarded for all metabolites.

Interestingly, it was obtained that 4-aminobutanoic remarkably changed over different ripening times. It is reported that cheese starters produce 4-aminobutanoic acid during ripening (Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998). Particularly, Nomura et al. reported that a cell extract of a Lactic Acid Bacterium (LAB) from the *Lactococcus lactis* strain, showed a significant glutamate decarboxylase activity, which catalyzes the decarboxylation of glutamate into 4-aminobutanoic acid. Therefore, it can be inferred that during the 75-day ripening period between cheeses subjected to 105 days of maturation and those ripened for 180 days, the decarboxylation reactions may have been a key factor in the production of 4-aminobutanoic acid. Also, the level of tyramine was identified to change significantly with temperature, indicating that thermal milk treatment significantly influences the level of this metabolite. More in detail, the analysis revealed that the tyramine level in raw milk cheeses was moderate and importantly increased in cheeses made from milk heated at 57 °C (HCA). Conversely, a marked decrease was observed in the tyramine content of HCB samples. These findings are consistent with the research conducted by

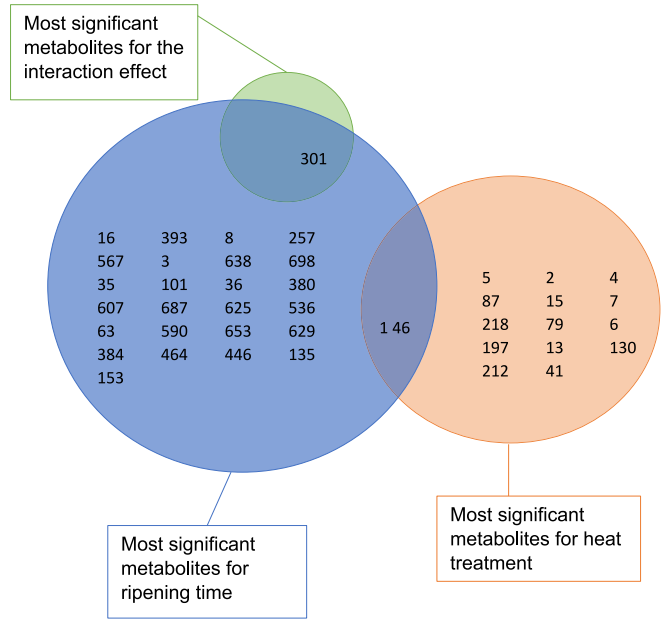

Fig. 3. Venn diagram displaying the metabolites for which the individual factors and interaction effect resulted to be significant ($p < 0.01$) from the 2-way ANOVA test. The metabolite number is shown in ascendent order in the *p*-value.

Table 1
List of the metabolites detected with the two-way ANOVA test with a significance level a = 0.01.*

Label	Factors	Metabolite	Label	Factors	Metabolite
1	A and B	Tyramine	197	B	**Unknown**
2	B	Cadaverine	212	B	Lactose 8TMS
3	A	4-aminobutanoic acid	218	B	Lactose 8TMS
4	B	Phenylalanine	257	A	Proline
5	B	Malic acid	301	A and AB	Arachidonoyl amide
6	B	**Unknown** Methyl glycocholate	380	A	Myo-Inositol 6TMS
7	B	3TMS	384	A	Valine
8	A	Citric acid	393	A	Phenylpropanoic acid
13	B	**Unknown**	446	A	Myo-Inositol
15	B	Leucine	464	A	Citric acid 2TMS
16	A	Glucose	536	A	Galactose
35	A	Erythrose	567	A	Lactic acid
36	A	**Unknown**	590	A	**Unknown**
41	B	Glutamine	607	A	**Unknown**
46	A and B	Urea	625	A	**Unknown**
63	A	4-aminobutanoic acid 3 TMS	629	A	**Unknown**
79	B	Urea 2TMS	638	A	Succinic acid
87	B	Lactose	653	A	Maltose 8TMS
101	A	D-Fructose	687	A	**Unknown**
130	B	Glycerol 3TMS	698	A	**Unknown**
135	A	Turanose			
153	A	**Unknown**			

* Factor A is the ripening time; Factor B is the thermal treatment; the term AB represents the interaction between the two factors.

Table 2
Results of the Benjamini-Hochberg procedure, showing metabolite codes and corresponding p-values.

Ripening time		Thermal treatment	
Metabolite	p-value	Metabolite	p-value
(4-aminobutanoic acid)	0.0014	Tyramine	8.734e-05
Citric acid	0.0005		
Glucose	0.0003		
Urea	0.0011		
Proline	0.0009		
Arachidonoyl amide	1.581e-05		
Phenylpropanoic acid	0.0005		
Lactic acid	0.0014		

Kebary et al., who found a similar trend when comparing raw milk cheese to cheese made from milk heated to 70 °C, 75 °C, and 80 °C (Kebary, El-Sonbaty, & Badawi, 1999). The increased tyramine content in the lower-temperature heated milk cheese may be due to the favourable conditions created by such heat treatments for the growth of proteolytic bacteria and/or the activation of proteolytic enzymes (Kebary et al., 1999). Conversely, higher temperatures may have reduced proteolytic bacteria concentration and denatured decarboxylase enzymes, resulting in lower tyramine levels. Similarly, according to the ANOVA test, cadaverine, the second most significant metabolite in terms of thermal treatment effect, decreased abruptly with HCB. However, there was no significant difference between raw milk cheese and cheese made from 57 °C heated milk for such a metabolite. Noteworthy is that arachidonoyl amide exhibited the lowest *p*-value for ripening time analysis. Recent works reported that several fermented food matrices like cheese are rich in endocannabinoid metabolites (e.g., arachidonoyl amide) due to the action of microbial activity (Yılmaz, Kocadağlı, & Gökmen, 2023). Other compounds like long chain-carboxylic acids and proline are known to vary over different ripening

times (Becchi, Rocchetti, Vezzulli, Lambri, & Lucini, 2023; Conte et al., 2021; Tekin & Güler, 2019). While the formers are known to show complex behavior along different ripening times (Caboni, Maxia, et al., 2019; Caboni, Murgia, et al., 2019), other studies reported the upregulation of proline in cheeses subjected to prolonged ripening times (Becchi et al., 2023; Singh et al., 2023).

Similar analytical and statistical approaches were reported by Rocchetti et al. (Rocchetti, Michelini, Pizzamiglio, Masoero, & Lucini, 2021) using an untargeted metabolomics technique to differentiate Parmigiano Reggiano PDO during ripening stages. Additionally, Zhang X. et al. reported an untargeted metabolomics method to discriminate Mongolian cheese during storage.

The application of both univariate and multivariate classification models to classify cheese samples and identify the metabolites accountable for such classification allows us to validate the significance of the compounds shown in Table 3. The employment of ANOVA coupled with the False Discovery Rate (FDR) correction served as an initial screening process for identifying a subset of metabolites considered relevant to this study.

3.2. Classification of cheese samples

Following the detection of relevant metabolites, two different classification techniques were applied to discriminate cheese samples according to the ripening time and thermal treatment. In order to minimize data variances and enhance the classifier performances, a pairwise approach was adopted during analysis, meaning that comparisons were made between ripening time classes within the same thermal treatment level and vice versa. This process was performed for each metabolite, and the ones that exhibited the highest accuracy are documented in Table 3, which presents the classification metrics obtained through 3-fold cross-validation. Specifically, the metabolites that yielded the best performance metrics when subjected to *t*-test classification, as well as groups of metabolites whose accuracy values surpassed a threshold ranging from 70% to 80%, are reported. The choice of the accuracy threshold value depended on the overall classification results for the specific pairwise discrimination. For each pairwise classification, if at least one metabolite exhibited an accuracy that exceeded a threshold value, the specific metabolite is considered performant for a given comparison. In order to achieve a reasonable number of significant metabolites, an 80% threshold value is chosen. The same operation is followed when the accuracy is 75% or 70%.

Notably, the employment of the *t*-test classifier demonstrated good efficacy in classifying samples based on ripening time, as accuracy was >75% in all cases. On the other hand, results show that classification performance was less favourable in terms of thermal treatment, particularly in comparison between RC and HCA, indicating that the average peak values between these classes were not considerably different given the same ripening time. Indeed, no relevant metabolites resulted in good candidates for discerning between these thermal treatments, regardless of the ripening time. A lower treatment temperature likely led to fewer noticeable differences in metabolites content between the HCB and raw milk cheese when they were compared. Results show that acceptable performances (Maroco et al., 2011) were obtained for arachidonoyl amide and phenylpropanoic acid, for which the effect of different ripening times was statistically significant from the two-way ANOVA test and whose null hypothesis was also rejected following FDR correction, validating their significant variation over different ripening time. Furthermore, the 4-aminobutanoic acid also showed a notable result in discriminating HCB180 from HCB105, thus confirming the importance of this compound during cheese ripening. Concerning the heat treatment classification, the tyramine based t-test classifier was found to be the most effective for two pairwise classifications, thus validating the crucial role played by this compound in determining the thermal heating characteristics of cheese. Therefore, these results confirm the findings identified with the ANOVA tests and FDR corrections carried out by means of the Benjamini-Hochberg method. The findings indicated that metabolites that were considered to be discriminant for ripening time were mainly fatty acids, interestingly supplemented by one endocannabinoid, while biogenic amines such as tyramine, accompanied by two soluble sugars (i.e., maltose and lactose) resulted in predominantly discriminating samples on a thermization process basis.

The performance metrics were also evaluated using PLS-DA for sample classification. In this case, the impact of ripening time and thermal treatment on the metabolite content was not analyzed on a per-metabolite basis. Still, the collective information of the entire metabolic profile was utilized to construct a classification model. The results of this analysis are presented in Table 4, which reports the classification metrics obtained through a 3-fold Venetian blind cross-validation coupled with a PLS-DA model.

A comparison between Tables 3 and 4 illustrates that the PLS-DA

Table 3
Univariate classifier performance metrics.

Classes	Metabolites		Univariate classifier cross validation indices		
	Most performant metabolite	Performant metabolites	Accuracy given by performant metabolites	Best Accuracy[a]	Best FPR
RC180 vs RC105	Arachidonoyl amide	Arachidonoyl amide	>75%	76%	14%
HCA180 vs HCA105	Phenylpropanoic acid	Phenylpropanoic acid	>80%	81%	19%
HCB180 vs HCB105	4-aminobutanoic acid	4-aminobutanoic acid, Glutamic acid, Erythrose, Hydroxycaproic acid, Norleucine, Hydroxyisocaproic acid, Norvaline, Hydroxyisocaproic acid 2, U1	>75%	80%	26%
RC180 vs HCA180	U2	U2	>70%	73%	25%
RC180 vs HCB180	Tyramine	Tyramine, Lactic acid, U3, Glutamine, Urea, U5, Lactose	>70%	73%	32%
HCA180 vs HCB180	Tyramine	Tyramine	>75%	77%	32%
RC105 vs HCA105	Maltose	Maltose	>70%	72%	25%
RC105 vs HCB105	Glycilglutamic acid	Malic acid, Glycilglutamic acid	>75%	80%	27%
HCA105 vs HCB105	U6	U6,U7	>75%	75%	19%

[a] Accuracy correspondent to the metabolite whose univariate classification resulted in the best performance. U = not annotated metabolite. Frequently, mass spectrometry-based metabolic profiles encompass numerous peaks that remain unidentified, making it challenging to correlate them with the metabolic physiology of the system under investigation (Kanani, Chrysanthopoulos, & Klapa, 2008).

Table 4
PLS-DA performance metrics in cross validation.

Classes	Multivariate classifier Cross validation indices		Optimal number of latent variables
	FPR	Accuracy	
RC180 vs RC105	25%	73%	3
HCA180 vs HCA105	11%	88%	4
HCB180 vs HCB105	18%	83%	8
RC180 vs HCA180	25%	59%	7
RC180 vs HCB180	36%	62%	7
HCA180 vs HCB180	35%	66%	3
RC105 vs HCA105	31%	69%	2
RC105 vs HCB105	25%	74%	10
HCA105 vs HCB105	29%	72%	1

classifier generally performs less accurately than the univariate classifier when assigned to discriminate cheese samples based on heat treatment. On the other hand, higher performances were recorded for ripening time, especially for samples taken from heat-treated milk (i.e., HCA and HCB). Overall, the univariate classifier performed better at classifying cheese samples. This result may be due to the presence of several uninformative metabolites, or the scarce correlation among the metabolites content (Saccenti, Hoefsloot, Smilde, Westerhuis, & Hendriks, 2014). To support the occurrence of these phenomena, the correlation between all pairs of variables was examined using a heatmap (Fig. S.2), complemented with the distribution of correlation coefficients (Fig. S.3), both reported in supplementary materials. From this analysis, it was obtained that only 17.6% of the examined correlation coefficients showed absolute values >0.5, suggesting that the overall behavior of the variables exhibits low correlation. Indeed, through the averaging of accuracies of the nine pairwise classifications, the univariate model yielded a value of 76%, whereas PLS-DA achieved 72%. Similar classification accuracy results were reported by other authors (Azcarate et al., 2015; Teixido-Orries, Molino, Femenias, Ramos, & Marín, 2023). Incidentally, Fig. 4 displays the algorithm iterations performed to determine the optimal number of latent variables for PLS-DA in the classification of HCA180 versus HCA105 samples. As depicted, the selection of 10 latent variables resulted in the minimum error rate in cross-validation. However, it was noted that about 50% of the samples were classified as unassigned. In order to balance the trade-off between error rate minimization and the number of unassigned samples, four latent variables were eventually selected as part of the model. For the samples analyzed in this study, utilizing the collective information from the entire metabolic profile may not be sufficient in accurately classifying samples from differently heated milk with the same ripening time. However, PLS-DA classification performances were adequate when the classifier was asked to distinguish samples based on ripening time with the same thermal treatment. These results suggest that ripening time exerts a more evident influence on metabolites profile under the same thermal conditions, thus offering more details into the dynamics of the ripening process.

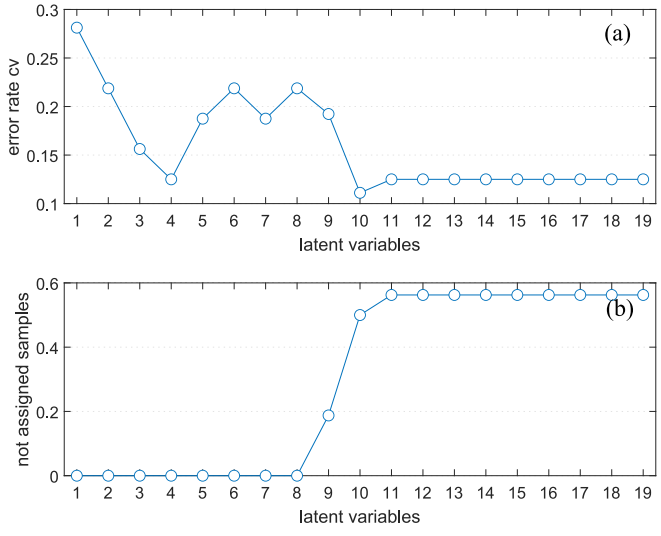

Fig. 4. Error rate (a) and ratio of not assigned samples (b) in cross validation as a function of the latent variables number included in the PLS-DA model using a Bayesian assignation criterion.

3.3. *Future perspectives*

For several years in Sardinia (Italy), producers of "Fiore Sardo DOP" sheep's cheese have raised concerns about unfair competition from producers flooding the market with counterfeit Fiore Sardo cheeses, notably those bearing the Fiore Sardo DOP label made from milk treated thermally (through thermization or pasteurization). Such counterfeiting practices would undoubtedly diminish the revenue, particularly for smaller producers, and inflict significant harm on the Protection Consortium. The relevance of this study lies in its potential practical applications to discriminate different cheesemaking practices, such as thermal treatment. This distinction is of crucial importance in ensuring compliance with PDO regulations and safeguarding the authenticity and quality of traditional cheeses.

The metabolic markers identified in this study, which were also confirmed by other works, show promise in differentiating various thermization processes and ripening times of cheese. However, further validation and replication of these results are necessary to establish their reliability across different types of cheese. Moreover, the study presented herein can support future research efforts in employing robust classification tools to reckon the authenticity of a broader range of PDO food products.

4. Conclusions

This study aimed to identify which metabolites were mainly responsible for the discrimination of cheese samples based on ripening time and thermal treatment. Two-way ANOVA was employed to test the hypothesis, showing that 60 metabolites were statistically significant in discriminating at least one of the two factors, comprising the interaction effect. The Benjamini-Hochberg method was applied to assess, by means of a more conservative approach, the significance of content variations of metabolites over different ripening times and thermal treatments. As a result of the analysis, 9 metabolites exhibited significant changes over the course of ripening, while only one metabolite underwent a significant change due to thermal treatment. Proline and 4-aminobutanoic acid were found to be upregulated in late ripening cheese, as confirmed by the statistical significance downstream FDR correction. The concentration of compounds belonging to biogenic amines, saccharides and carboxylic acids classes resulted to significantly change when milk was treated at 68 °C, compared to raw milk or milk treated at 57 °C. A univariate *t*-test classifier was then utilized to classify the test samples and showed improved accuracy compared to PLS-DA in terms of thermal treatment classification. Conversely, multivariate classifier performances were higher when it was tasked with ripening time-based classification. The results of this work support the use of a univariate approach in classifying the cheese samples under investigation. For this case study, the analysis of individual metabolites may provide precise information and be an effective tool for sample classification. In contrast, joint information from the entire metabolic profile did not yield optimal results in terms of an accurate overall classification. This outcome may be due to the scarce multicollinearity among the predictor variables (i.e. metabolite content) for which the multivariate model did not fit experimental data as accurately as the univariate approach. Therefore, it is important to consider the predictor variables correlation

and the analysis's purpose when deciding whether to use a univariate or multivariate model.

In conclusion, the application of an untargeted metabolomic proved to be a promising tool for a comprehensive understanding of the influence of thermal treatment and ripening time on the metabolic profile of Fiore Sardo cheese. Identifying the key metabolites responsible for such effects permitted the development of a classification tool capable of identifying cheese loaves subjected to different treatments.

CRediT authorship contribution statement

Leonardo Sibono: Writing – review & editing, Writing – original draft, Software, Data curation. **Cristina Manis:** Writing – review & editing, Writing – original draft, Methodology. **Francesca Zucca:** Data curation. **Luigi Atzori:** Writing – review & editing. **Stefania Tronci:** Data curation. **Mattia Casula:** Data curation. **Massimo Pes:** Methodology. **Pierluigi Caboni:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Massimiliano Grosso:** Data curation.

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 request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139930.

References

Anedda, R., Melis, R., & Curti, E. (2021). Quality control in Fiore Sardo PDO cheese: Detection of heat treatment application and production chain by MRI Relaxometry and image analysis. *Dairy, 2*(2), 270–287. https://doi.org/10.3390/dairy2020023

Atasoy, A. F., & Türkoğlu, H. (2008). Changes of composition and free fatty acid contents of Urfa cheeses (a white-brined Turkish cheese) during ripening: Effects of heat treatments and starter cultures. *Food Chemistry, 110*(3), 598–604. https://doi.org/10.1016/j.foodchem.2008.02.046

Azcarate, S. M., De Araújo Gomes, A., Alcaraz, M. R., Ugulino De Araújo, M. C., Camiña, J. M., & Goicoechea, H. C. (2015). Modeling excitation-emission fluorescence matrices with pattern recognition algorithms for classification of Argentine white wines according grape variety. *Food Chemistry, 184*, 214–219. https://doi.org/10.1016/j.foodchem.2015.03.081

Ballabio, D., & Consonni, V. (2013). Classification tools in chemistry. Part 1: Linear models. PLS-DA. *Analytical Methods, 5*(16), 3790–3798. https://doi.org/10.1039/c3ay40582f

Becchi, P. P., Rocchetti, G., Vezzulli, F., Lambri, M., & Lucini, L. (2023). The integrated metabolomics and sensory analyses unravel the peculiarities of mountain grassland-based cheese production: The case of Parmigiano Reggiano PDO. *Food Chemistry, 428*. https://doi.org/10.1016/j.foodchem.2023.136803

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B: Methodological, 57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Benkerroum, N. (2016). Biogenic amines in dairy products: Origin, incidence, and control means. *Comprehensive Reviews in Food Science and Food Safety, 15*(4), 801–826. https://doi.org/10.1111/1541-4337.12212

Berrar, D. (2018). Cross-validation. *Encyclopedia of Bioinformatics and Computational Biology: ABC of Bioinformatics, 1–3*, 542–545. https://doi.org/10.1016/B978-0-12-809633-8.20349-X

Brereton, R. G. (2009). *Chemometrics for Pattern Recognition. Chemometrics for pattern recognition*. https://doi.org/10.1002/9780470746462

Caboni, P., Maxia, D., Scano, P., Addis, M., Dedola, A., Pes, M., … Pirisi, A. (2019). A gas chromatography-mass spectrometry untargeted metabolomics approach to discriminate Fiore Sardo cheese produced from raw or thermized ovine milk. *Journal of Dairy Science, 102*(6), 5005–5018. https://doi.org/10.3168/jds.2018-15885

Caboni, P., Murgia, A., Porcu, A., Demuru, M., Pulina, G., & Nudda, A. (2016). Gas chromatography-mass spectrometry metabolomics of goat milk with different polymorphism at the αS1-casein genotype locus. *Journal of Dairy Science, 99*(8), 6046–6051. https://doi.org/10.3168/jds.2015-10537

Caboni, P., Murgia, A., Porcu, A., Manis, C., Ibba, I., Contu, M., & Scano, P. (2019). A metabolomics comparison between sheep's and goat's milk. *Food Research International, 119*, 869–875. https://doi.org/10.1016/j.foodres.2018.10.071

Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. *International Dairy Journal, 13*(11), 841–866. https://doi.org/10.1016/S0958-6946(03)00109-2

Combarros-Fuertes, P., Fernández, D., Arenas, R., Diezhandino, I., Tornadijo, M. E., & Fresno, J. M. (2016). Biogenic amines in Zamorano cheese: Factors involved in their accumulation. *Journal of the Science of Food and Agriculture, 96*(1), 295–305. https://doi.org/10.1002/jsfa.7093

Conte, P., Cinquanta, L., Lo Meo, P., Mazza, F., Micalizzi, A., & Corona, O. (2021). Fast field cycling NMR relaxometry as a tool to monitor Parmigiano Reggiano cheese ripening. *Food Research International, 139*. https://doi.org/10.1016/j.foodres.2020.109845

Dedola, A. S., Piras, L., Addis, M., Pirisi, A., Piredda, G., Mara, A., & Sanna, G. (2020). New analytical tools for unmasking frauds in raw milk-based dairy products: Assessment, validation and application to fiore sardo PDO cheese of a RP-HPLC method for the evaluation of the α-l-fucosidase activity. *Separations, 7*(3), 1–11. https://doi.org/10.3390/separations7030040

FAO. (2023). Gateway to dairy production and products. Retrieved March 20, 2011, from https://www.fao.org/dairy-production-products/products/en/.

Ferrante, M. C., & Mercogliano, R. (2023). Focus on histamine production during cheese manufacture and processing: A review. *Food Chemistry, 419*. https://doi.org/10.1016/j.foodchem.2023.136046

Hassan, F. A. M., Abd El-Gawad, M. A. M., & Enab, A. K. (2012). Flavour compounds in cheese (review). *International Journal of Academic Research, 4*(5), 169–181. https://doi.org/10.7813/2075-4124.2012/4-5/a.20

Idle, J. R., & Gonzalez, F. J. (2007). Metabolomics. *Cell Metabolism, 6*(5), 348–351. https://doi.org/10.1016/j.cmet.2007.10.005

Kanani, H., Chrysanthopoulos, P. K., & Klapa, M. I. (2008). Standardizing GC-MS metabolomics. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 871*(2), 191–201. https://doi.org/10.1016/j.jchromb.2008.04.049

Kandasamy, S., Yoo, J., Yun, J., Kang, H. B., Seol, K. H., & Ham, J. S. (2021). Quantitative analysis of biogenic amines in different cheese varieties obtained from the korean domestic and retail markets. *Metabolites, 11*(1), 1–16. https://doi.org/10.3390/metabo11010031

Kebary, K. M. K., El-Sonbaty, A. H., & Badawi, R. M. (1999). Effects of heating milk and accelerating ripening of low fat Ras cheese on biogenic amines and free amino acids development. *Food Chemistry, 64*(1), 67–75. https://doi.org/10.1016/S0308-8146(98)00085-5

Khattab, A. R., Guirguis, H. A., Tawfik, S. M., & Farag, M. A. (2019). Cheese ripening: A review on modern technologies towards flavor enhancement, process acceleration and improved quality assessment. *Trends in Food Science and Technology, 88*, 343–360. https://doi.org/10.1016/j.tifs.2019.03.009

Kováts, E. (1958). Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone. *Helvetica Chimica Acta, 41*(7), 1915–1932. https://doi.org/10.1002/hlca.19580410703

Lee, S., & Lee, D. K. (2018). What is the proper way to apply the multiple comparison test? *Korean Journal of Anesthesiology, 71*(5), 353–360. https://doi.org/10.4097/kja.d.18.00242

Liao, S. M., & Akritas, M. (2007). Test-based classification: A linkage between classification and statistical testing. *Statistics & Probability Letters, 77*(12), 1269–1281. https://doi.org/10.1016/j.spl.2007.03.012

Maroco, J., Silva, D., Rodrigues, A., Guerreiro, M., Santana, I., & De Mendonça, A. (2011). Data mining methods in the prediction of dementia: A real-data comparison of the accuracy, sensitivity and specificity of linear discriminant analysis, logistic regression, neural networks, support vector machines, classification trees and random forests. *BMC Research Notes, 4*. https://doi.org/10.1186/1756-0500-4-299

Moniente, M., Botello-Morte, L., García-Gonzalo, D., Virto, R., Pagán, R., Ferreira, V., & Ontañón, I. (2023). Combination of SPE and fluorescent detection of AQC-derivatives for the determination at sub-mg/L levels of biogenic amines in dairy products. *Food Research International, 165*. https://doi.org/10.1016/j.foodres.2022.112448

Montgomery, D. (2013). *Design and analysis of experiments*. John Wiley \& Sons, Inc.

Natrella, G., Gambacorta, G., Squeo, G., & Faccia, M. (2023). Impact of Milk Thermization on the quality characteristics of P.D.O. "Canestrato Pugliese" ovine hard cheese. *Foods, 12*(5). https://doi.org/10.3390/foods12051080

NIST. (2005). Mass spectral library (NIST/EPA/NIH). Retrieved from http://www.nist.gov/.

Nomura, M., Kimoto, H., Someya, Y., Furukawa, S., & Suzuki, I. (1998). Production of γ-aminobutyric acid by cheese starters during cheese ripening. *Journal of Dairy Science, 81*(6), 1486–1491. https://doi.org/10.3168/jds.S0022-0302(98)75714-5

OECD/FAO. (2021). *OECD-FAO Agricultural Outlook 2021–2030*. Retrieved from. https://doi.org/10.1787/19428846-en.

Oliver, S. G., Winson, M. K., Kell, D. B., & Baganz, F. (1998). Systematic functional analysis of the yeast genome. *Trends in Biotechnology, 16*(9), 373–378. https://doi.org/10.1016/S0167-7799(98)01214-1

Putri, S. P., Ikram, M. M. M., Sato, A., Dahlan, H. A., Rahmawati, D., Ohto, Y., & Fukusaki, E. (2022). Application of gas chromatography-mass spectrometry-based metabolomics in food science and technology. *Journal of Bioscience and Bioengineering, 133*(5), 425–435. https://doi.org/10.1016/j.jbiosc.2022.01.011

del Rio, B., Fernandez, M., Redruello, B., Ladero, V., & Alvarez, M. A. (2024). New insights into the toxicological effects of dietary biogenic amines. *Food Chemistry, 435*. https://doi.org/10.1016/j.foodchem.2023.137558

Rocchetti, G., Lucini, L., Gallo, A., Masoero, F., Trevisan, M., & Giuberti, G. (2018). Untargeted metabolomics reveals differences in chemical fingerprints between PDO and non-PDO Grana Padano cheeses. *Food Research International, 113*, 407–413. https://doi.org/10.1016/j.foodres.2018.07.029

Rocchetti, G., Michelini, S., Pizzamiglio, V., Masoero, F., & Lucini, L. (2021). A combined metabolomics and peptidomics approach to discriminate anomalous rind inclusion levels in Parmigiano Reggiano PDO grated hard cheese from different ripening stages. *Food Research International, 149*. https://doi.org/10.1016/j.foodres.2021.110654

Saccenti, E., Hoefsloot, H. C. J., Smilde, A. K., Westerhuis, J. A., & Hendriks, M. M. W. B. (2014). Reflections on univariate and multivariate analysis of metabolomics data. *Metabolomics, 10*(3), 361–374. https://doi.org/10.1007/s11306-013-0598-6

Santiago-López, L., Aguilar-Toalá, J. E., Hernández-Mendoza, A., Vallejo-Cordoba, B., Liceaga, A. M., & González-Córdova, A. F. (2018). Invited review: Bioactive compounds produced during cheese ripening and health effects associated with aged cheese consumption. *Journal of Dairy Science, 101*(5), 3742–3757. https://doi.org/10.3168/jds.2017-13465

Sibono, L., Grosso, M., Tronci, S., Errico, M., Addis, M., Vacca, M., … Caboni, P. (2023). Investigation of seasonal variation in fatty acid and mineral concentrations of pecorino Romano PDO cheese: Imputation of missing values for enhanced classification and metabolic profile reconstruction. *Metabolites, 13*(7). https://doi.org/10.3390/metabo13070877

Silva, H. L. A., Balthazar, C. F., Esmerino, E. A., Vieira, A. H., Cappato, L. P., Neto, R. P. C., … Cruz, A. G. (2017). Effect of sodium reduction and flavor enhancer addition on probiotic Prato cheese processing. *Food Research International, 99*, 247–255. https://doi.org/10.1016/j.foodres.2017.05.018

Singh, T. P., Arora, S., Borad, S. G., Bam, J., Paul, V., Thomas, R., & Sarkar, M. (2023). Fatty acid and amino acid profiling, antioxidant activity and other quality characteristics of vacuum packed cheddar style-yak milk cheese during ripening. *Food Bioscience, 51*. https://doi.org/10.1016/j.fbio.2022.102213

Teixido-Orries, I., Molino, F., Femenias, A., Ramos, A. J., & Marín, S. (2023). Quantification and classification of deoxynivalenol-contaminated oat samples by near-infrared hyperspectral imaging. *Food Chemistry, 417*. https://doi.org/10.1016/j.foodchem.2023.135924

Tekin, A., & Güler, Z. (2019). Glycolysis, lipolysis and proteolysis in raw sheep milk Tulum cheese during production and ripening: Effect of ripening materials. *Food Chemistry, 286*, 160–169. https://doi.org/10.1016/j.foodchem.2019.01.190

Varmuza, K., & Filzmoser, P. (2016). *Introduction to Multivariate Statistical Analysis in Chemometrics. Introduction to multivariate statistical analysis in Chemometrics*. https://doi.org/10.1201/9781420059496

Wold, S., Sjöström, M., & Eriksson, L. (2001). PLS-regression: A basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems, 58*(2), 109–130. https://doi.org/10.1016/S0169-7439(01)00155-1

Yılmaz, C., Kocadağlı, T., & Gökmen, V. (2023). Determination of endocannabinoids in fermented foods of animal and plant origin by liquid chromatography tandem mass spectrometry. *Food Chemistry, 427*. https://doi.org/10.1016/j.foodchem.2023.136766