Praca oryginalna *Original paper*

Biogenic amines content in Tuscan traditional products of animal origin*)

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Summary

Biogenic amines can be naturally present in many foods and they can also be produced in high amounts by the activity of amino acid decarboxylases of microorganisms. If ingested in significant amounts they may produce direct or indirect effects on a consumer's health. In food microbiology, their large presence has been associated to spoilage and fermentation processes. The aim of the present study was to assess the content of biogenic amines (single and total value) in Tuscan traditional cheeses and sausages. Thirty samples of these products were tested. Biogenic amines content was analyzed by a HPLC-UV method. Tyramine was, in all the matrices, the amine most often detected and quantified, followed by putrescine and cadaverine. In conclusion, except in dry sausages, the data obtained in the present study suggest these traditional foods have generally low biogenic amines total content.

Keywords: biogenic amines, cheese, sausage, food safety, traditional products

Biogenic amines (BAs) are basic nitrogenous compounds formed by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (19). They are organic bases with low molecular weight and are originated by microbial, vegetable and animal metabolisms. Histamine (HIS), tyramine (TYR), 2-phenylethylamine (2-PHEN), cadaverine (CAD), putrescine (PUT), tryptamine (TPT), spermidine (SPD) and spermine (SPR) have been found in different amounts in several cheeses, sausages and other fermented foods (3, 11, 13, 15, 19, 20). The accumulation of BAs in foods requires the availability of precursors (i.e. amino acids), the presence of microorganisms with amino acid decarboxylating enzymes and favorable conditions for both microbial growth and decarboxylating activity (22). Potentially, all foods that contain proteins or free amino acids, and that have undergone enabling microbial/biochemical activity conditions can be expected to contain BAs. The BAs could represent a potential hazard for human health if ingested in a significant amount by healthy subjects. They are also a risk at a lower dose when potentiating factors such as amine oxidase-inhibiting drugs, alcohol and some pathologies (e.g. gastrointestinal diseases) are present (15, 20).

Determining the exact toxicity threshold of BAs in individuals is extremely difficult, since the toxic dose is strongly dependent on both the amount and the kinds of BAs (23). Besides their toxicological effects, BAs are of concern in relation to food hygiene. High amounts of certain amines may be found in food as a consequence of the use of poor quality raw materials, contamination and inappropriate conditions during food processing and storage (5, 22).

The traditional food products, considered particular for their features, represent cultural, historical and economic aspects of a country. In the EU, the importance of foods with traditional characteristics is recognised. In the legislation it is laid down that Member States may grant derogations to assure that they can continue to be produced, with due regard to food health objectives (4). In Italy, regional lists of traditional products are periodically compiled, as stated by the law (12), to increase the visibility of their heritage, in order to valorise specialities that in some cases do not enable massive production and/or promotional investments.

*) Part of this paper has been communicated at the S.I.S.Vet Congress, 2009 (Udine, Italy).

The interest of food business operators and competent authorities is focused on guaranteeing the traditional products hygiene and safety, and the implementation of useful prevention tools related to specific artisanal productions. The law states that traditional products have to be manufactured in a homogeneous manner, according to traditional rules for at least 25 years (12).

Nevertheless, the absence of technological standardization and uncontrolled hygiene conditions can represent a potential circumstance for a high biogenic amines production, and thus to be of concern for the health's consumer (11). Nowadays these kinds of Italian typical foodstuffs can be available in distant countries, due to the modern world wide trade system. Hence it is especially important to know, at the international level, the features of these products.

Few papers concerning the total content of BAs in Italian traditional products are reported in literature: significant total contents of BAs in artisanal Salsiccia and Soppressata sausages (16), in Pecorino Abruzzese cheeses from pasteurized milk (11) and in „Lardellato" salami (18), have been found.

No papers concerning the content of BAs in Tuscan traditional products are present in literature. In this study different categories of traditional cheese and meat products of Tuscany are taken into account in order to assess the content of BAs (single and total value).

Material and methods

Sampling. Two groups of samples of Tuscan traditional products, cheeses and meat products, were taken into account. The former was divided in two categories: soft pecorino cheeses (SP, n = 8) and semi-hard pecorino cheeses (SHP, n = 7). The latter was divided in three categories: fresh sausages (*salsiccia fresca Toscana*) (S, n = 5), cooked sausages (CS, n = 5) and dry fermented sausages (DS, n = 5). All the products were manufactured in Tuscany and bought in supermarkets, in local stores or directly from the producers.

The SP category is produced with sheep's milk submitted to a pasteurization or thermization process and coagulated after the inoculation of a lactic acid starter with calf or lamb rennet. The curd is cut with dimensions from maize to hazelnut, molded, drained and dry or brine salted, with a final ripening phase on typical wood shelves, usually for 15-30 days.

The SHP category is produced with raw sheep's milk, without the addition of a lactic acid starter. The curd is cut in size ranging from rice to maize, molded and submitted to larger whey than SP and dry or brine salted. This product is ripened on typical wood shelves for 45-90 days.

The *salsiccia fresca Toscana* is one of the most appreciated traditional sausages produced in Italy, mostly produced by small family plants or in butcher's shops. It is obtained from chopped meat stuffed in small casings. The mixture consists of pork's meat and fat, salt, pepper and spices, added with nitrates and nitrites. No wide sugar fermentation, and drying process are present. Its shelf-life ranges between 8 to 10 days.

CS are produced from pig's blood and fat (*Buristo*) or from the second cut of pork meat (*Soppressata*). CS are submitted to a cooking process and then stored under refrigerated temperatures.

DS are prepared with pork's meat and lard, salt, sugar, spices; antioxidants (ascorbic acid) and preservatives (potassium nitrate and sodium nitrite) are also added to the mixture. No starter cultures are added. DS are dried for 1-2 days in a drying room and then ripened in conditioned room for 30-45 days.

Chemicals. HIS, TYR, 2-PHEN, CAD, PUT, TPT, SPD, SPR, 1.7-diaminoheptane (internal standard, IS), dansyl chloride (derivatization agent, DCl) and proline were purchased from Sigma-Aldrich Inc (Saint Louis, MO, USA); reagent grade hydrochloric acid (HCl), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH) trichloracetic acid (TCA) and diethyl ether from Carlo Erba (Milan, Italy); HPLC grade acetonitrile, acetone and methanol from Lab Scan (Dublin, Ireland). Ultrapure water was obtained with a Milli-Q system (Millipore, Milan, Italy).

Stock solutions. Stock solutions of BA(s) and IS were prepared by dissolving the substances in distilled water (1000 µg/mL). All stock solutions were stored at –20°C and working solutions were prepared daily through dilution. The different working solutions were used for the determination of the calibration curves, recovery, intra-/inter-day accuracy, limit of detection (LOD) and limit of quantification (LOQ).

Preparation of samples. The preparation of samples was based on a previous procedure (Innocente et al. 2007), partially modified. Briefly, 10 g of cheese or sausage were accurately weighed in a centrifuge tube, added with 20 mL of 0.1 M HCl or 5% TCA (Moret and Conte 1996), respectively, containing 1 mL (50 µg/mL) of IS and then homogenized with Ultra-Turrax® T25 Basic (Ika equipment, Staufen, Germany) for 5 min. The homogenate was centrifuged at 12,000 g for 20 min at 4°C, the aqueous layer was collected and the pellet was re-extracted using the same procedure. The two aqueous extracts were combined, diluted to 50 mL with 0.1 M HCl or 5% TCA, filtered under vacuum and centrifuged 5 min at 14,000 g to eliminate residual food particles.

For the derivative preparation, 1 mL of saturated $NaHCO_3$ solution was added to a 2 mL aliquot of the diluted extract and adjusted to pH 11.5 by adding 1 M NaOH. This mixture was added with 2 mL of DCl (5 mg/mL) incubated at 40°C for 60 min and occasionally shaken. In order to eliminate the excess of DCl the mixture was treated with 400 µL of a L-proline solution (100 mg/mL), vortexed for 1 min and left to react in the dark for 15 min at room temperature. The sample was then added with 2 mL of diethyl ether, mixed and centrifuged at 2,000 g for 5 min. The organic layer was collected and the procedure repeated once again. The organic layers were combined and dried under nitrogen flow at room temperature. The residue was re-dissolved in 2 mL of acetonitrile and transferred into a vial for HPLC analysis.

Apparatus and chromatographic conditions. HPLC analyses were performed with a Thermo Finnigan HPLC (Walthan, MA, USA) equipped with a gradient pump (P 2000),

UV detector (UV 2000) set at 254 nm, auto-sampler/-processor with a variable (1-100 µL) loop (AS 3000) controlled by Chromquest 4.1 software. The column was a reversed-phase Hypersil GOLD C18 (150 mm × 4.6 mm, 3 µm) (Superchrom, Milan, Italy). Acetonitrile (A) and water (B) were used as solvents: the elution programme was held at 65% of B for 1 min, ramped at 80% (10 min), 90% (12 min), 100% of B (16 min) and held until the end of the run (23 min) with a flow rate of 0.8 mL/min (13).

The intra-/inter-day precision and accuracy were determined from 3 replicate samples at LOQ, low, medium and high concentrations of pure analytes, during a period of 7 days. Recovery was tested by the standard addition procedure using three addition levels (1, 5, 10 mg/L) for each amine in each kind of sample (cheese and meat products). Three determinations were carried out for each addition level. The recovery of each amine was calculated by comparing the chromatographic areas of the peaks with those obtained for a directly derivatized standard solution of the same concentration. These values were subtracted from the initial content quantizated in the un-spiked matrices (blanks). LOD and LOQ were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10, respectively (1).

The data obtained were evaluated according to the Kruskal-Wallis test, analyzing the differences between the groups. Differences were considered significant if associated with a probability level of less than 0.05.

Tab. 1. Average amine recoveries (%) in cheeses and meat products ($\bar{x}$ ± SD)

BA	Cheeses		Meat products		
	SP	SHP	S	CS	DS
TPT	72.5 ± 4.0	75.2 ± 6.2	69.5 ± 5.7	75.7 ± 6.7	73.7 ± 4.7
2-PHEN	69.5 ± 5.1	67.9 ± 5.5	71.5 ± 3.5	67.9 ± 5.6	70.2 ± 6.5
PUT	99.4 ± 3.5	98.4 ± 4.5	96.4 ± 5.6	98.6 ± 4.5	97.5 ± 5.4
CAD	99.1 ± 5.6	100.5 ± 4.5	97.7 ± 3.4	99.6 ± 4.5	101.4 ± 3.4
HIS	77.8 ± 5.7	74.5 ± 6.8	75.8 ± 3.9	72.4 ± 5.6	77.2 ± 6.1
IS	96.2 ± 6.6	98.4 ± 4.2	95.9 ± 5.7	97.4 ± 4.1	96.7 ± 5.5
TYR	83.5 ± 3.6	80.5 ± 4.5	79.7 ± 5.3	83.2 ± 4.5	85.7 ± 5.9
SPD	80.6 ± 2.5	83.3 ± 5.8	80.7 ± 3.8	79.1 ± 5.3	77.5 ± 5.7
SPR	60.7 ± 7.2	67.3 ± 5.9	65.7 ± 4.5	71.1 ± 5.4	67.9 ± 4.7

Explanations: Tryptamine (TPT), 2-phenylethylamine (2-PHEN), putrescine (PUT), cadaverine (CAD), histamine (HIS), tyramine (TYR), spermidine (SPD), spermine (SPR) and internal standard (IS). Soft pecorino cheese (SP) and semi-hard pecorino cheese (SHP). Fresh sausages (S), cooked sausages (CS), dry fermented sausages (DS)

Results and discussion

In the present study the analytical method previously reported to detect BAs in cheese and meat products (8), was not suitable because of the occurrence of gel during the extraction procedure that drastically reduced the recoveries of the analytes. The two new steps (filtration under vacuum and high-speed centrifugation) added to the procedure prevented the production of the third gel phase. The LOQ and LOD of the modified method were 1 and 0.3 mg/kg, respectively. The maximum value of the variability coefficient of the intra-/inter-day precision was 7%. Table 1 shows the recoveries of the individual amines in the different matrices tested.

Figure 1 shows the BAs profile in cheeses. The most concentrated BAs were TYR (125.64 ± 114.54 mg/kg SHP; 27.13 ± 37.58 mg/kg SP) and PUT (95.81 ± 103.41 mg/kg SHP; 24.78 ± 18.30 mg/kg SP). CAD, 2-PHEN, TPT and HIS were detected in smaller amounts, whereas SPD and SPR only as traces. SP and SHP showed a similar BAs presence. The wide variability in BAs value, reported in cheeses belonging to the same category, made the difference in the overall BAs content between SHP (307.61 ± 334.01 mg/kg) and SP (78.59 ± 62.52 mg/kg) not significant, despite the large variation reported between the average values.

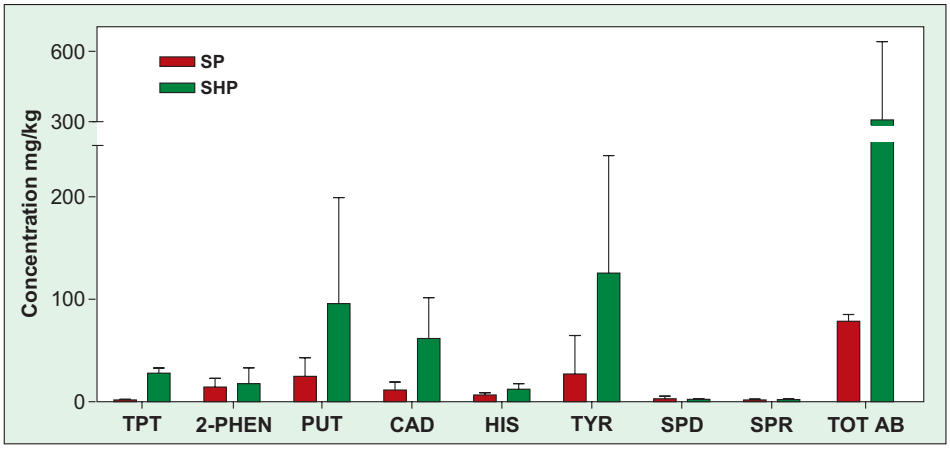

Fig. 1. Mean concentrations of biogenic amines in soft pecorino cheese (sp, red columns) and semi-hard pecorino cheese (shp, green columns). Bars represent standard deviation. No significant difference was detected between the two groups of samples

Figure 2 depicts the BAs detected in sausages. The average values of the main BAs quantified were: TYR (23.38 ± 24.66 mg/kg S; 12.93 ± 12.17 mg/kg CS; 615.33 ± 619.44 mg/kg DS), PUT (14.56 ± 12.00 mg/kg S; 19.53 ± 11.30 mg/kg CS; 308.38 ± 182.39 mg/kg DS) and CAD (13.82 ± 14.05 mg/kg S; 10.43 ± 7.09 mg/kg CS; 268.06 ± 277.16 mg/kg DS). HIS (91.83 ± 100.13 mg/kg), 2-PHEN (70.97 ± 97.11 mg/kg) and TPT (68.83 ± 100.84 mg/kg) were de-

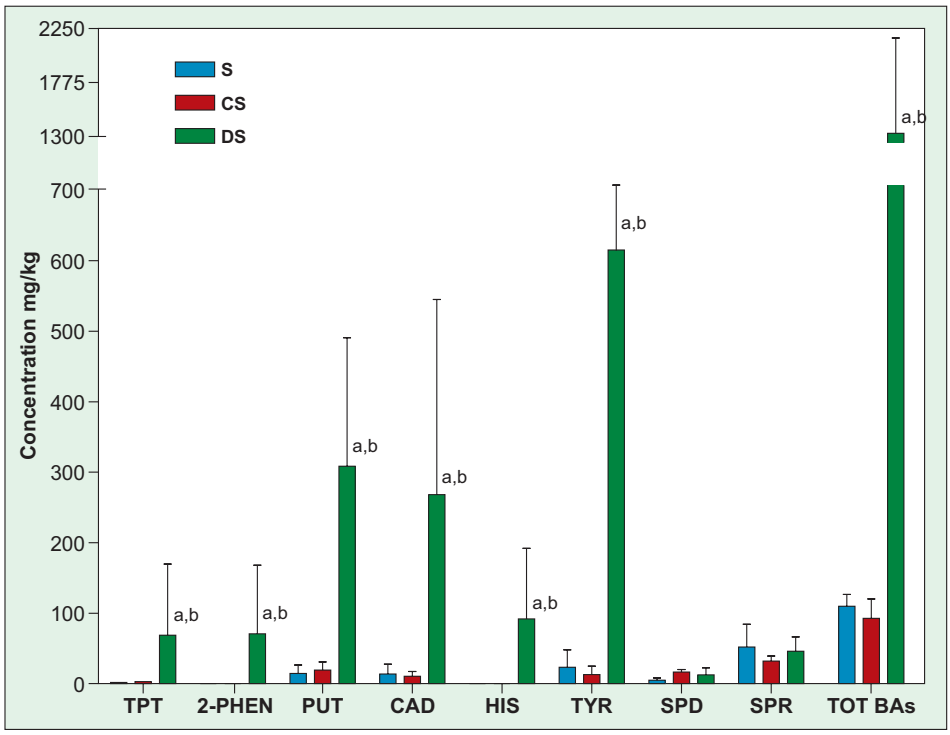

Fig. 2. Mean concentrations of biogenic amines in fresh sausages (s, blue columns), cooked sausages (cs, red columns) and dry fermented sausages (ds, green columns). Bars represent standard deviation. a = significantly different ($p < 0.05$) from s value; b = significantly different ($p < 0.05$) from cs value

tected only in DS samples. The DS products showed significant total BAs. This value was 13 times higher than those found in S and CS. No significant differences were reported in single and total BAs between S and CS. Concerning the total amount of BAs, DS (1340.87 ± 838.94 mg/kg) was four times more contaminated than SHP (307.71 ± 334.01 mg/kg).

The analytical method carried out in this study was based on a previous procedure (8) partially modified, because of the presence of a third gel phase responsible for the reduction of the recoveries. This occurrence was not reported in Innocente et al. (8). It could be due to the high presence of fat in the cheese and sausage samples tested in the present study.

The consumption of food containing a high concentration of BAs may cause toxic effects: values higher than 1000 mg/kg for total BAs (21), 800 mg/Kg for TYR, 100 mg/Kg for HIS and 30 mg/kg for 2-PHEN (5, 22), have been reported as toxic doses in food. It is usual to consume fermented foodstuffs together during meals, thereby enhancing the possibility of having undesired effects. According to EFSA (2), the effect of BAs in humans is underestimated and in order to quantify them, more research in this field is necessary.

The wide variability of BA concentrations detected in cheeses could be due to the heterogeneity of samples and is in line with previous studies concerning similar products (11, 20). Factors such as raw milk quality, hygiene of processing technology, microbial populations and the environment could be responsible for these variations in pecorino cheese within the same category.

The total content of BAs in SP lower than SHP could be due to a decrease in the number and types of different bacteria – including many categories of decarboxylase-positive microorganisms – caused by the heat-treatment of milk (15, 19). In contrast, other studies speculated that pasteurization does not reduce the quantity of TYR in Pecorino Abruzzese (11) and Emmental (9).

TYR was the most concentrated BA in both types of cheese, followed by PUT and CAD, in accordance with previous studies (7, 15, 19). In both types of cheese the amount of TYR was always well below 800 mg/kg; the amount of HIS was below 100 mg/kg, disagreeing with an earlier study (11). The amounts of diamines (PUT and CAD) and polyamines (SPD and SPR) in cheeses were quite low and almost nil, respectively, suggesting a negligible or poor risk on consumer's health.

In meat products, TYR, PUT and CAD were the most frequently occurring BAs according to Parente et al. (16) and Rea et al. (18). In DS, BAs total content resulted over the threshold of 1,000 mg/kg, while S and CS showed a lower value in contrast to Hernandez-Jover et al. (6).

In this study the HIS quantity was significant in DS compared to S and CS, despite the average value being lower than the supposed toxic threshold of 100 mg/kg (22). The amounts of CAD, PUT, 2-PHEN and TPT were significantly higher in DS than in the other two types of sausages. Given that cooking reduces the number and types of different bacteria, a large amount of BAs is not expected in CS. At any rate, their content could be due to other factors such as the quality of the raw meat material (17), the hygiene of processing technology and storage conditions (10). Similarly, the poor amount of BAs reported in S could be attributable to the short shelf-life that does not allow bacteria to produce a large amount of BAs. The high levels of BAs found in DS are considered inevitable as the technology of production promotes a large presence of free amino acids and BAs producing microorganisms. The subsequent long-lasting ripening time allows both the growth of bacteria and the decarboxilation activity (22), leading to the formation of BAs.

Although PUT and CAD are well known not to cause directly adverse effects on human health, when subjected to heat they can give rise to the formation of

secondary amines, and with the presence of nitrites, these can generate nitrosamines, chemical agents considered to possess major carcinogenetic properties (15, 19, 24). This issue is particularly important in fermented products that may contain high BA levels in the presence of nitrates and nitrites, used as food preservatives. Hence, the significant amounts of PUT and CAD recovered in DS in the present study might be of concern for the consumer. Evidently, there are difficulties and limitations in determining the true level of toxicity, but considering the findings reported by various authors, there are undoubtedly a large number of cheeses and meat products containing BAs. Unfortunately, there is a lack of specific legislation setting limits on BAs in cheese and meat products, despite some authors making general recommendations to set a limit for HIS in foods.

In conclusion, except in the case of DS the data obtained in the present study shows that Tuscan traditional products have a generally safe BAs total content. Therefore, the evaluation of BAs in Tuscan traditional products could be an important tool for determining the food quality, providing additional guarantees of safety for consumers.

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