Biogenic amines content in Fiore Sardo cheese in relation to free amino acids and physicochemical characteristics

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Abstract

Fiore Sardo is a Protected Designation of Origin (PDO) cheese produced in Sardinia (Italy) from raw sheep's milk, presenting risk factors due to an accumulation of Biogenic Amines (BA). A total of 37 Fiore Sardo cheese samples produced in 19 dairy farms were collected from local retail stores to evaluate BA content and its relationship with free amino acids (FAA) and composition. The following were determined for each sample: pH, water activity, composition (moisture, dry matter, NaCl, protein and fat content). FAA and BA, after extraction, were determined by HPLC-FL. The total BA content in Fiore Sardo PDO cheese samples was 127±87 mg 100 g^{-1}, ranging between 6 and 366 mg 100 g^{-1}. Tyramine showed the highest concentration (82±51 mg 100 g^{-1}), followed by putrescine (21±26 mg 100 g^{-1}). Moreover, cadaverine, histamine, β-phenylethylamine and tryptamine were detected at concentrations lower than 10 mg 100 g^{-1}. Overall 54% of the samples analysed exceeded the threshold of 90 mg 100 g^{-1} for total BA content, posing a potential risk for consumers. BA, total FAA (2233±764 mg 100 g^{-1}) and pH were positively correlated (P≤0.01) between themselves, whereas BA content was not correlated with a_w, humidity and percentage of NaCl. The hierarchical cluster analysis results, considering 37 samples and 6 variables, detected four different groups. Samples with BA ≥200 mg 100 g^{-1} were distributed in two groups characterized by a higher proteolysis indicator levels (FAA, pH) but significantly different for a_w, humidity and NaCl concentration. The results showed that high levels of BA were detectable in some samples of Fiore Sardo PDO cheese, suggesting that effective technological conditions at production should be adopted.

Introduction

Biogenic amines (BA) are low-molecular-weight nitrogenous compounds which at low concentrations are essential for natural metabolic and physiological functions in animals, plants and microorganisms (Premont *et al*., 2001). Nevertheless, BA accumulation occurring in food may have a toxicological effect and pose a foods safety risk, especially for susceptible consumers. Though BAs are usually detoxified by monoamine oxidase (MAO) and diamine oxidase (DAO) in the small intestine, toxic effects may arise through amine oxidase oversaturation due to high concentrations of BA or when detoxifying activity is impaired, due to the use of monoamine oxidase inhibitors (MAOI), alcohol consumption or gastrointestinal disorders (Shalaby, 1996). Tyramine, histamine, tryptamine, β-phenylethylamine, putrescine, cadaverine, spermidine and spermine are the most common biogenic amines occurring in foods. Fermented foods, meat, beverages, fish and fish products can accumulate high BA concentrations (Ladero *et al*., 2010; Doeun *et al*., 2017). Fish and cheese are the foods most commonly associated with amine poisoning (EFSA, 2011). Excessive consumption of foods with a high content of histamine, such as fish and fish products, may result in scombroid poisoning with symptoms similar to allergic reactions, such as dyspnea, headache, hives, diarrhea and hypotension (Taylor, 1986; Ladero *et al.*, 2010). Cheese may contain potentially harmful levels of BA, especially tyramine, a vasoactive amine responsible for the so-called "cheese reaction", with symptoms such as a rise in blood pressure, severe headaches, a hypertensive crisis and heart failure (Smith, 1981; Ladero *et al.*, 2010). In addition, polyamines react with nitrite to form carcinogenic nitrosamines (Kim *et al.*, 2009). The formation of biogenic amines can occur in cheese as a result of bacterial decarboxylation of the corresponding amino acid precursors resulting from the proteolysis process, through substrate-specific decarboxylase enzymes or by deamination and transamination of aldehydes and ketones (Ten Brink *et al*., 1990; Linares *et al.,* 2012). In cheese, decarboxylase-producing strains could be provided by raw milk, some starter cultures or microbial contamination. Proteolysis, with the consequent release of free amino acid precursors of BA, is affected by rennet and enzyme activity or microbial fermentation during cheese production and ripening. The BA accumulation rate and concentration in cheese is then influenced by the following factors: raw milk hygiene; processing conditions, like milk heat treatment, starter cultures and enzymes, ripening temperature and duration; intrinsic (a_w, pH, NaCl%) and extrinsic factors (oxygen concentration, relative humidity) of the product (Silla Santos, 1996; Santos *et al.*, 2003; Linares *et al.*, 2012; Loizzo *et al*., 2013; Gardini *et al.*, 2016; Doeun *et al*., 2017).

The presence of BA could be a risk factor for artisanal cheeses (Martuscelli *et al*., 2005; Mascaro *et al*., 2010; Schirone *et al.*, 2011; Schirone *et al.*, 2012; Loizzo *et al*., 2013; Manca *et al*., 2015). The artisanal Fiore Sardo cheese is a Protected Designation of Origin (PDO) cheese, produced in Sardinia (Italy), in which the use of raw sheep's milk, the intensive fermentation activity carried out by natural autochthonous microorganisms (Mannu *et al.*, 2000; Pisano *et al*., 2006; Pisano *et al*., 2007), ripening duration (>3.5 months,

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often even more) and storage temperature, can be identified as risk factors for the formation and accumulation of BA. Therefore, the aim of this research is to assess BA content in Fiore Sardo cheese and evaluate the relationship between the BA content and product parameters such as proteolysis level, pH, NaCl concentration, water activity and relative humidity.

Materials and Methods

Cheese making technology

Fiore Sardo PDO cheese, as recorded in Regulation (EC) No 1263/96, is manufactured from raw ewe's milk obtained from one or two milkings (evening and morning milking). The cheesemaking process follows traditional technological procedures, and fermentation is carried out solely by the indigenous microbial flora present in the milk or by the addition of natural autochthonous microorganisms by starter culture. The milk is coagulated by adding lamb's milk or kid rennet at 34-36°C (during spring or wintertime, respectively). The curd is cut for about three minutes until a millet-seed size is reached. The grains are left to rest for five minutes in the bottom of the vat and the curd, after extraction from the whey, is moulded to obtain the typical wheel, whereby a "mule-back" lateral face is obtained by overlapping two truncated conic shapes. Briefly scalding of the crust may be carried out using hot water or "scotta" (the whey obtained after ricotta cheese production), to obtain a smooth, regular surface. A casein disc is placed on the crust to mark the cheese wheel, which is salted by brining for not more than 48 hours. The first ripening step lasts as long as two weeks, at 18-20°C in rooms where the cheese wheels are placed on grids, usually subjected to natural smoking, for about two hours/day. The second ripening steps take place at temperatures between 10-15°C, usually at farmstead level. During the third ripening step the environmental temperature does not exceed 15° degrees. During ripening the wheels are turned upside down and their surface is treated with olive oil, vinegar and salt. Ripening time is not less than 3.5 months and 6 months, respectively, for the table and grating types.

Samples and analysis procedures

A total of 37 Fiore Sardo PDO cheese samples were randomly purchased from local retail stores in Sardinia (Italy). With ripening periods ranging between six and twelve months, the samples were produced by 19 local farms during 2017-18 milking. One to three batch samples were collected per producer.

The cheeses were brought to the laboratory by refrigerated transport, cut into pieces, discarding up to 1.5 cm from the rind, and grated. The chemical and physicochemical analyses were carried out on the fresh product, as was the extraction of free amino acids and biogenic amines. pH values were determined by direct reading using a GLP22 pH-meter (Crison instruments, Barcelona, Spain) and a_w by Aqualab 4TE (Decagon, Pullman, WA, USA). The FOSS FoodScan NIT (FOSS, Eden Prairie, MN, USA) was used to measure moisture, dry matter, NaCl, protein and fat content. To extract FAA and BA, 2 g of ground cheese were mixed with 1 ml of a solution containing the internal standards (α-Methyl-DL-phenylalanine 5 g/L and 1,7-Diaminoheptane 0.2 g/L in HCL 0.1 M) and 50 ml of a solution of HCl 0.1 M, containing 0.2% of 3,3′-Thiodipropionic acid (TDPA). The mixture was homogenised in an ULTRA TURRAX homogeniser (Zipperer, Staufen, Germany) for five minutes. The cheese slurry obtained was centrifuged (ALC PK131R; ALC International S.r.l., Milan, Italy) at 1250 x *g* (4000 rpm) for 20 min at 4°C. The supernatant was recovered and the residue reextracted using 40 ml of HCl 0.1 M. Then the two acid extracts were combined, and the volume adjusted to 100 ml with trichloroacetic acid 1M. The extract was derivatised with Dns-Cl following the Eerola method (1993), with some modifications, such as a reduction in the amount of sodium carbonate and small changes in temperature and time. 50 µL of sodium carbonate 1 M, and 400 µL of dansyl chloride solution (1% w/v in acetone) were added to 250 µL of standard solutions or sample extracts. The vial containing the reaction mixture was capped, vortexed, then incubated at 50°C for 50 min under stirring in an SH 2000-DX Thermo mixer (Finepcr, Seoul, Korea). In order to remove the Dns-Cl excess, the mixture was treated with 30 µL of NaOH 5 M, then, to remove the excess of carbonate, 30 µL of glacial acetic acid, and 250 µL of acetonitrile was added. The solution was filtered through a 0.22 µm PVDF syringe filter (Millipore, Bedford, MA, USA) and then 10 µL were injected in high-performance liquid chromatography. FAA and BA were determined through a Varian (Walnut Creek, CA, USA) chromatography system equipped with a ProStar 230 Solvent Delivery System, a ProStar 410 autosampler, and an LC 305 fluorescent detector (Jasco, Hachioji, Japan). The system was controlled by Varian Star Chromatography Workstation software (Version 5.31). The column used was an Alltima C_{18}, 150 x 4.6 mm, 2.6 µm [Alltech Italia, Sedriano (MI)] fitted with an Alltima C_{18} guard column, 7.5 mm x 4.6 mm x 5 µm, thermostated at 33°C. Detection was carried out with the fluorescent detector operating at 340 and 510 nm as excitation and emission wavelengths, respectively. Chromatographic analysis was conducted following the Minocha & Long (2004) method, modified as previously described by Manca (2015). Identification of the nitrogenous compounds was performed by comparing the retention times of peaks in the samples with those of standard solutions and by adding the suspected compound to the samples. The target compounds were quantified using the internal standard method. The HPLC-FL method was validated in terms of linearity, recovery percentage, limit of detection (LoD) and limit of quantification (LoQ), calculated from the amount of BAs required to give a signal-to-noise ratio of 3, and a signal-to-noise ratio of 10 respectively (Table 1).

Statistical analyses

The minimum, maximum, average and standard deviation were calculated for each parameter. The results were expressed as means of two replications. One-way ANOVA (followed by Tukey's test) was used to compare mean values. Pearson correlation analysis was conducted to determine the relationship between the different

Table 1. Results from method validation for quantification of the dansylated biogenic amines.

Amines	Linearity Range (R^2)	Recovery (%±SD)	LoD*	LoQ*
Tryptamine	1-250 (0.997)	90±6	0.3	1.0
β-Phenylethylamine	0.6-250 (0.998)	92±5	0.2	0.6
Putrescine	0.2-250 (0.999)	97±3	0.06	0.2
Cadaverine	0.2-250 (0.999)	95±4	0.08	0.02
Histamine	10-250 (0.996)	88±8	1	3
Tyramine	1.2-250 (0.998)	96±3	0.04	1.2
Spermidine	0.2-250 (0.999)	94±5	0.06	0.2
Spermine	0.2-250 (0.999)	92±6	0.06	0.2

*Values expressed as mg 100 g^{-1}.

parameters considered. Statistical significance was declared at P≤0.01. Data were also subjected to hierarchical cluster analysis (HCA), a multivariate procedure to detect groupings in the data. The HCA was done by unweighted pair-group average linkage (between groups) using the squared Euclidean distance. All statistical analyses were performed with the software package SPSS 14 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

The results of physicochemical parameters, composition and total free amino acid content, measured in the 37 samples of Fiore Sardo PDO cheese, are shown in Table 2. The values for pH, moisture and NaCl were within the range of those found by Pisano (2006) in the same type of cheese after 6-9 months of ripening. In comparison with the data reported by this latter author there was, however, a discrepancy for a_w values which Pisano quantified between 0.75±0.01 and 0.79±0.00, whereas the mean value found in our study was 0.88±0.04. Indeed, only one sample, showing a very hard and dry texture showed an a_w value of 0.78 close to that found by Pisano (2006). The fat percentage in dry matter was always higher than 40%, in accordance with the Technical Specifications (EC, 1996), and protein in dry matter had mean values of around 41%, as observed by Pisano (2006). The degree of proteolysis in Fiore Sardo cheeses was ascertained by measuring the total amount of FAA (calculated as the sum of the individual FAA), with a mean±sd of 2233±764 mg 100 g^{-1}, close to the value (2229±1671 mg 100 g^{-1}) found in artisanal Pecorino made with raw ewe's milk (Manca *et al*., 2015). The average of this parameter was higher than that found in Fiore Sardo (1259 mg/100ml) by De Pasquale (2016); however, it should be emphasised that the results of this latter author referred to cheeses after 4 months of ripening and obtained from a single producer. The amino acid profile was similar to that found in other artisanal sheep's milk cheeses (Izco *et al*., 2000; Pappa *et al*., 2008; Manca *et al*., 2015; De Pasquale *et al*., 2016). Glutamic acid, leucine, valine and lysine were the amino acids mostly frequently present. These are the most abundant FAA when lactococci and lactobacilli constitute the dominant microflora, as observed in other raw ewe's milk cheeses (Pintado *et al*. 2008) as well as in Fiore Sardo (Pisano *et al*. 2006). Total BA content was 127±87 mg 100 g^{-1} (Table 3), higher than 70±40 mg 100 g^{-1} found in a previous work on Fiore Sardo (Zazzu *et al*, 2019). As observed in this latter work, which considered cheeses obtained from four producers only, our results, based on products from a greater number of producers, confirmed the large variability in BA content in Fiore Sardo cheese. The use of raw milk with different degrees of hygienic quality and sometimes with uncontrolled temperature and humidity storage conditions, combined with different cheesemaking practices, can affect the conditions promoting the growth and activity of certain groups of microorganisms involved in proteolytic degradation and biogenic amine synthesis (Novella-Rodriguez *et al*., 2003; Pintado *et al*., 2008). The level of BA found in this study was similar to that reported by other authors for different types of raw ewe's milk cheeses, such as the Sardinian Pecorino (Manca *et al*., 2015), Formaggio di Fossa (Mascaro *et al*., 2010), Pecorino di Farindola (Schirone *et al*., 2011) and Pecorino Abruzzese (Martuscelli *et al*., 2005). Tyramine was the main biogenic amine in the samples analysed (Table 3), as in the other types of cheese quoted above. Tyramine concentration was 82±51 mg 100 g^{-1}, representing 64% of the mean total BA content, followed by putrescine (mean value 21±26 mg 100 g^{-1}). Cadaverine, histamine, β-phenylethylamine and tryptamine were generally detectable at concentrations lower than 10 mg 100 g^{-1}. These latter amines were sometime absent; histamine in particular was not detected in around 60% of the samples considered. Spermidine and spermine were never found, as observed in other types of raw ewe's milk cheese (Martuscelli *et al*., 2005; Manca *et al*., 2015). Currently, no legal limit has been established for BA in cheese, but previous studies suggested safety limits for tyramine at 100–800 mg kg^{-1}, for histamine at 50-100 mg kg^{-1} (Nout, 1994), and for total BA content at 900 mg kg^{-1} (Shalaby, 1996).

Considering the lower and upper thresholds suggested for tyramine and histamine, the Fiore Sardo samples that exceeded the proposed limits ranged between 89-46%

Table 2. Physicochemical parameters, composition and total free amino acid content in Fiore Sardo PDO cheese samples.

Parameters	Mean	SD	Min	Max
a_w	0.884	0.037	0.780	0.948
pH	5.14	0.18	4.81	5.52
*Humidity	28.70	3.79	17.41	36.20
*Dry matter	71.30	3.79	63.8	82.59
*Fat	35.65	3.12	29.19	45.35
*Fat/DM	50.00	3.34	42.82	55.68
*Protein	29.26	1.90	25.78	33.08
*Protein/DM	41.05	1.66	36.16	44.82
*Salt	3.29	0.94	1.75	5.05
*Salt/DM	4.62	1.31	2.52	6.88
**ΣFAA	2233	764	694	4214
**ΣFAA/DM	3146	1112	933	6192

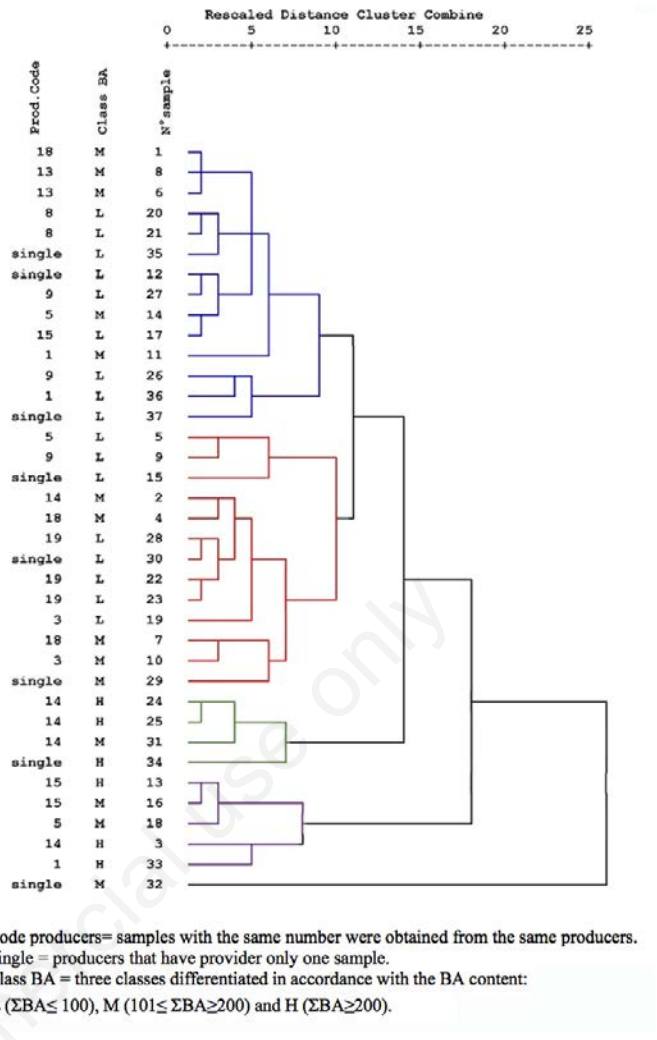
*Values expressed as g 100 g^{-1}; **values expressed as mg 100 g^{-1}. DM= dry matter.

Table 3. Biogenic amine content in Fiore Sardo PDO cheeses. Values expressed as mg 100 g^{-1}.

Parameters	Mean	SD	Min	Max
Tryptamine	3	2	n.d.	8
β-Phenylethylamine	7	9	n.d.	46
Putrescine	21	26	n.d.	95
Cadaverine	6	7	n.d.	33
Histamine	8	13	n.d.	65
Tyramine	82	51	3	200
Spermidine	n.d.	n.d.	n.d.	n.d.
Spermine	n.d.	n.d.	n.d.	n.d.
ΣBA	127	87	6	366
ΣBA/DM	179	122	8	501

DM= dry matter.

and 40-24%, respectively. On the basis of the sum of BAs (Σ_{BA}), and in view of the recommended limit, 56% of the samples exceeded the limit of 900 mg kg^{-1} and this could represent a possible risk for consumers. In order to evaluate the possible relationship between BA content and the variables a_w, pH, NaCl and total FAA content, Pearson's test was used (Table 4). A significant positive correlation between total FAA and total BA content, as well as the single biogenic amines was found. This result confirmed that the availability of free amino acid, ensured by proteolysis, supports the formation of biogenic amines in Fiore Sardo, as highlighted in other types of cheese (Mascaro *et al*., 2010; Loizzo *et al*., 2013; Manca *et al*., 2015). As a consequence of proteolysis, total FAA and BA were positively correlated with pH. This correlation has been observed in other cheeses, such as Terrincho cheese (Pintado *et al*., 2008) after a short ripening time, where low pH was correlated with the lowest tyramine concentrations. The biogenic amines analysed were not correlated with NaCl concentration, with the exception of cadaverine that had a weak positive correlation ($P \leq 0.05$) with this variable. A high concentration of NaCl could inhibit the growth or decarboxylase activity of some species of bacteria (Santos *et al*., 2003; Linares *et al*., 2012; Gardini *et al*., 2016). In Fiore Sardo the salt content did not seem to influence either proteolytic activity or BA formation. Biogenic amines were not correlated with the variables a_w and humidity. Considering that these latter tend to be related to ripening time, the duration of maturation did not



Code producers= samples with the same number were obtained from the same producers.
Single = producers that have provider only one sample.
Class BA = three classes differentiated in accordance with the BA content:
L (ΣBA≤ 100), M (101≤ ΣBA≥200) and H (ΣBA≥200).

Figure 1. Dendrogram obtained by Hierarchical cluster analysis using data set of six variables and 37 cheese samples.

Table 4. Pearson correlation coefficients among the physicochemical parameters, total free amino acid and biogenic amine content measured in Fiore Sardo PDO cheeses.

	a_w	pH	Humidity	Fat	Protein	Salt	Dry matter	Tryptamine	β-Phenyl-ethylamine	Putrescine	Cadaverine	Histamine	Tyramine	ΣBA	ΣFAA
a_w	1	00.386*	0.697**	-0.070	-0.731**	-0.742**	-0.697**	-0.115	-0.29	-0.01	-0.119	0.023	-0.098	-0.100	-0.084
pH	0.386*	1	0.255	-0.105	-0.049	-0.499**	-0.255	0.157	0.213	0.276	0.013	0.292	0.465**	0.428**	0.635**
Humidity	0.697**	0.255	1	-0.643**	-0.776**	-0.165	-10.000**	-0.074	-0.239	0.159	0.226	0.020	-0.076	-0.001	0.106
Fat	-0.070	-0.105	-0.643**	1	0.089	-0.420**	0.643**	0.075	-0.027	-0.228	-0.471**	0.235	0.030	-0.055	-0.288
Protein	-0.731**	-0.049	-0.776**	0.089	1	0.318	0.776**	0.123	0.401*	-0.006	0.021	-0.150	0.176	0.125	0.206
Salt	-0.742**	-0.499**	-0.165	-0.420**	0.318	1	0.165	-0.097	0.105	0.028	0.332*	-0.234	-0.117	-0.060	-0.107
Dry matter	-0.697**	-0.255	-1.000**	0.643**	0.776**	0.165	1	0.074	0.239	-0.159	-0.227	-0.019	0.076	0.001	-0.106
Tryptamine	-0.115	0.157	-0.074	0.075	0.123	-0.097	0.074	1	0.172	0.018	-0.046	0.205	0.367*	0.293	0.433**
B-Phenylethylamine	-0.290	0.213	-0.239	-0.027	0.401*	0.105	0.239	0.172	1	639**	0.383*	0.100	0.740**	0.779**	0.359*
Putrescine	-0.010	0.276	0.159	-0.228	-0.006	0.028	-0.159	0.018	0.639**	1	0.576**	0.218	0.587**	0.790**	0.412*
Cadaverine	-0.119	0.013	0.226	-0.471**	0.021	0.332*	-0.227	-0.046	0.383*	576**	1	-0.098	0.262	0.433**	0.142
Histamine	0.023	0.292	0.020	0.235	-0.15	-0.234	-0.019	0.205	0.100	0.218	-0.098	1	0.519**	0.532**	0.429**
Tyramine	-0.098	0.465**	-0.076	0.030	0.176	-0.117	0.076	0.367*	0.740**	587**	0.262	0.519**	1	0.951**	0.596**
ΣBA	-0.100	0.428**	-0.001	-0.055	0.125	-0.060	0.001	0.293	0.779**	790**	0.433**	0.532**	0.951**	1	0.599**
ΣFAA	-0.084	0.635**	0.106	-0.288	0.206	-0.107	-0.106	0.433**	0.359*	0.412*	0.142	0.429**	0.596**	0.599**	1

BA, biogenic amines; FAA free amino acids. **Correlation is significant at the P< 0.01. *Correlation is significant at the P< 0.05.

seem to be one of the main factors affecting biogenic amine formation in Fiore Sardo, as highlighted in other raw ewe's milk cheese (Manca *et al* 2015). Given the variability of the parameters measured, hierarchical cluster analysis (HCA) was performed to identify similarities and correlations between the samples of cheeses, using 37 objects and 6 variables (a_w, humidity, pH and the content in dry matter of NaCl, total FAA and total BA). The hierarchical clustering results can be seen in Figure 1. To get a clearer view of the dendrogram, the samples were divided into three classes, according to their total BA content: L ($\Sigma_{BA} \leq$ 90 mg 100 g^{-1}), M (91$\leq\Sigma_{BA}\geq$200 mg 100 g^{-1}) and H ($\Sigma_{BA}\geq$200 mg 100 g^{-1}). In the dendrogram four groups of cheeses and one outlier (sample N°32 that presented the lowest a_w value recorded) were identified. The samples with $\Sigma_{BA}\geq$200 mg 100 g^{-1}were distributed in two groups (N°s 3 and 4) and those with $\Sigma_{BA}\leq$90 and 91$\leq\Sigma_{BA}\geq$200 mg 100 g^{-1} in the other two (N°s 1 and 2). To highlight the differences between the groups identified by HCA, in Table 5 the mean, standard deviation and Tukey's test results of the parameters measured were shown. Higher pH levels were found in the samples belonging to group N° 3 and, to a lesser extent, to those of group N° 4 (mean values 5.4 ÷ 0.1 and 5.2 ÷ 0.1, respectively), confirming that this variable was clearly related to proteolysis and BA formation. The samples of groups N° 2 and 4 presented a higher level of NaCl (4.23 ÷ 0.73 and 3.64 ÷ 0.67 mg 100 g^{-1}, respectively) but significant differences in total BA content (111±42 and 279±73 mg 100 g^{-1}, respectively) and FAA (2360±629 and 2960±161 mg 100 g^{-1}, respectively). Groups N° 3 and 4 also showed the highest levels of the single amines, with the exception of histamine content that was higher in group 3, characterised by significant higher levels of pH and humidity and a lower content of NaCl. HCA highlighted that Fiore Sardo PDO is marketed with rather different compositional characteristics that could influence its organoleptic and wholesome quality. The variability observed for the parameters measured may be due to differences in milk composition and/or manufacturing processes (Pisano *et al*., 2006). In fact, the Technical Specifications allow the use of various technological options: different type of rennet (lamb or goat), addition or not of starters, different duration of ripening and related conditions (e.g. temperature), factors that are able to influence the characteristics of the cheese (Vicente *et al* 2001; Pisano *et al*. 2007; Pappa *et al*., 2008; Doeum *et al*., 2017). In addition, by means of HCA it was possible to assess how the manufacturing processes differ from one producer to another; however, they seem quite constant for the single producers over time. In fact, as shown in Figure 1, almost all the samples from the same producer were included in the same group.

Conclusions

Great variability was observed for a_w, pH, humidity and NaCl concentration as well as for total free amino acid and biogenic amine content. Considering the total BA content, 54% of the samples exceeded the threshold of 90 mg 100 g^{-1} and could represent a possible risk for consumers. As observed in other types of raw sheep's milk cheese, tyramine was the most frequently present amine, followed by putrescine (64% and 16% of the total BA content, respectively). The Pearson correlation test highlighted a positive correlation between the total content of BA and that of FAA. In Fiore Sardo both ripening time and proteolysis activity promote the availability of amino acid precursors that are fundamental for the formation of BA. The content of these nitrogenous compounds was consistently related with a higher pH, and it was also affected by proteolytic activity. A_w, humidity and NaCl content were not related to BA, indicating that they did not represent a risk-limiting factor. These results were confirmed by the HCA, which clustered the samples with the highest level of biogenic amines in two groups with high levels of pH in common, but significant different levels of a_w, humidity and salt. In addition, HCA showed that samples from the same producer were usually placed in the same group for BA levels, giving evidence of repeatability of the processes. A comparison between the process conditions used by the groups of producers identified could give indications to pick out factors affecting BA content. In fact, due to the role of autochthonous bacteria in determining the organoleptic and qualitative characteristics of Fiore Sardo PDO cheese, it is difficult to suggest interventions involving the microbiota to reduce the risks related to BA. More appropriate combinations of other technological factors should therefore be evaluated.

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Table 5. Mean and standard deviation of the physicochemical parameters, total free amino acid and biogenic amine content of the four groups identified by means of hierarchical cluster analysis (HCA).

Parameters	Group			
	1	2	3	4
a_w	0,90±0,03^{b}	0,86±0,02^{a}	0,92±0,01^{b}	0,85±0,01^{a}
pH	5,1±0,2^{a}	5,1±0,1^{a}	5,4±0,1^{b}	5,2±0,1^{ab}
*Humidity	28,7±3,6^{ab}	29,7±2,7^{ab}	31,3±1,1^{b}	25,3±3,0^{a}
*Dry matter	71,3±3,6^{ab}	70,3±2,7^{ab}	68,7±1,1^{a}	74,7±3,0^{b}
*Fat	36,7±2,1^{ab}	33,2±2,7^{a}	36,0±0,6^{ab}	35,5±3,4^{ab}
*Fat/DM	51,5±2,0^{b}	47,2±3,4^{a}	52,4±1,4^{b}	47,4±2,6^{a}
*Protein	28,8±1,7^{a}	29,2±1,8^{a}	28,1±1,2^{a}	31,9±0,9^{b}
*Protein/DM	40,5±1,4^{a}	41,7±1,8^{ab}	40,8±1,1^{ab}	42,8±0,9^{b}
*Salt	2,90±0,60^{ab}	4,23±0,73^{c}	2,15±0,42^{a}	3,64±0,67^{bc}
*Salt/DM	4,07±0,81^{ab}	6,01±0,97^{c}	3,14±0,65^{a}	4,91±1,04^{bc}
**ΣFAA	1725±523^{a}	2360±629^{ab}	3133±165^{b}	2960±161^{b}
**ΣFAA/DM	2421±729^{a}	3368±926^{ab}	4566±1051^{b}	3969±259^{b}
**ΣBA	71±43^{a}	111±42^{a}	223±69^{b}	279±73^{b}
**ΣBA/DM	100±60^{a}	158±61^{a}	325±105^{b}	375±104^{b}
**Tryptamine	2±1^{a}	3±3^{a}	4±3^{a}	4±2^{a}
**β-Phenylethylamine	4±3^{a}	4±2^{a}	9±4^{a}	26±17^{b}
**Putrescine	9±14^{a}	19±19^{ab}	45±21^{bc}	53±43^{c}
**Cadaverine	3±3^{a}	9±9^{ab}	6±8^{ab}	14±9^{b}
**Histamine	3±4^{a}	7±9^{a}	25±27^{c}	11±13^{bc}
**Tyramine	51±33^{a}	69±26^{a}	136±33^{b}	171±37^{b}

*Values expressed as g 100 g^{-1}. **Values expressed as mg 100 g^{-1}. DM= dry matter. ^{a,b,c}Tukey's test results, mean values within columns with a different letter are significantly different (P≤0.05).

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