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Biogenic Amines in Traditional *Fiore Sardo* PDO Sheep Cheese: Assessment, Validation and Application of an RP-HPLC-DAD-UV Method

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Abstract: This contribution aimed to measure for the first time the amount of biogenic amines (BAs) in one of the most ancient and traditional sheep cheese produced in Sardinia, Italy: the Protected Designation of Origin (PDO) Fiore Sardo. To achieve this, an original RP-HPLC-DAD-UV method has been developed that was completely validated in terms of LoD, LoQ, linearity, precision and trueness, and tested on 36 real Fiore Sardo PDO cheese samples produced by four different cheesemakers and marketed by four stores. The average total concentration of the eight BAs (i.e., tyramine, tryptamine, histidine, putrescine, cadaverine, 2-phenylethylamine, spermine and spermidine) measured in Fiore Sardo cheese was 700 mg/kg, with a range between 170 mg/kg and 1,100 mg/kg. A great variability in the total amount of BAs has been evidenced among the Fiore Sardo marketed in the four stores as well as for the cheeses purchased in different times in the same store. Tyramine (350 mg/kg), putrescine (150 mg/kg), histamine (80 mg/kg) and cadaverine (30 mg/kg) are the most abundant BAs found in this matrix. Among the many factors concurring, the dominant microflora of Fiore Sardo PDO is likely the principal cause of the qualitative and quantitative distribution of BAs in this matrix. Finally, the total amount of BAs found in Fiore Sardo PDO is not able to cause any health alert situation for consumers.

Keywords: Fiore Sardo PDO; biogenic amines; sheep's cheese; RP-HPLC

1. Introduction

Biogenic amines (BAs) are organic bases characterized by a low molecular weight, a medium polarity and the presence of at least one aminic group bonded to an aliphatic, aromatic or heterocyclic moiety. BAs can be formed in food containing mainly proteins (or, simply, free amino acids) or also carbonylic compounds such as aldehydes or ketones. As a matter of fact, it is well known that the bacterial decarboxylation of amino acids or transamination of simple carbonylic compounds lead to the formation of BAs [1–3]. Many BAs show severe adverse effects towards human health: tyramine (Tyr) is cytotoxic, causing the necrosis of the HT29 intestinal cells [4], again Tyr, tryptamine (Trp) and 2-phenylethylamine (Phe) are vasoactive [5], whereas histamine (His), putrescine (Put) and cadaverine (Cad) are toxic towards the nervous system [5]. The contemporary presence of Put and Cad in food potentiate the toxic action of His [6]. In addition, it has been demonstrated that BAs are possible precursors for the formation of carcinogenic N-nitroso species [7]. Finally, the polyamines spermine (Spm) and spermidine (Spd) are often naturally present in food

[8]. However, also their excess has been recently associated with potential health risks [9,10]. Despite their recognized toxicity, the limit concentrations of BAs in fermented foodstuffs are far from being adequately standardized by regulatory agencies.

Among the foodstuffs prone to containing meaningful amounts of BAs, it is worth noting dairy products [2,7,8,11–14], fish [15–17] and fish products [18,19], meat [20,21] and meat products [20–22], soy derivatives [6], fresh and fermented products by vegetables [23–25], chocolate [25], honey [1] and fermented beverages like wine [1,26–30] or beer [29,31].

As a matter of fact, cheese, mainly aged cheese, provides an excellent substrate for the production of BAs [32–36]. The constant presence of both a not sterile environment and casein proteolysis during all cheese making activities ensures the constant availability of free amino acids. Furthermore, the profile and the concentration of BAs in cheese are associated with a number of technological factors: the use of raw (or pasteurized) milk [37–39], the presence of native (or inoculated by a starter culture) bacteria belonging to *Pseudomonas*, *Enterobacter*, *Micrococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus* genera [35,40], the pH [35,41], the amount of NaCl added during the cheese making phases [35,41], but also the ripening [38,42], storage conditions [32,41,43] and post-ripening technological processes (e.g., cutting, slicing, grating) [44,45] play a key role in determining the amount of these compounds. All these variables concur to justify the enormous differences observed in the amount of the most common BAs when varying the type of cheese. Although it should be possible to find cheeses in which the most common BAs are very low in concentration (also below the mg/kg level) [2,46], it is quite normal measuring, for each one, concentration levels in cheeses ranging between some tens of mg/kg, but it is not rare that the amounts found can also exceed the levels of hundreds [2,47–49] or even the level of thousands [46,50,51] of mg/kg for His [46], Tyr [50,51], Put [46] and Cad [50]. Details of the different analytical approaches used for determining BAs in cheese have been extensively discussed in a number of excellent reviews on this topic [6,32,33,36,52,53]. Among others, liquid chromatography nowadays still represents the most preferred analytical choice [7,8,12,40,42,46,54,55], even sometimes coupled with a mass spectrometry detection [11,56,57]. Other chromatographic methods have also been used for this aim, like ion-exchange chromatography [51,58], ion chromatography [59] and gas-chromatography coupled with mass spectrometry [60]. Apart from chromatography, several analytical techniques can also be succesfully applied for measuring selected BAs: controlled-potential methods [22], enzyme-linked immunosorbent assay methods [61] and capillary electrophoresis techniques [62] have been proposed for the His determination, whereas amperometric biosensors were used for the determination of Tyr [63] and square wave adsorbpitive stripping voltammetry for the determination of Trp [64].

Traditional Fiore Sardo PDO (henceforward called simply "Fiore Sardo") is an uncooked and long-ripening hard cheese, produced only from raw whole milk by Sarda breed sheep. Its cheese making technique, unchanged for centuries, makes it the most ancient sheep cheese produced in Sardinia. The coagulation process of sheep milk is promoted by lamb or kid rennet paste, and this induces, during the ripening of the cheese, an intense lipolysis [65,66], whereas proteolysis is meaningful only in the first months of the ripening. By a microbiological viewpoint, the mesophilic lactic acid bacteria (*Lactococcus lactis*) and enterococci (*Enterococcus faecium*) dominates the microflora of Fiore Sardo cheese [67–70]. The concomitant total absence of any thermal treatment underwent by the sheep's milk and the long ripening of Fiore Sardo cheese before marketing, means that the amount of BAs in the final product may be worthy of some attention.

To the best of our knowledge, no previous study has addressed the establishment of the presence of BAs in this cheese. While one contribution has been published containing data on the amounts of BAs in other sheep's cheeses from Sardinia [71], unfortunately the validation part of the analytical method is missing. For this reason, and on the basis of the previous results that our research group has achieved in last years in the assessment, validation and application of new methods for the determination of analytes of specific interest in sheep cheeses [65,67,72–74], the principal aim of this study is to develop and validate an original RP-HPLC method devoted to the

determination of eight BAs (i.e., Tyr, Trp, His, Put, Cad, Phe, Spm, Spd, all reported in Figure 1) in Fiore Sardo sheep's cheese.

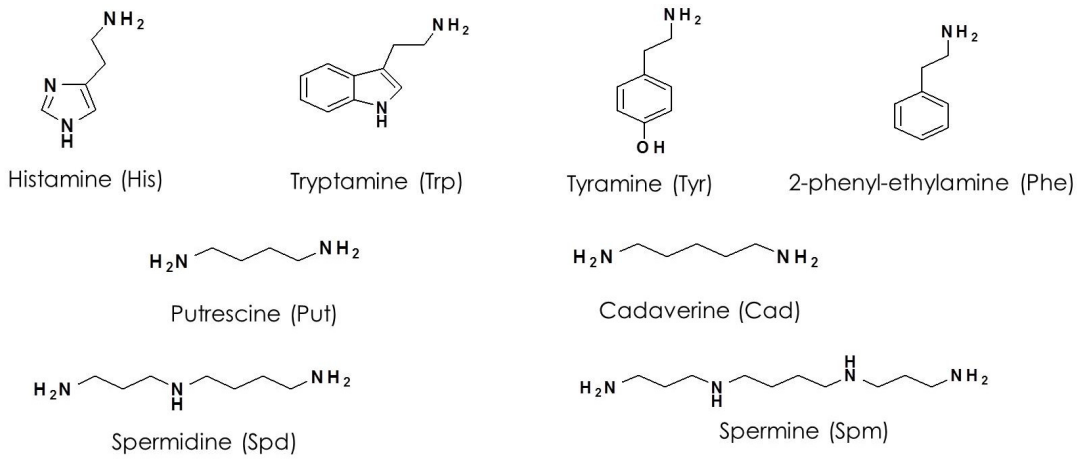

Figure 1. The eight BAs object of study.

2. Materials and Methods

2.1. Sampling

A total of 36 Fiore Sardo cheese samples (400 g ca. each), produced by four artisanal cheesemakers, were purchased in four different stores located in Italy. Each store was always supplied by the same cheesemaker. In order to give account for the intrinsic variability of samples produced by each cheesemaker, three samples were taken from each store every 15 days. Hence, a total of nine samples per store was collected. Samples were carried to the laboratory at 4 °C and stored at the same temperature until analysis.

2.2. Chemicals

Tyr (99%), Trp (99%), His (>99%), Put (>99%), Cad (>99%), Phe (99%), Spm (>99.5%), Spd (99.5%), 1,7-diaminoheptane (>98.0%) and 5-(dimethylamino)-naphthalene-1-sulfonyl chloride (>99.0%) were from Sigma-Aldrich (Milan, Italy). All BAs, except for 1,7-diaminoheptane, were purchased as hydrochloride salts, and the amounts weighted were corrected on the basis of their purity and referred to the free amine. 0.1 mol/L aqueous solution of hydrochloric acid, 28% aqueous solution of ammonia, diethyl ether and sodium bicarbonate were analytical grade reagents from Carlo Erba (Milan, Italy), whereas HPLC grade acetonitrile was from Merck (Milan, Italy). Ultrapure (Type 1) water (specific resistance > 18 MΩ) was always used throughout the analyses.

2.3. Equipment

The chromatographic apparatus used in this study was an Agilent Series 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a Guard-RP, HI-5RP-10C5/K pre-column (Agilent Technologies) a Kromasil 100-5 C18, 250 mm × 4.6 mm × 5 μm particle size column (CPS Analitica, Milan, Italy) and a Diode Array Detector model DAD G 1315B (Agilent Technologies).

2.4. Biogenic Amine Analysis

2.4.1. Exctraction and Derivatisation

Cheese (2 g) was homogenized in 20 mL of a solution containing the internal standard (20 mg/L of 1,7-diaminoheptane in 0.1 mol/L HCl) and centrifuged at 1,780 × g for 20 min at 4 °C. Extraction was repeated with 20 mL of 0.1 mol/L HCl. Combined extracts were made up to 50 mL with 0.1 mol/L HCl and then filtered on Whatman 42 paper.

As proposed by Innocente et al. [12], derivatisation was achieved by mixing 1 mL of acid extract with 0.5 mL of saturated $NaHCO_3$ solution and derivatising reagent (5-(dimethylamino)-naphthalene-1-sulfonyl chloride, dansyl chloride, DCl) solution (10 mg, dissolved in 1 mL of acetone). The mixture was left for 60 min at 40 °C. The residual DCl was removed by adding 200 µL of 28% ammonia aqueous solution, vortexed for 1 min and allowed to react in the dark for 30 min at room temperature. The DC derivates of BAs were then extracted three times with 1 mL of diethyl ether [7]. The combined extracts were dried under air flow, the residue was re-dissolved in 1 mL of acetonitrile and filtered through a 0.22 µm PTFE filter before analysis.

2.4.2. HPLC Separation

10 µL of the DCl derivatized BAs solution was injected in the HPLC apparatus. The column was termostated at 30 °C and flux was set at 0.8 mL/min. Elution solvents were acetonitrile and water. The elution program used in the analysis is shown in Table 1. All analytes were eluted within ca. 20 min, and each run was 35 min long.

Table 1. Elution program for the HPLC gradient separation of the biogenic amines DCl derivates.

Time (min)	Acetonitrile (%)	Water (%)
0	65	35
1	65	35
10	80	20
12	90	10
16	100	0
30	100	0
Post run		
30.1	65	30
35	65	30

2.4.3. Wavelength optimization

Literature findings suggested the detection of dansyl derivates of biogenic amine at 254 nm [12]. However, the analysis of DAD spectrum of dansyl derivates of all biogenic amines standards at 5 µg/mL revealed a maximum of absorbance at 218 nm for all analytes (Figure 2). Figure 3 shows the chromatographic profile of dansyl derivates of biogenic amines standards at 5 mg/L detecting both at λ = 254 nm (Figure 3A) and at λ = 218 nm (Figure 3B). A higher signal-to-noise ratio was observed at the lower wavelength, hence the 218 nm wavelength was used in this study.

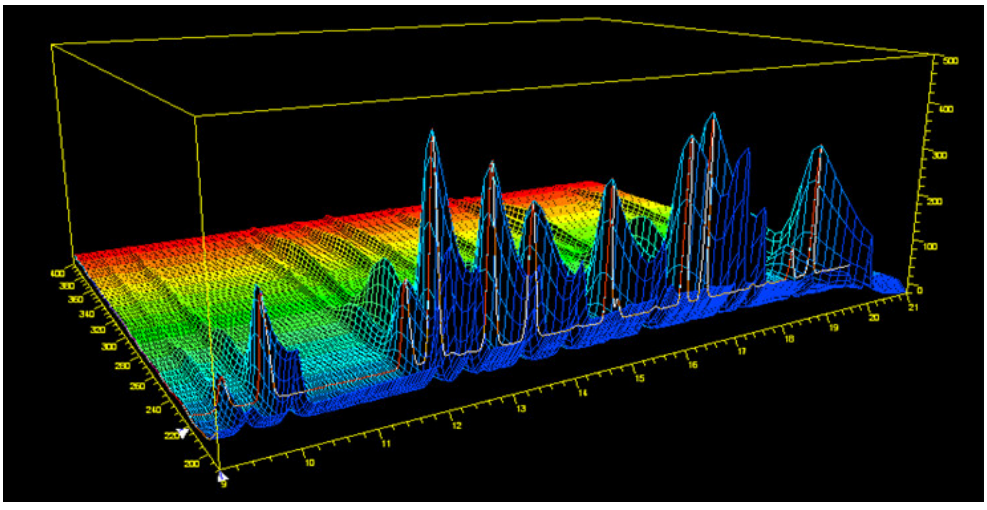

Figure 2. UV absorption of dansyl derivates of BA standard solutions (concentration: 5 mg/L each) as a function of the chromatographic run. λ = 218 nm.

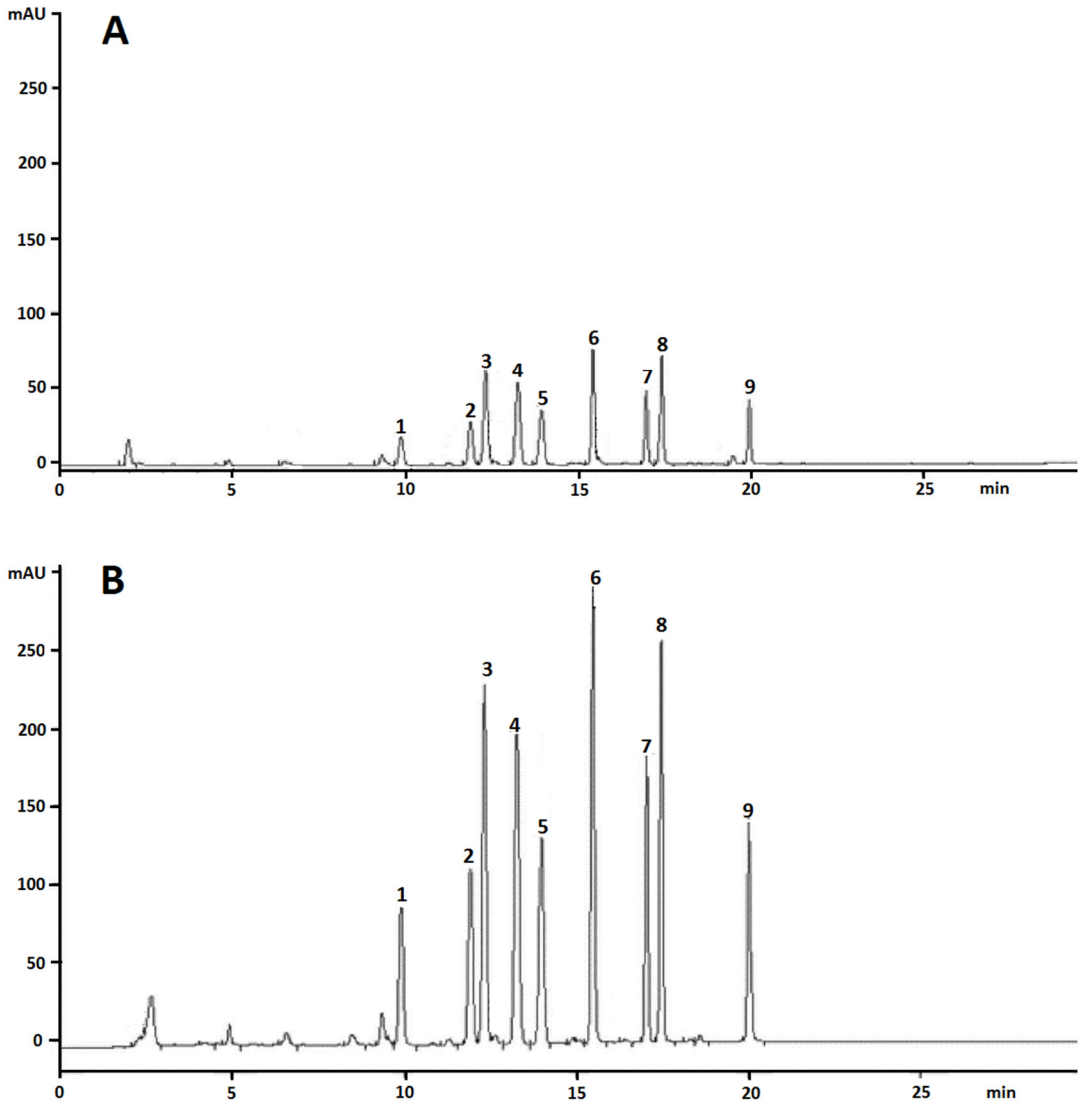

Figure 3. Chromatographic profile of dansyl derivates of standard solution (concentration: 5 mg/L each) of BAs. (**A**) λ = 254 nm; (**B**) λ = 218 nm. Peak sequence: 1 = Trp; 2 = Phe; 3 = Put; 4 = Cad; 5 = His; 6 = 1,7-diaminoheptane, internal standard; 7 = Tyr; 8 = Spd; 9 = Spm.

2.4.4. Identification and Quantification of the Biogenic Amines

The peaks of dansy derivates of BAs were identified by comparing retention times with those of standard solutions. Quantification was accomplished by internal calibration on different concentration levels in the relevant linearity interval of each analyte. A 1,000 mg/L stock solution containing each of the biogenic amines was properly diluted to obtain working solutions at concentrations ranging between 50 and 0.1 mg/L, in order to build a calibration curve ranging between 2.5 and 1,250 mg/kg of cheese. A known amount of internal standard (1,7-diaminoheptane in 0.1 mol/L HCl) has been added to each working solution, and the mixture has been firstly subdued for the derivatization step and, next, the HPLC analysis was been performed three times. Figure 4 reports a typical chromatogram obtained by a sample of Fiore Sardo cheese.

Since an overestimation effect was substantiated in the quantification of biogenic amines in Castelmagno, Raschera and Toma cow cheeses from Piedmont, Italy [11], a comparison between analytical data from the proposed method and a multiple standard addition method was performed for all BAs in two different samples of Fiore Sardo cheese (i.e., the first from the A store, and the second from the C store). No significant differences between results obtained from both methods were found for all analytes (criteria: two-tail t-test, $p = 0.95$).

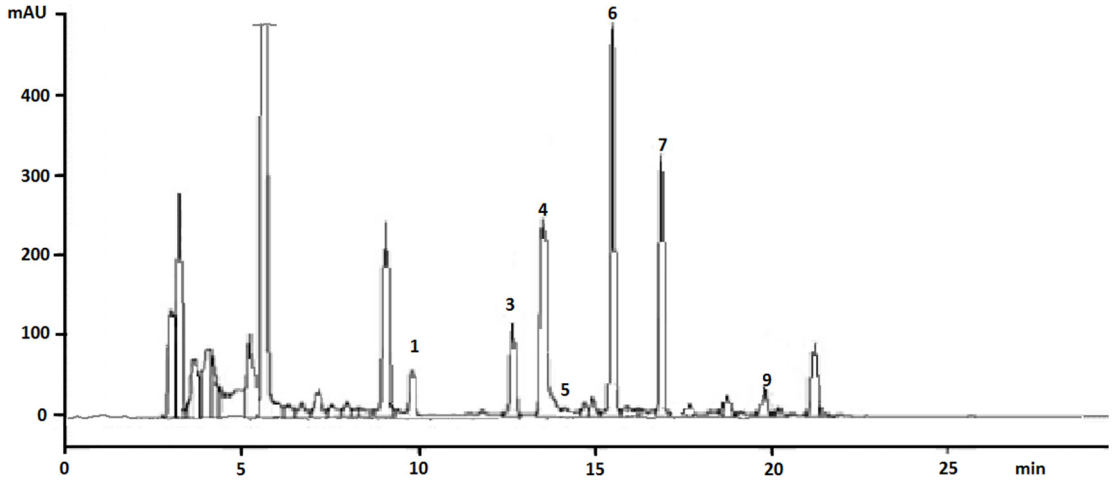

Figure 4. HPLC profile of the biogenic amines contained in a sample of Fiore Sardo cheese (D store). Peak sequence: 1 = Trp; 3 = Put, 4 = Cad, 5 = His, 6 = 1,7-diaminoheptane, internal standard, 7 = Tyr, 9 = Spm.

2.4.5. Analytical Method Validation

Validation of the proposed method was accomplished on the basis of the limit of detection (LoD), limit of quantification (LoQ), linearity, precision and trueness. LoD was calculated according to the Upper Limit Approach (ULA1) approved by IUPAC [75]. For each biogenic amine, four different solutions at increasing concentrations close to the expected LoD (i.e., between 0.01 and 0.50 mg L^{-1} for each analyte) were prepared and analyzed. Each measurement was performed in triplicate. In addition, the ULA1 approach recommends that the LoQ value is three times the relevant LoD value. Linearity was checked on at least two orders of magnitude of concentration, as a function of the relative abundance of each biogenic amine, typically in the range 0.1–50 mg/L (i.e., 2.5–1,250 mg/kg of cheese). Precision was evaluated in terms of both repeatability (i.e., the CV measured for ten consecutive replicates of the same sample in the same analytical session) and intermediate precision (i.e., the CV obtained for the analysis of aliquots of the same sample by four different operators in different analytical sessions). The acceptability of these precision scores was checked in terms of HorRat ratio values (i.e., the ratio between experimental and theoretical CV measured on the basis of the Horwitz's theory) [76]. Due to the lack of any certified reference materials, trueness was estimated through recovery tests. Four aliquots of a Fiore Sardo cheese sample were submitted to the overall analytical procedure after the addition of increasing amounts of each BA to three of them, whereas the fourth was analyzed as it is (i.e., without any addition of BA). For each analyte, a plot of the analytical concentration, $\mathbf{C}_{exp,n}$ ($0 \le n \le 3$) measured after each addition of analyte (y axis) versus the $\mathbf{q}_i/\mathbf{m}_i$ ratio (x axis), where $\mathbf{q}_i$ is the mass of the i-th addition of analyte ($1 \le i \le 3$) and $\mathbf{m}_i$ is the mass of the i-th sample of cheese, was accomplished. The recovery is represented by the percent slope value of the regression line in this way obtained. The estimation of bias was made in triplicate. The presence/absence of bias has been evaluated by means of a two-tail t-test ($p = 0.95$).

2.5. Statistical Analysis

The statistical package Minitab 16 (Minitab 16 Statistical Software (2010), Minitab, Inc., State College, PA, USA) was used for the statistical analysis. GLM (general linear model) analysis and Tukey test for multiple comparison of means were used for comparing cheeses purchased at different stores. The model included the effects of store/cheesemaker (F, 4 levels: A, B, C, D).

3. Results and Discussion

3.1. Assessment of the Chromatographic Method

BAs determination still remains one of the more demanding challenges in the field of the food analysis, and this is due to a number of factors. Firstly, the high polarity and the quite low molecular weight of the BAs favors their solubility in water rather than in the organic solvents commonly used for extraction by a matrix which may be also very complex. In addition, the contemporary presence of many analytes in a concentration range wide more than six orders of magnitude (typically between the g/kg and the μg/kg), the absence of intrinsic properties that may facilitate their detection with both spectroscopic or electrochemical methods, and the presence of a number of potential interfering species makes this determination a very intriguing, but extremely complex analytical task. An extraction step is undoubtly needed in order to separate the BAs by potential interfering substances and increase the concentration of analytes in the extracts, but very often this shrewdness is not sufficient to ensure values of LoQ low enough to also quantify traces of BAs. Hence, also an additional step of derivatization of the analytes is frequently required. Unfortunately, both extraction and derivatization steps complicate the analytical method and may also be responsible for meaningful losses of analytes. In the specific case of cheese, the extraction of BAs has been accomplished by repeated treatment of the grated sample with aliquots of 0.1 mol/L aqueous solution of HCl, which proved in the past to be a very good choice for the extraction of biogenic amines from this matrix [54]. The approach chosen for developing the proposed method is the direct derivatization of the acidic extracts [12]. It represents a very convenient option in order to maximize the accuracy of the method reducing at the same time the lenght of the analysis and avoiding the unnecessary dilution of samples. Derivatization of BAs reduce the polarity of the analytes and improve their separation in RP columns. The most frequently used derivatizing agents adopted for BAs in cheese are the O-phthalaldehyde and—mainly—the dansyl chloride, and each of them is characterized by a number of advantages and drawbacks. One of the key advantages of O-phthalaldehyde is its rapid reaction with BAs that are also at room temperature. These derivatives are fluorescent, and this allow the reaching of very low LoD values (up to fmol levels). On the other hand, the principal drawback of the O-phthalaldehyde derivatives with BAs is a rather poor stability, mainly in comparison to the dansyl derivatives. Furthermore, the analytical column used for their separation has to be resistant to alkaline mediums. For these reasons, the most common reagent used in the last years in the derivatization of foods containing high amounts of BAs is dansyl chloride. It provides stable derivatives that can be easily detected by a UV, DAD, fluorescence or MS detector, as a function of the expected concentration of BAs in the matrix considered. In addition, dansylation can be directly accomplished on the acidic extract of the cheese matrix [12], and this improves the overall accuracy of the method. The most meaningful drawback is the slowness of the reaction: the dansylation reaction is not quantitative at 20 °C, at 40 °C it takes usually 60 min to accomplish the completeness, but the reaction time can be further reduced by heating the reagents to 70 °C. For these reasons, direct derivatization of the acidic extracts of cheese with dansyl chloride was the technique chosen for the proposed method. The nature of the cheese matrix, presumably rich in free amino acids, encourages inserting an extraction step with diethyl ether before chromatographic analysis, , designed to separate the dansylated free amino acid (solubilized in the aqueous layer) from the dansyl derivatives of Bas that are more affine to the organic layer [12]. The abundant literature relative to the RP-HPLC evaluation of BAs in cheeses of different origin offer many analytical insights in order to optimize the principal features of the RP-HPLC method. Reviews and articles covering this topic show that the C18 columns are most frequently used for this task. Among others, Hypersil BDS C18 [54] and Kromasil 100-5 C18 columns [12,24] are frequently adopted for cheese analysis. The comparative evaluation of the performance of both columns substantiated a worse resolution and an almost double duration of the chromatographic run for the Hypersil BDS C18 compared for the Kromasil 100-5 C18 column. For these reasons, this last column has been chosen for developing the proposed method. Finally, the optimization of the gradient of

water and acetonitrile (i.e., the most preferred solvents previously used for this aim [12,24,77–80]) proposed by Torracca et al. [77] completed the chromatographic method developed in this study.

3.2. Validation Parameters

Table 2 shows the results obtained on the validation of the proposed method.

Table 2. Data obtained in the validation of the HPLC method for the biogenic amines analysis.

			Linearity	Precision		Trueness
Analyte	**LoD (mg/kg)**	**LoQ (mg/kg)**	**Linearity range, mg/kg, (R^2)**	**Repeatability CV% $_{exper(r)}$ ($HorRat_r$)**	**Intermediate precision CV% $_{exper(IP)}$ ($HorRat_{IP}$)**	**Recovery (%±SD)**
Trp	0.20	0.60	0.60–100 (0.9997) 2.5–1250 (0.9993)	6.5 (0.4)	14.3 (0.9)	99 ± 2
Phe	0.10	0.30	0.30–125 (0.9998) 2.5–1250 (0.9996)	6.8 (0.4)	10.7 (0.7)	94 ± 2
Put	0.07	0.20	0.20–125 (0.9960) 2.5–1250 (0.9905)	4.4 (0.3)	7.0 (0.4)	87 ± 9
Cad	0.13	0.40	0.40–250 (0.9997) 2.5–1250 (0.9949)	4.1 (0.3)	6.4 (0.4)	95 ± 12
His	0.20	0. 60	0.60–500 (0.9994) 2.5–1250 (0.9994)	3.8 (0.2)	9.2 (0.6)	85 ± 5
Tyr	0.17	0.50	0.50–125 (0.9985) 2.5–1250 (0.9979)	5.0 (0.3)	4.9 (0.3)	90 ± 6
Spd	0.23	0.70	0.70–125 (0.9980) 2.5–1250 (0.9945)	-	-	-
Spm	0.07	0.20	0.25–100 (0.9999) 2.5–1250 (0.9949)	8.6 (0.5)	16.1 (1.0)	82 ± 8

LoD and LoQ values lie in the range between 0.07 mg/kg and 0.23 mg/kg and between 0.20 mg/kg and 0.70 mg/kg, respectively. Data are consistent with those already published [53,78]. In principle, linearity was evaluated along more than two orders of magnitude of concentration, ranging between 2.5 mg/kg and 1,250 mg/kg. However, the analysis of the samples revealed that the concentration levels of BAs in few Fiore Sardo samples were below the lowest concentration value considered in the linearity interval. Therefore a second calibration curve was taken into account for those analytes, where the least concentration was equal to the LoQ, obtaining in this case R^2 values between 0.9960 and 0.9999. Consequently, linearity is only sufficient for Put, acceptable for Spd, Cad (2.5–1,250 mg/kg range) and Spm (2.5–1,250 mg/kg range), good for Tyr and excellent for the remaining analytes/concentration range couples. Precision data are between 3.8% (His) and 8.6% (Spm) for repeatability, and between 4.9% (Tyr) and 16.1% (Spm) for the intermediate precision measurement. All of them can be considered acceptable for the concentration range considered according the Horwitz's theory [76], as proved by the relevant HorRat ratios, always below the threshold value of 1.5. Finally, the amounts of the recovery values measured in this study are in good agreement with those already reported in literature [12,53,54,78]. The two tail t-test ($p = 0.95$) substantiates that all values obtained are not statistically different from the quantitative recovery (t_{exp} between 0.71, Trp, and 4.24, Phe and His, $t_{tab} = 4.30$), hence accounting for a general absence of bias of the method proposed.

3.3. Biogenic Amines in Fiore Sardo Sheep Cheese

The proposed analytical method was used to determine the biogenic amine content in 36 Fiore Sardo cheese samples, produced by four cheesemakers and purchased in four diffent stores. The results obtained are shown in Table 3, in which the mean value of the nine samples of every local store/cheesemaker, the standard deviation of their distribution and their range are indicated for each biogenic amine.

Table 3. Mean amount (±sd) and range of biogenic amine content in Fiore Sardo cheese samples. Data are expressed in mg/kg of cheese.

Stores	Trp		Phe		Put		Cad	
	Mean ± sd	range	Mean ± sd	range	Mean ± sd	range	Mean ± sd	range
A	0.4 ± 0.4	<0.2–1.2	2 ^{b} ± 2	<0.3–6.3	<0.2 ^{c}		6 ^{b} ± 4	1.3–13
B	3 ± 3	0.2–8.2	16 ^{a} ± 8	1.8–25	450 ^{a} ± 200	88–730	20 ^{ab} ± 30	1.0–94
C	4.4 ± 3.5	0.3–10.5	15 ^{a} ± 10	9.3–42	130 ^{b} ± 70	36–210	40 ^{a} ± 20	6.7–70
D	5.5 ± 6.5	<0.2–16.9	9 ^{ab} ± 10	1.3–27	20 ^{bc} ± 20	<0.2–49	40 ^{a} ± 25	7.3–83
Total	**3 ± 8**	**<0.2–16.9**	**10 ± 20**	**<0.3–42**	**150 ± 200**	**<0.2–730**	**30 ± 50**	**1.0–94**
Store Effect	ns		**		***		*	

Stores	His		Tyr		Spm		ΣBAs
	Mean ± sd	range	Mean ± sd	range	Mean ± sd	range	Mean ± sd
A	4 ^{c} ± 6	<0.65–19	60 ^{c} ± 70	<0.5–180	100 ^{a} ± 30	8–160	170 ± 80
B	85 ^{b} ± 75	5.2–250	400 ^{b} ± 100	170–530	30 ^{b} ± 30	10–88	1000 ± 200
C	210 ^{a} ± 20	190–250	700 ^{a} ± 100	445–800	30 ^{b} ± 30	17–115	1100 ± 100
D	10 ^{c} ± 10	<0.65–33	300 ^{b} ± 300	110–770	50 ^{b} ± 10	29–65	500 ± 300
Total	**80 ± 80**	**<0.65–250**	**350 ± 300**	**<0.5–800**	**50 ± 60**	**8–160**	**700 ± 400**
Store Effect	***		***		***		

Mean values and sd have been calculated on the basis of not-rounded analytical data. **ΣBAs** = Total mean BAs amount for each store. Amounts in *italic* character are below the relevant LoD. Spd amount was always below the LoD. Values within column not sharing a common superscript are significantly different. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The average total concentration of the eight BAs measured in Fiore Sardo cheese is 700 mg/kg. The great variability in the total amount of BAs, evidenced by the wide range (between the 170 mg/kg for the Fiore Sardo samples purchased in store A and the 1,100 mg/kg for the cheeses purchased in store C) is also reflected in the very high variability in BAs concentration found for the cheeses purchased in different times in the same store. Also the concentration of each BA measured in Fiore Sardo cheese is extremely variable among the different stores. Spm is the most abundant BA in Fiore Sardo purchased in store A and its content is significantly higher respect to that determined in cheeses purchased in the other stores ($p < 0.001$). In cheeses A, Spm is followed by Tyr, whereas the remaining BAs show average concentrations less than 10 mg/kg each. Interestingly, Put is always below the relevant LoD in cheeses A, whereas it is often abundant in the remaining cheeses. On the other hand, Tyr is the most abundant BA in Fiore Sardo purchased in the remaining stores (mainly for store C, $p < 0.001$), followed by Put (mainly for store B, $p < 0.001$)) and His (mainly for store C, $p < 0.001$)). Spm and Cad are constanty present in these matrices at average levels of few tens of mg/kg, whereas still less abundant are Phe and Trp. Finally, Spd was always found to be below its LoD. The remarkable differences among producers in the application of a cheese making process that is still almost artisanal today and—mainly—the differences in the technique of preparation and use of the rennet paste are likely responsible of the extremely wide dispersion of data measured for each BA in this study.

For the reader's convenience, Table 4 summarizes the results of the determination of BAs in a wide number of sheep's cheeses produced worldwide. In particular, literature reported data also obtained on different sheep's cheeses produced in Sardinia [71]. The results here obtained are in good agreement with those reported in such study for the Pecorino cheese produced from farmhouses. As well as for the Fiore Sardo, this cheese is also made from raw milk, coagulated without the presence of any starter culture, and its ripening takes place in rooms with no control over humidity or temperature. On the other hand, the farmhouse Pecorino is a semicooked cheese that is coagulated with calf rennet (and not with lamb or kid rennet, like happens for Fiore Sardo cheese).

Table 4. Concentrations (mean and/or range) of selected biogenic amines in sheep cheeses. Data are expressed in mg/kg of cheese.

Sheep cheese	Key features	Trp	Phe	Put	Cad	His	Tyr	Spm	Spd	Reference
Zamorano, Spain	Cheese made from raw milk; BAs have been measured between 1 day and 10 months of ripening. Data are inferred by the Figure 8 of the study.	3–50	3–120	10–190	5–35	1–55	1–85	0–20	0–115	[8]
Azeitão cheese	Cheese made from raw milk using an exctract of Cardoon as rennet. The length of ripening is at least of 20 days.	NM	NM	ND–137	161–260	414–818	72.3–445	17.2–81.6	NM	[47]
Pecorino Carmasciano, Italy	Cheese obtained from raw milk, coagulated at 40–45 °C with lamb curdle and ripened for 201 days. Data are inferred by the Figure 4 of the study.	NM	NM	100	120	65.5	136.41	350	110	[48]
Pecorino di Farindola, Italy	Cheese obtained from raw milk, coagulated at 30–35 °C with pig rennet and ripened for 90 days.	NM	0.0–127.1	9.9–394.1	26.8–276.1	0.0–21.8	52.3–1171.3	NM	36.3–143.9	[49]
1) Pecorino Sardo PDO 2) Pecorino 3) Casu Marzu	(1) Pecorino Sardo PDO is semi-cooked cheese made from thermised sheep milk inoculated with a starter culture and coagulated with calf rennet, ripening time between 1 and 12 months. (2) Pecorino is semi-cooked cheese made from raw sheep milk without a starter culture and coagulated with calf rennet, ripening time between 2 and 12 months. (3) Casu Marzu is a sheep's	(1) ND–9.3 (2) ND–13.5 (3) ND–41.8	(1) ND (2) ND–8.6 (3) ND–90.9	(1) 0.1–0.8 (2) ND–92.7 (3) 1.9–165.8	(1) 0.1–9.7 (2) ND–137.0 (3) 3.1–470.7	(1) ND–7.2 (2) ND–128.4 (3) ND–126	(1) ND–19.3 (2) 1.6–93.0 (3) ND–231.4	(1) ND–7.2 (2) ND (3) ND	(1) 0.2–5.4 (2) ND (3) ND	[71]

	cheese produced with the use of larvae of the cheese fly *Piophila casei*. Ripened for 2–3 months in warmer rooms.									
Pecorino Abruzzese, Italy	Two kinds of cheese: (a) raw milk, no starter culture; (b) Thermized milk with the addition of starter culture. Ripening time: 60 days. Data are inferred by the Figure 2 of the study.	(a) 25 [a]; 0–50 (b) 15 [a]; 10–20	(a) 40 [a]; 35–45 (b) 300 [a]; 30–600	(a) 60 [a]; 40–80 (b) 150 [a]; 40–250	(a) 20 [a]; 10–35 (b) 50 [a]; 0–120	(a) 270 [a]; 130–360 (b) 60 [a]; 30–100	(a) 170 [a]; 20–330 (b) 240 [a]; 100–400	(a) 30 [a]; 10–60 (b) 20 [a] 10–30	(a) ND (b) ND	[79]
Pecorino Toscano, Italy	Four kinds of sheep's cheeses, different each among other by the nature of thermal treatment (two from raw milk, two from pasteurized milk), the nature of the starter culture (two different types), the weight of the rind (between 1 and 2.4 kg) and the conditions and the length of the ripening.	11–88	24–144	22–512	2–262	ND–23	147–1132	ND–2	ND	[80]
Feta, Greece	Cheese from thermized milk constituted by 70% of sheep milk and 30% of goat milk, ripened and stored in brine. BAs have been measured during 4 months after production.	4.39–5.74	3.51–4.94	1.62–193	0.27–82.8	2.40–84.6	0–246	NM	NM	[81]

Manchego, Spain [82,84] and Venezuela [83]	Cheese made from raw milk; BAs have been measured after 3 and 6 months of ripening	3 months: <LoD–2.8 6 months: <LoD–15.3	NM	NM	NM	3 months: 24.5–50.3 6 months: 54.1–229	3 months: 233–304.1 6 months: 402.4–533	NM	NM	[82]
	Commercial samples, no details on the production phase	53.78 [a] 44.22–63.35	NM	104.66 [a] 101.05–108.28	52.48 [a] 38.25–66.72	98.93 [a] 76.70–121.18	43.10 [a] 38.57–47.62	110.36 [a] 93.05–127.67	120.22 [a] 117.40–123.05	[83]
	Cheese made from raw milk; BAs have been measured between 3 and 8 months of ripening.	ND	ND–49.8	215.4–668.3	289.9–803.1	60.0–100.1	109.4–326.8	NM	NM	[84]
Pecorino, Italy	(a) high pressure homogenization of raw milk, (b) raw milk, (c) thermized milk. Ripening time: 21 days.	NM	(a) 19.5 (b) 63.3 (c) 155	(a) 14.80 (b) 29.28 (c) 70.92	(a) 20.3 (b) 107 (c) 257	(a) 3.35 (b) 6.32 (c) 23.92	(a) 62.8 (b) 162 (c) 350	(a) 0 (b) 1.49 (c) 0	(a) 10.4 (b) 9.03 (c) 15.9	[85]
Terrincho; Portugal	Cheese obtained from raw milk, coagulated with calf rennet and ripened for 30 days.	35.4–172.9	12.9–237.8	82.6–446.5	48.6–239.6	0.0–10.9	0.0–283.1	NM	NM	[86]
Formaggio di fossa, Italy	Sheep cheese characterized by a ripening performed into pits dug in the sandstone for 90 days.	NM	173.0	579.6	1302.86	24.11	461.62	0	16.49	[87]
(1) Bryndza (2) Smoked cheese (3) Fresh cheese (4) Unripened (fresh) cheese (5) Pasta filata type cheese (6) Brined cheese (7) Flavored cheese	Cheeses 1–3 were from raw ewe's milk, whereas cheeses 4–7 were from pasteurized ewe's milk.	NM	NM	(1) ND–60.9 (2) 16.2–99.9 (3) ND–20.7 (4) ND–118.2 (5) ND (6) ND–229.5 (7) ND–108.8	(1) ND–42.6 (2) ND–80.7 (3) ND–19.6 (4) ND–35.8 (5) ND (6) ND–125.6 (7) ND	(1) ND–24.2 (2) ND (3) ND (4) ND (5) ND (6) ND (7) ND	(1) 34.6–107.4 (2) 8.9–38.3 (3) ND (4) ND–11.1 (5) ND (6) 23.1–174.6 (7) ND–114.7	(1) ND–9.7 (2) ND (3) ND (4) ND (5) ND–13.0 (6) ND–14.7 (7) ND	NM	[88]
Blue-veined cheese	Cheese made from pasteurized sheep's milk and ripened for 6 to 12 months.	61.8–71.11	13.25–61.44	17.28–33.46	NM	NM	7.15–52.20	NM	NM	[89]

12 cheeses from Abruzzo, Italy	(1) 3 cheeses were from sheep's and cow's milk, ripening time between 3 and 8 months. (2) 9 cheeses were only from sheep's milk, ripening time between 3 and 10 months.	NM	(1) 26.0–232.4 (2) ND–44.4	(1) 8.9–986.0 (2) ND–377.7	(1) ND–2172.6 (2) ND–116.4	(1) 200–743.3 (2) 10.3–761.4	(1) 312.1–1771.3 (2) ND–702.4	NM	NM	[90]
Fiore Sardo cheese, Italy	Cheese produced from raw milk, minimum ripening time: 3.5 months.	3 [a]; <0.2–16.9	10 [a]; <0.3–42	150 [a]; <0.2–730	30 [a]; 1.0–94	80 [a]; <0.65–250	350 [a]; <0.5–800	50 [a]; 10–160	<0.23	This study

BA = biogenic amines; NM = not measured; ND = not detectable [a] =mean value.

Results obtained in this study are not too different from those obtained for Feta (Valsamaki et al., 2000) [81] and for some sheep's cheeses produced in different regions of Italy (Abruzzo, Schirone et al., 2013 [90] and Tuscany, Torracca et al., 2015 [80]). However, it is interesting to note that the Feta is not a pure sheep cheese, because it is obtained by a mixture of sheep and goat milk, and also the sheep's cheeses from Tuscany and Abruzzo are often obtained by adopting other technological procedures (i.e., the milk has been undergone to thermal treatments, a starter culture has been used, there are differences on the nature of the rennet and of the ripening technique). On the basis of the data obtained, it is possible to suggest that the total BAs amount found in Fiore Sardo is relatively low also in comparison to other sheep's cheeses [87,90], and is surely not able to lead to any significant health alert situation for consumers.

However, the comparative exam of BAs amounts measured on the sheep's cheeses reported in Table 4 allowed to indicate at least two general trends: (i) in many sheep's cheeses Tyr was very often the most abundant BA and (ii) also Put and Cad were frequently found in high concentrations. The principal microbial groups found in sheep's cheeses are mesophilic and thermophilic lactobacilli, streptococci and enterococci, all within the range 10^5–10^7 cfu/g. In particular, the dominant microflora of Fiore Sardo is constituted by lactococci, always associated with a minor amount of enterococci [66,67,91]. Both micro-organisms remain also during the ripening of the cheese, and they are likely responsible of the high amounts always measured for Tyr, whereas the enterococci strains, able to decarboxylize aminoacids [92], may be responsible not only to the accumulation of α,ω-diamines like Put and Cad, but also of meaningful amounts of His [93]. Obviously, a number of additional parameters like the nature and the technique of preparation of the rennet, the water activity, the pH, the NaCl concentration, the conditions of storage and the time and the temperature of ripening, all still not strictly codificated by the specification of the Fiore Sardo sheep cheese, surely play a role in defining the extreme variability of the distribution of the BAs in this ancient and unique cheese from Sardinia.

4. Conclusion

For the first time, the concentration of eight biogenic amines (i.e., Tyr, Trp, His, Put, Cad, Phe, Spm, Spd) has been measured on a reliable sampling of the most ancient raw sheep cheese produced in Sardinia, Italy: the traditional Fiore Sardo. For do this, a new RP-HPLC-DAD-UV method has been developed, validated and tested on 36 real samples produced by four different cheesemakers and marketed by four stores. Validation was accomplished on the basis of the limit of detection, limit of quantification, linearity, precision and trueness. Quantification limits have been always below 0.75 mg/kg, linearity, measured always on a range of at least two orders of magnitude of concentration, has been sufficient for Put, but is excellent for more of the half of the analytes and concentration ranges considered. The evaluation of precision and trueness gave account for the overall accuracy of the method proposed. The average amount of the sum of the eight biogenic amines measured in Fiore Sardo sheep cheese is 700 mg/kg, whereas the range is between 170 mg/kg and 1,100 mg/kg. If compared with much more higher amounts often reported in literature, these concentrations seem unable to justify any health alert situation for potential consumers. In order of decreasing concentration, Tyr, Put, His and Cad have been the most abundant analytes measured in this matrix, with average concentrations of 350 mg/kg, 150 mg/kg, 80 mg/kg and 30 mg/kg, respectively, whereas Spd was always found below its LoD. The amount of each biogenic amine varied greatly not only among different stores (and, hence, among different producers), but also among different samples provided in different times by the same producer to the same store. Beyond the dominant microflora (mesophilic and thermophilic lactobacilli, streptococci and enterococci) characterizing the raw sheep milk and—consequently—the Fiore Sardo cheese, it is likely that such a wide variability in the amount of BAs is also affected by a number of technological parameters, like the nature and the technique of preparation of the rennet, the water activity, the pH, the NaCl concentration, the conditions of storage and the time and the temperature of ripening, all of which are still not strictly codificated by the specification of the Fiore Sardo sheep cheese.

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