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~~Biogenic amines in typical Italian cheeses: comprehensive correlation of their content with processing and nutritional characteristics~~

Chromatographic determination of biogenic amines in four typical Italian cheeses for a comprehensive correlation with processing and nutritional characteristics through chemometric approaches

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ABSTRACT

BACKGROUND: The presence of biogenic amines (BAs) in fermented food is well ascertained. ~~Biogenic amines~~ BAs can may affect food quality, ~~and can pose~~ ing risk health effects ~~risks~~.

In this work four typical Italian cheeses exported worldwide, ~~Primo sale siciliano, Grana Padano, Gorgonzola and Parmigiano Reggiano~~, were analyzed to determine the possible presence of ~~BAs, biogenic amines (ethanolamine, ethylamine, putrescine, cadaverine, histamine, spermidine, and spermine)~~. The cheese samples were analyzed as such and having been subjected to common or incorrect consumer handling (domestic grating, unrefrigerated storage ~~et al.~~).

RESULTS: A ~~chromatographic-amperometric~~ eation exchange method, ~~using methanesulfonic as the eluent, coupled with integrated amperometric detection~~ was developed, and validated ~~the performance of the method was assessed~~. Extraction of BAs ~~biogenic amines~~ was performed by the addition of methanesulfonic acid ~~eluent~~, determining matrix effect and recovery of biogenic amines directly within the cheeses.

~~Biogenic amine~~ BAs were present in the range of 0.019-0.044 mg/kg⁻¹, well below the current EU ~~limit currently set by the EU~~. Home-manipulation confirmed recontamination of the cheese. Through the multivariate statistical analysis (principal component analysis), the contents of BAs ~~biogenic amines~~ were correlated to the main processing parameters and to the nutritional properties of the four cheeses. This is believed to be the first study that presents this correlation.

CONCLUSION: ~~This study highlights correlations among~~ BAs and carbohydrates and anticorrelations with pH and in a less extent with moisture. ~~cadaverine, putrescine, and spermidine~~. Interestingly, ~~these biogenic amines~~ BAs are correlated with fat content. This correlation was confirmed by a new principal component analysis performed on our data set with additional data from the literature.

KEYWORDS: biogenic amines; ion chromatography; cheese; principal component analysis; fat correlation.

INTRODUCTION

Biogenic amines (BAs) are basic compounds with biological activity that can be produced in food due to the decarboxylation of amino acids. In cheese, the degradation of casein during maturation leads to the accumulation of free amino acids which can be converted into BAs by the bacterial decarboxylases of the contaminating microflora ¹. Among BAs, ethanolamine, ethylamine, putrescine, cadaverine, histamine, spermidine and spermine are primary and secondary biogenic amines which can be formed in cheese according to the producer microorganism ²⁻⁴.

The presence of BAs in any cheese is related to its manufacturing, ripening and storage conditions ⁵. Salt concentration, pH, bacterial activity, humidity, storage temperature, ripening time, and type of microorganisms are well-known factors which affect the formation of biogenic amines ⁶. Generally, the currently available literature studies on the formation of BAs only deal with one of the factors mentioned above ⁶. Comprehensive approaches that analyzes multiple factors and conditions that originate the BAs are usually under-investigated ⁷ and need better attention.

Although the presence of some biogenic amines is also related to the flavor properties of the final cheese product ⁸, it may also be responsible for the well-documented toxicological effects ^{9, 10}. Therefore, the determination of BAs in a cheese is necessary to determine the toxicological risk associated with its consumption; but it also estimates the quality of the cheese.

The determination of BAs can be carried out with various analytical methods based on separation techniques, such as gas chromatography ¹¹, thin-layer chromatography ¹², capillary electrophoresis ¹³ and, above all, high-performance liquid chromatography (HPLC) ^{8, 14}.

By far, HPLC methods, including reversed-phase and ion-exchange mechanisms ¹⁵, are the most widely employed approaches for the analysis of BAs, but they have the intrinsic disadvantage that pre-column ¹⁶ or post-column derivatization ¹⁵ is needed to obtain sensitive instrumental detection even with mass spectrometry ¹⁷. Recently, to improve the sensitivity, in comparison to the more conventional HPLC-fluorescence technique, innovative approaches based on HPLC combined with thermal lens spectrometry (TLS) have been investigated ¹⁸.

Screening techniques based on nuclear magnetic resonance analysis ¹⁷ or on TLS after enzymatic reaction ¹⁸, allow determination of total content of BA, while suitable biosensoristic techniques ¹⁹ allow the determination of single amines.

Screening techniques based on nuclear magnetic resonance analysis or on TLS after enzymatic reaction allow to determine the total BA content, while specific biosensors are developed to determine a specific amine. However, the availability of direct and simple analytical methods capable of discriminating and simultaneously determining each individual BA is of great utility for food processing and control.

~~In light of the above considerations,~~ In the light of the considerations mentioned above, this manuscript deals with many aspects concerning the presence of BAs in Italian cheese.

~~For this work, the purpose of this work is to measure the content of BA in~~ four typical Italian cheeses (Primo Sicilian salt, Grana Padano, Gorgonzola, Parmigiano Reggiano) were chosen since ~~that they~~ are produced and sold under very different conditions which give them typicality and uniqueness. ~~The~~Seven BAs ~~were~~ investigated ~~were~~ (i.e.: ethanolamine, ethylamine, putrescine, cadaverine, histamine, spermidine and spermine) ~~and they were~~ chosen according to their possible ² or documented ^{1,3} occurrence in cheese-³.

After measuring the BA concentrations in the four cheeses, ~~Additionally,~~ the variation of the BA content in the same cheeses was studied after simulating typical consumer behaviours (domestic grid, storage outside the refrigerator), in order to highlight any change in BAs content after purchase. For analysis, BAs were extracted by methanesulfonic acid and the extract was analysed by ion chromatography (IC) with integrated pulsed amperometric detection (IPAD).

Because of the limited information concerning the effect of the variables inherent in the cheese production process on the formation of biogenic amines, the study was also addressed to the investigation of these aspects.

As a further novel aspect, this research aims to investigate whether the presence of biogenic amines in the cheese may be correlated to the nutritional properties of the cheese itself. For this purpose, c

Correlations and similarities among the BAs found in cheeses with the main characteristics (pH, humidity, ripening time, ash) and nutritional properties (salt, fats, proteins, carbohydrates) of the cheeses themselves were investigated by applying the Principal Component Analysis (PCA).

To self-test the results obtained by PCA, data collected from a recent published study were added to the results obtained in this study and a new PCA was derived and discussed.

MATERIALS AND METHODS

Reagents and solutions

All reagents were of analytical grade and obtained from Sigma Aldrich (Chemie, Steinheim, DE). For eluent and standard solution preparation, ultrapure water (18.2 MΩcm at 25°C) was obtained by a Milli-Q Academic system (Millipore, Billerica, MA, USA).

Instrumentation

Biogenic amines were determined using an ICS-3000 (Dionex, Thermofisher, USA) ion chromatograph equipped with a 10 μL-loop and with an AD40 Electrochemical Detector (Dionex, Thermo Scientific), with a Ag/AgCl reference electrode and a gold (Au) working electrode, imposing amine waveform²⁰, with minor modifications: 0 see, 0.130 V; 0.04 see, 0.130 V; 0.05 see, 0.33 V; 0.21 see, 0.33 V; 0.22 see, 0.55 V; 0.46 see, 0.55 V; 0.47 see, 0.33 V; 0.56 see, 0.33 V; 0.57 see, -1.67 V; 0.58 see, -1.67 V; 0.59 see, 0.93 V; 0.60 see, 0.13 V. Signal was integrated between 0.21 and 0.47 seconds.

For the chromatographic separation, an IonPac CG18 (2 x 50 mm) guard column and an IonPac CS18 (2 x 250 mm) analytical column from Thermofisher were used. After testing the separation of the amines with isocratic elution, the following gradient conditions were used. Eluent: methanesulfonic acid, MSA, 0.3 mL min⁻¹; t=0-6 min 3 mM MSA; t=6-10 min 3-10 mM MSA; t=10-22 min 10-15 mM MSA; t=22-28 min 15 mM MSA; t=28-35 min 15-30 mM MSA; t=35-45 min 30-45 mM MSA.

After the chromatographic elution and before detection, the eluate was basified with 0.1 M NaOH (driven at 0.25 mL min⁻¹) through a 3-way mixing tee, to neutralize the amines.

Chromatographic and amperometric data were collected and elaborated by the software Chromeleon 6.80 (Dionex, Thermofisher).

Cheese samples

Four cheese samples were purchased for this work: Primo Sale Siciliano, Grana Padano, Gorgonzola and Parmigiano Reggiano pre-grated packed in a zippered envelope.

Primo Sale Siciliano was bought in an open street market in Sicily (South Italy) and transported to Turin, in northern Italy.

Grana Padano, Gorgonzola and pre-grated Parmigiano Reggiano were purchased from a local Turin supermarket.

To evaluate the variation of the BA content originating from the most common consumer habits, the following experimental design was followed.

- Primo sale and Gorgonzola cheeses were analysed as such;
- Grana Padano was analysed as such and after home grating;
- Parmigiano Reggiano was analyzed after proper storage of 3 days in the refrigerator and after an improper storage period of 3 days unrefrigerated after opening.

The physico-chemical and nutritional characteristics of each cheese have been obtained from the sheets provided by the producers and are summarized in Table 1. These data were used in the PCA analysis (performed by the XLStat software, version 2016).

Extraction of BAs from cheese samples and analysis by IC

The extraction procedure, performed in triplicate was the following: 2 g of each cheese sample were crumbled, put in a test tube and added with 15 mL of 20 mM MSA. The test tube was posed in an ultrasound bath for 15 min and frozen for 25 min. Afterwards, the test tube was centrifuged at 4500 \times g for 10 min. The supernatant was collected, filtered into 0.45 μ m-mixed cellulose esters filters, diluted to 25 mL with ultrapure water and injected for IC analysis.

Quantitation of BAs was performed by the standard addition method.

RESULTS AND DISCUSSION

Chromatographic separation of BAs and figures of merit

The determination of BAs is based on ion chromatography coupled with integrated pulsed amperometric detection (IPAD). This detection technique provides a direct analysis of BA without the need for a derivatization step and is highly selective, thus improving the already high selectivity performance of the chromatographic separation²¹⁻²³.

To elute the seven selected BAs, several isocratic elutions with MSA were tested. At all the investigated MSA concentrations, the observed elution order (ethanolamine, ethylamine, putrescine, cadaverine, histamine, spermidine and spermine) roughly followed the number of amine groups and hydrophobicity of the molecule. The first eluted compounds, i.e.: ethanolamine and ethylamine, have a similar selectivity and require low eluting power to be resolved. Conversely, the two most hydrophobic divalent amines, namely spermidine and spermine, need a concentrated mobile phase to elute as symmetrical peaks at reasonable retention times. The gradient elution used (see Materials and Methods section) allowed good separation among the considered BAs (Figure 1).

At these experimental conditions, linearity was verified by injecting five different solutions containing the seven BAs. For each calibration level, injections were performed in triplicates. Limits of detection (LODs) and limits of quantitation (LOQs) were calculated as follow: $LOD = 3 \times SD_{xy}/b$ and $LOQ = 10 \times SD_{xy}/b$ (where SD_{xy} is the standard deviation of the response on the y axis over the whole calibration curve and b is the slope of the calibration curve)²⁴. The linearity of the method was verified by Root Mean Square Error (RMSE), which was found to be lower of at least two orders of magnitude than chromatographic peak areas. Results are summarized in Table 2.

The LOD values obtained are in line with those observed for BAs analysed with HPLC and fluorescence detection (same injection loop volume), after pre-column derivatization with 2,3-dicarboxaldehyde¹⁸.

Analysis of Italian cheeses by IC-IPAD

Recovery and Matrix effect (ME) evaluation

Recovery and ME were determined by adding putrescine, as a model compound, to two cheeses in which the absence of such amine was previously verified. The procedure was as follows: BAs were extracted from the cheeses as detailed in the Materials and Methods section; post-extracted solutions were added with 5 mg L^{-1} putrescine and injected for IC-IPAD analysis. The obtained chromatographic area ($A_{std,matrix}$) was compared with that obtained by adding the same amount of analyte in the extraction solvent ($A_{std,solvent}$); ME was calculated according to the following equation $ME (\%) = 100 \cdot (A_{std,matrix} - A_{std,solvent}) / A_{std,solvent}$ and was 22.2%.

Since it is commonly considered that a $ME > |20\%|$ may have a significant impact on the performance of the method ²⁵, the standard addition approach was preferred for quantification.

To evaluate the extraction recovery, 2 g of each cheese were spiked and homogenized with 1 mL of 100 mg L^{-1} putrescine, to give a nominal concentration of 5 mg L^{-1} in the extract. Afterwards the cheeses were extracted as detailed in the Materials and Methods section and injected for IC-IPAD analysis ($A_{\text{spiked cheese}}$). The percentage extraction recovery (ER%), calculated according to the following equation $ER\% = 100 \cdot (A_{\text{spiked cheese}} / A_{\text{std.matrix}})$, was 70.4%. Considering the extraction procedure used, the method detection limits thus range from about 0.007 (ethylamine, spermine) to about 0.007 (ethanolamine, putrescine) $mg \cdot kg^{-1}$.

BAs in Italian cheeses

The concentrations of BA found in the four Italian cheeses after IC-IPAD analysis of the relative extracts are shown in Table 3. As shown, sweet Gorgonzola is the only cheese in which the BAs were not found.

As far as Gorgonzola cheese is concerned, few studies are currently available in the literature and do not specify the type of Gorgonzola analyzed ¹. It is therefore believed, this is the first study in which the content of BA has been analyzed in a sweet Gorgonzola cheese type.

The content of ethanolamine in Grana Padano and Parmigiano Reggiano is in agreement with the one observed in other studies ¹. Other amines were not detected below the quantitation limits. It is interesting to note that the common user's habit to grate cheese at home almost doubled the amount of the only BA detected (ethanolamine). This observation is in agreement with the microbiological recontamination of food products, following the use of cutting boards or utensils ²⁶, well documented in the case of histamine for home-made Emmental cheese ²⁷. Finally, improper storage outside the refrigerator has increased the content of the only detected BA (ethanolamine) of about 63%. This behavior is consistent with the effect of temperature on BA accumulation ²⁸.

Primo Sale, a hard cheese from Sicily, is the cheese with the highest BA number. At this time, there does not appear to be any comparison possible with data concerning the presence of BA in this type of cheese. However, it is known that Gram-negative bacteria (mainly Enterobacteriaceae) which may be present in milk are able to produce histamine, putrescine and cadaverine ²⁸. Alternatively, a survey of fifty samples of Primo Sale, purchased from four historical markets in Sicily and from retail sale premises along the market streets, highlighted that 54% of

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the samples were of unsatisfactory or borderline microbiological quality according to criteria in EC Regulation 2005/2073/EC. Indeed, taking into account the levels of Enterobacteriaceae, 42% of the tested samples were characterized by bad hygienic quality²⁹. There is a presumption that selling, and transportation conditions could explain the presence of the detected BAs (i.e. putrescine, cadaverine and spermidine).

Currently regulatory, maximum limit for BAs in cheese has been established. The European Union limit of 0.100 mg/kg^{-1} is established only for histamine in fish by Directive 2073/2005. However, an intake greater than 0.040 mg of BA per meal was considered potentially toxic³⁰. In our study, the sum of the concentrations of BAs in cheeses varies from 0.019 to 0.044 mg/kg^{-1} , clearly showing that the content of BA in all cheeses is well below the thresholds mentioned above.

Correlations between concentration of BAs and processing and nutritional parameters of Italian cheeses

Principal Component Analysis (PCA) is a powerful multivariate technique in which linear combinations of the variables is performed to calculate new not correlated variables, called principal components.

PCA was initially performed considering the BA concentrations measured in the four cheeses and the physico-chemical and nutritional properties listed in Table 1.

A correct chemometric approach requires that variables represented less than 50% in objects must be discarded or incorporated into a single variable³¹. For three out of four cheeses (PCA objects), putrescine, cadaverine and spermidine were under the detection limit. Conversely, ethanolamine was detected in all cheese samples. For this reason, putrescine, cadaverine and spermidine were introduced in the PCA as a sum together with ethanolamine; moreover, ethanolamine was considered as a variable, singularly. Ethylamine, histamine and spermine were not detected in all the samples, and hence they were not included in the chemometric analysis.

Data on the content of BA in grated Grana Padano and Parmigiano Reggiano stored outside the refrigerator were not included in the PCA because the physico-chemical and nutritional properties of the cheeses could be different from those of the original fresh products. Prior to PCA, data were autoscaled. The scree plot analysis showed that the greatest part of the information (variance) of the original dataset is contained in the first 2 principal components (7678.43%),

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which were therefore considered for the graphical representation (F1 vs F2, Figure 2). The biplot in Figure 2 combines the plots of the scores (coordinates of the objects on the new variables F1, F2) and of the loadings (weights of original variables on the linear combination principal components), allowing to identify groups of objects with similar ~~behaviour~~behavior as well as correlation among the original variables. Some considerations are reported below. The plot of the scores identifies four well separate groups, coherently with the four types of cheese. It can be observed that, Ethanolamine ~~ethanolamine~~ is ~~uncorrelated with cadaverine, putrescine and spermidine~~ but correlated with ripening time, ash, salt, ~~sugars~~ and proteins, but ~~Ethanolamine~~ is anti-correlated with humidity. The loadings of the total sum of BAs, Cadaverine, putrescine and spermidine are highly correlated ~~which included also cadaverine, putrescine and spermidine, is instead highly correlated with fats, carbohydrates and hence energy.~~ ~~Although multivariate analysis on BAs in cheeses is very scarce, a statistic correlation between putrescine and cadaverine was reported⁷. Increases in putrescine and cadaverine concentrations are considered to correlate with bacterial starter activities and counts in fermented foods³².~~

~~Cadaverine, putrescine and spermidine are correlated to fats, carbohydrates and hence energy.~~ The correlation of carbohydrates with ~~these three biogenic~~ amines may be explained by the fact that the presence of fermentable carbohydrate enhances amino acid decarboxylase activity in bacteria³².

There is no prevalence in current literature relating to the dependence of the biogenic amine content with the fat content in cheese or dairy products. However, a decrease in the concentration of cadaverine, putrescine and spermidine with the decrease in fat content in cheese milk was observed by Kebary³³, who attributed this phenomenon to the minor activity of water derived from high salt content and lower humidity. In our work, these BAs are well represented by the sum of BAs loadings.

The data shown in Figure 2 ~~is~~ are very informative and in accordance with previous statements, since they reveal: (i) anti-correlation between salt and moisture; (ii) anti ~~non~~-correlation between ~~cadaverine, putrescine, spermidine~~ the sum of BAs and salt; ~~(ii) anti correlation between cadaverine, putrescine, spermidine and salt;~~ (iii) a partial anti ~~anti~~ correlation between ~~cadaverine, putrescine, spermidine~~ the sum of BAs and moisture. This latter behavior is in good agreement to what reported by Swelam and Mehanma in their study on correlation of some properties of Egyptian Ras cheese with chemical constituents³⁴.

The data in Figure 2 shows ~~an no apparent~~anti-correlation between the BA content and the pH. Indeed, the effect of pH is inherent to the fermentation process ~~and-~~ mMany authors agree that low pH values ~~favour~~favor the accumulation of BAs ³⁵ ~~even if low pH values can inhibit the growth of decarboxylating microorganisms, thus reducing the formation of BAs~~ ²⁸.

To the best of our knowledge, this is the first study in which the PCA also includes nutritional properties for the complete characterization of cheeses in relation to the content of BAs. Indeed, no direct comparison is currently available to discuss any similarity or difference with previous studies.

To verify the considerations derived from the PCA and discussed above, a new PCA was performed, adding to our data, those available from Marijan and coworkers ⁶ related to a hard cheese (Livno) at different stages of ripeness. In detail, the new data introduced in the PCA include physico-chemical parameters (pH, humidity, ash) and nutritional parameters (salts, fats, proteins) common to those considered in our present study as well as the content of three BAs in common with our study (putrescine, cadaverine, spermidine, expressed again a sum).

Although a larger number of objects (cheese samples) and new data for each variable were added to the PCA, the new biplot chart (Figure 3) confirms the results previously obtained and discussed (Figure 2). ~~i~~In detail, the correlation between putrescine, spermine and cadaverine is still present. The above mentioned the sum of BAs are-is still correlated with fat content and anticorrelated with pH. Anticorrelation between ash and humidity is still evident.

With regard to the content of BAs as a function of the ripening time, it is very difficult to highlight a well-defined anti-correlation and/or non-correlation in the two PCAs. These results do not contradict those obtained by Guarcello and colleagues ⁷, which showed an anti-correlation between the putrescine and cadaverine content with the ripening time. Finally, as expected, cheese samples from Marijan study are in a ~~well defined~~separated cluster since the new objects belong to the same kind of cheese (Livno).

CONCLUSION

The evaluation of the content of biogenic amines in cheeses is important to define a possible health risk and to evaluate the effectiveness of the storage conditions. Ion chromatography coupled with integrated pulsed amperometric detection allows the direct determination of biogenic amines in cheese without derivatization. With this analytical approach, four typical Italian cheeses

were analyzed to determine the BA content in freshly purchased cheeses and subjected to domestic manipulation immediately after purchase. The grating of the cheese seems to increase the content of BAs due to the possible microbiological contamination of cooking utensils. ~~The PCA analysis of our data alone or integrated with those obtained from the literature, indicates a strong correlation for the presence of cadaverine, putrescine and spermidine.~~ For the first time, the correlations between the BA content and nutritional properties through PCA analysis are shown here. The multivariate analysis shows a correlation of the sum of BAs ~~three amines~~ with fats and carbohydrates, thus introducing further indications on the presence of BAs expected, depending on the nutritional qualities of the cheeses. Unlike what is usually reported in the literature, the maturation time is not always a specific parameter that influences the BA content.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

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Table 1. Physico-chemical and nutritional properties of the cheeses analysed, according to manufacture indications.

Property	Primo Sale	Grana Padano	Sweet Gorgonzola	Parmigiano Reggiano
<i>pH</i>	5.25	5.35	6.00	5.40
<i>Ripening time (days)</i>	15	360	60	360
<i>Humidity (%)</i>	46.3	32	49	30.8
<i>Salt (%)</i>	1.55	5	1.81	1.6
<i>Fats (%)</i>	32.8	28	27	28.4
<i>Saturated fats</i>	19.8	18	19.4	20.9
<i>Carbohydrates (%)</i>	3.8	0	0.9	4.1
<i>Sugars (%)</i>	0.07	0	0.1	0.9
<i>Proteins (%)</i>	29	33	19.47	32
<i>Energy (Kcal)</i>	436	395	330	399

Table 2. Linearity, LOD and LOQ values expressed in $\mu\text{g/L}^{-1}$ for the IC-IPAD method.

	Slope	Intercept	R²	RMSE	LOD	LOQ
<i>Ethanolamine</i>	2.977	4.333	0.9996	0.0829	91	278
<i>Ethylamine</i>	0.322	0.443	0.9999	0.0009	9	28
<i>Putrescine</i>	1.846	2.644	0.9998	0.0610	109	330
<i>Cadaverine</i>	0.752	3.255	0.9999	0.0136	60	183
<i>Histamine</i>	1.666	9.509	0.9995	0.0164	32	106
<i>Spermidine</i>	2.089	3.245	0.9999	0.0175	27	82
<i>Spermine</i>	1.174	3.072	0.9995	0.0345	10	33

RMSE: root mean square error. It represents the standard deviation of the differences between predicted y-values and observed y-values.

Table 3. Content of biogenic amines in four typical Italian cheeses evaluated by IC-IPAD.

Cheese	Biogenic amines (mg/kg^{-1})							Σ
	Ethanolamine	Ethylamine	Putrescine	Cadaverine	Histamine	Spermidine	Spermine	
<i>Primo Sale Siciliano</i>	<u>0.004</u> \pm <u>0.005</u>	n.d.	<u>0.021</u> \pm <u>0.001</u> \pm 4	<u>0.009</u> \pm <u>0.001</u> \pm 0	n.d.	<u>0.002</u> \pm <u>0.0002</u>	n.d.	<u>0.037</u> \pm 1
<i>Grana Padano</i> ^{a)}	<u>0.019</u> \pm <u>0.001</u> \pm 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<u>0.019</u> \pm 0
<i>Grana Padano</i> ^{b)}	<u>0.037</u> \pm <u>0.002</u> \pm 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<u>0.037</u> \pm 6
<i>Sweet Gorgonzola</i>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
<i>Parmigiano reggiano</i> ^{c)}	<u>0.026</u> \pm <u>0.002</u> \pm 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<u>0.026</u> \pm 9
<i>Parmigiano reggiano</i> ^{d)}	<u>0.043</u> \pm <u>0.003</u> \pm 8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<u>0.043</u> \pm 9

^{a)} As purchased

^{b)} Home-grated

^{c)} Correctly stored in fridge

^{d)} Improperly stored, for 3 days out of fridge

n.d.: not detected below the method detection limits (see text)

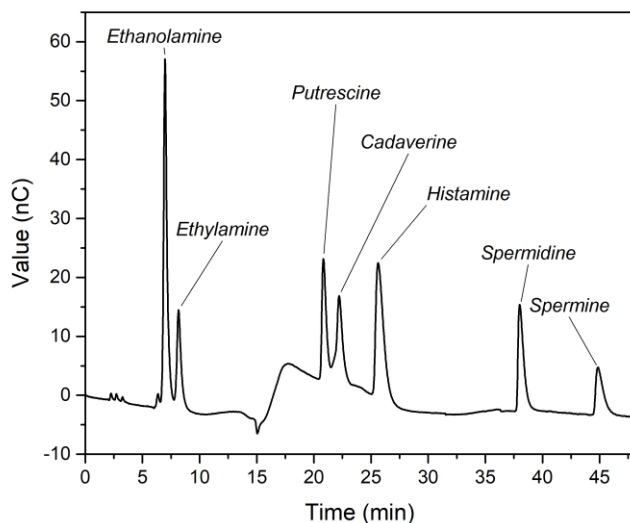
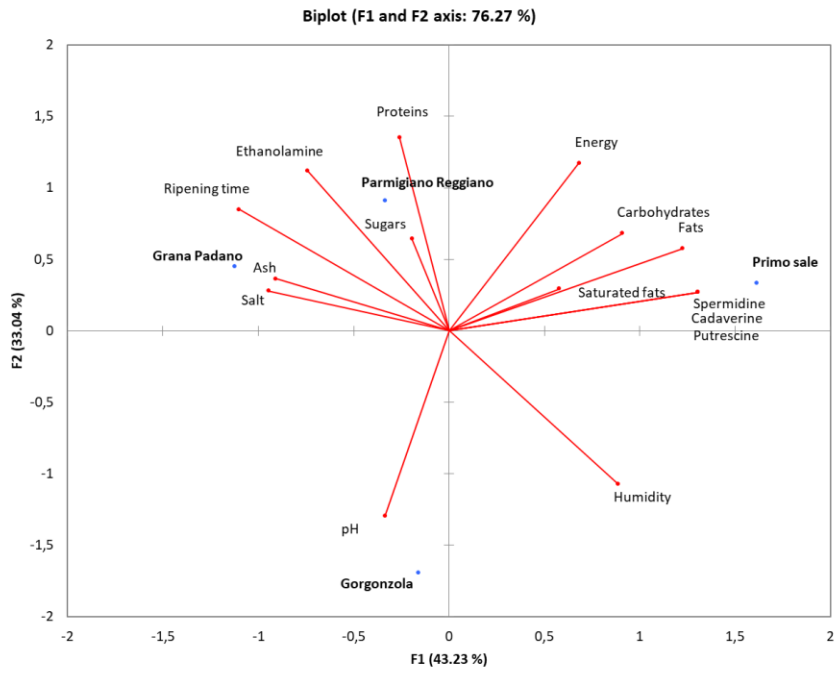


Figure 1. Determination of the seven BAs by ion chromatography with integrated pulsed amperometric detection. Column: IonPac CG 18 (2 x 50 mm) and IonPac CS18 (2 x 250 mm). Gradient conditions (eluent methanesulfonic acid, MSA, 0.3 mL min⁻¹): t=0-6 min 3 mM MSA; t=6-10 min 3-10 mM MSA; t=10-22 min 10-15 mM MSA; t=22-28 min 15 mM MSA; t=28-35 min 15-30 mM MSA; t=35-45 min 30-45 mM MSA. Waveform for detection at Au working electrode (Ag/AgCl reference electrode): 0 sec, 0.130 V; 0.04 sec, 0.130 V; 0.05 sec, 0.33 V; 0.21 sec, 0.33 V; 0.22 sec, 0.55 V; 0.46 sec, 0.55 V; 0.47 sec, 0.33 V; 0.56 sec, 0.33 V; 0.57 sec, -1.67 V; 0.58 sec, -1.67 V; 0.59 sec, 0.93 V; 0.60 sec, 0.13 V. Signal was integrated between 0.21 and 0.47 seconds.

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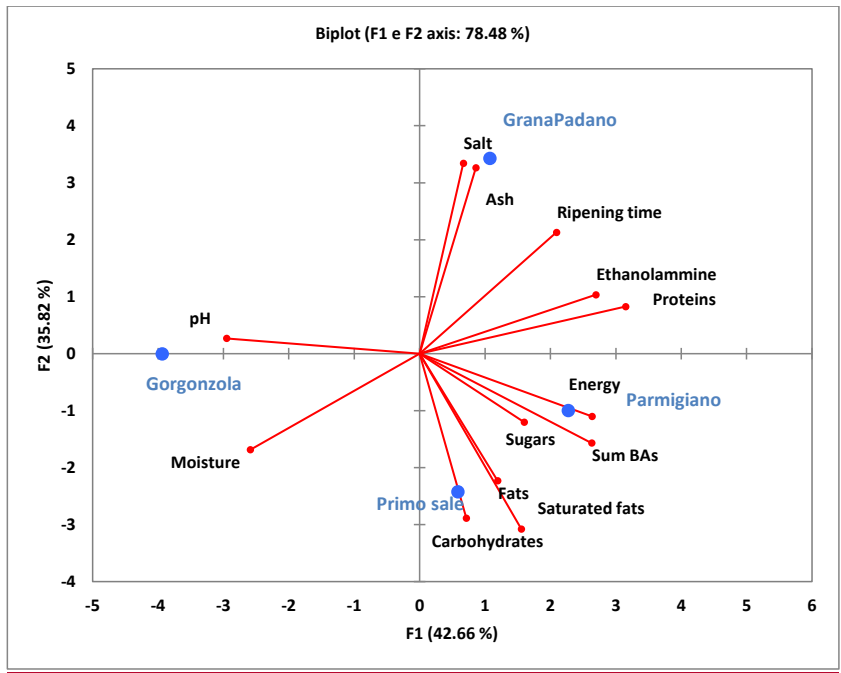
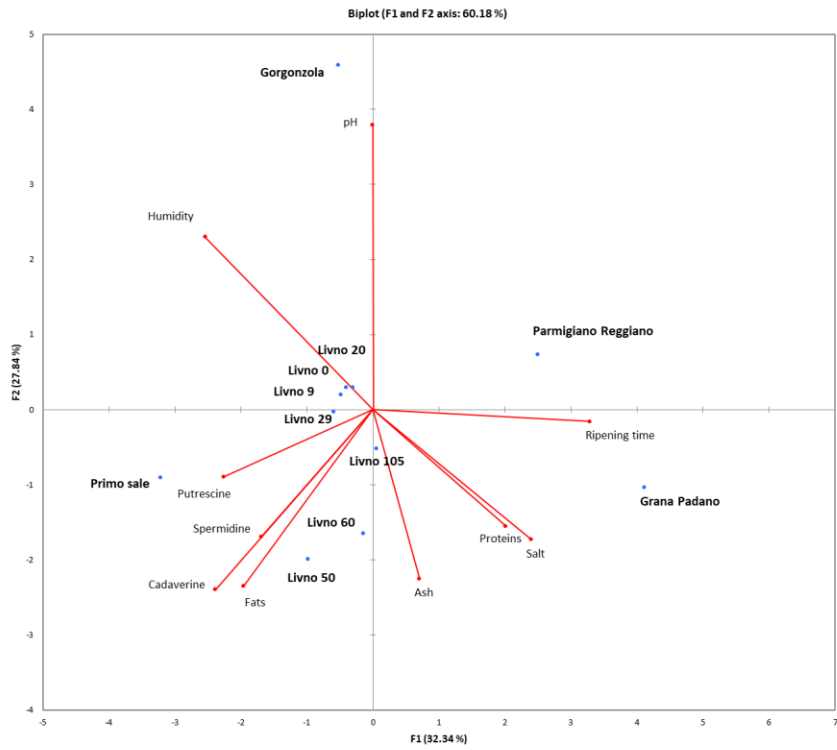


Figure 2. Biplot graph of the objects (cheeses) and of the loadings (red lines: BAs content, pH, humidity, ripening time, ash, salts, fats, carbohydrates, proteins) after PCA.



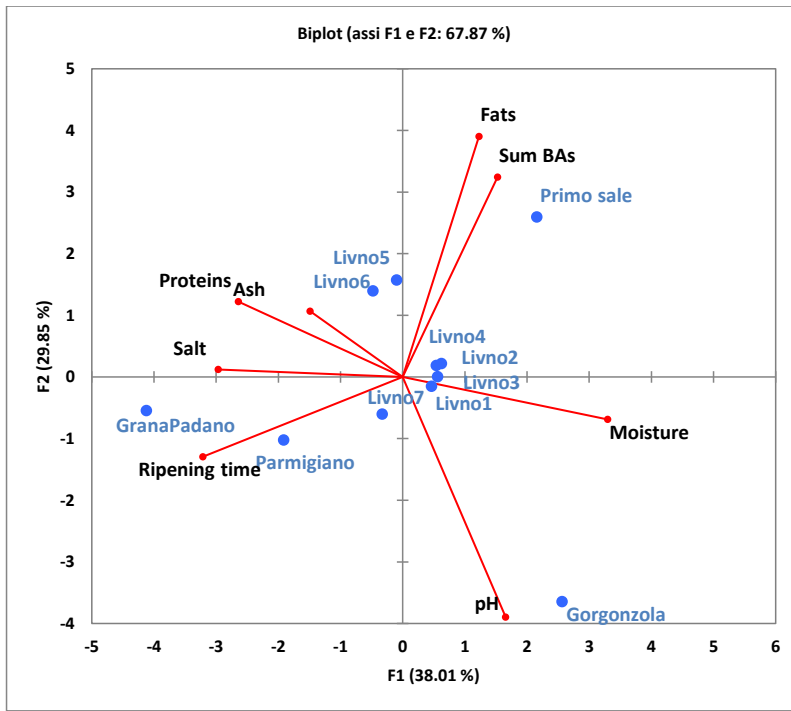


Figure 3. PCA on BAs content, pH, humidity, ripening time, ash, salts, fats, proteins available from this study (Primo sale, Parmigiano Reggiano, Grana Padano and Gorgonzola) and from Marijan and coworkers ⁶ (Livno). The numbers within the Livno cheese identify the number of ripening days.