Biogenic Amines Profile of Serbian Traditional Sausage in Relation to Raw Material and Production Conditions

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Abstract: Petrovská klobása is a traditional dry fermented sausage produced in the Autonomous Province of Vojvodina. Formation of nine biogenic amines was determined in three sausage groups produced from hot deboned (A1) and cold meat (B1, B3) during drying and ripening in traditional room (A1, B1) and in industrial ripening chamber (B3). Dansyl chloride derived amines were analysed by high-performance liquid chromatography with diode-array detector (HPLC-DAD) on Eclipse XDB-C18 column. Histamine, the most important amine from food safety point of view, was not found in any of the analyzed samples. Serotonin and spermidine were also not detected in any samples, while spermin was determined in all analyzed samples. At the end of drying, phenylethylamine was the predominant amine in A1 sausage group (51.6 mg/kg), and tryptamine in B1 (38.1 mg/kg) and B3 (28.7 mg/kg) sausage groups. At the end of ripening, tryptamine was the predominant biogenic amine in all sausage groups (133, 121 and 39.8 mg/kg in A1, B1 and B3 groups, respectively). Total level of biogenic amines in all investigated sausages did not exceed 174 mg/kg at the end of drying and 238 mg/kg at the end of ripening period. Tryptamine and cadaverine may be used as indicators of total biogenic amines for sausages produced in traditional and industrial conditions from hot deboned and cold meat.

Key words: Biogenic amines, traditional dry fermented sausage Petrovská klobása, raw material, production conditions.

1. Introduction

Biogenic amines are organic bases naturally present in many foods [1, 2]. They are formed during ripening processes by microorganisms with ability to synthesize amino acid decarboxylase and high proteolytic potential or they are formed as a result of food spoilage [1, 3-5]. Consumption of food containing high amounts of biogenic amines may be harmful [4, 6-9]. High levels of biogenic amines present in foods may cause migraine, headache, gastric and intestinal problems and pseudo allergic responses [3, 6].

Histamine is considered as the most toxic biogenic amine [1, 2, 6, 8]. It has vasoactive properties and may cause urticaria, hypotension, headache, flushing, abdominal cramps and other problems for human health [10]. Thus, it is important to monitor biogenic amines in fresh and processed foods, not only due to their toxicity, but also because they can be a useful index of spoilage or ripening stage [1, 4, 11-14].

Petrovská klobása is a traditional dry fermented sausage which production dates back to 250 years ago. It has been produced in the area nearby Bački Petrovac town in the Autonomous Province of Vojvodina, Republic of Serbia. Production is carried out in small household enterprises in December, when temperatures are around 0 °C and lower, using traditional methods. This product has been protected with designation of origin (PDO) according to the Serbian legislation due to its specific and recognizable characteristics [15-17].

Traditionally, homemade Petrovská klobása is produced exclusively from pork meat and fat, red hot paprika powder, salt, crushed garlic, caraway and sugar. Ripen sausage is characterized by specific hot taste, aromatic and spicy flavor, dark red color and
hard consistency [15-17]. Traditional production of Petrovská klobása does neither include the use of starter cultures nor additives (nitrite, gluconodeltalactone, etc.), in contrary to industrial production of fermented sausages. Fermentation and ripening processes, as well as final quality of the product, are influenced by indigenous microflora of raw materials and environment. During ripening process, activity of present microflora causes decarboxylation of amino acids and formation of biogenic amines.

Great fluctuations of biogenic amine content are reported in the same type of products. These differences depend on numerous variables: qualitative and quantitative composition of microflora, chemical and physical factors, hygienic procedure adopted during production and availability of amino acids as precursors. Large variability in type and quantity of biogenic amines, even in sausages produced under controlled conditions, indicates that their formation depends on a complex interaction of factors [2, 8]. Dry fermented sausages can be a source of biogenic amines, due to high amount of proteins present in these products, and they are precursors for decarboxylation which is occurring during ripening [2].

Large variations in biogenic amine content and composition were found in traditional fermented sausages produced in European countries, even though usually tyramine is the most important biogenic amine [18-20]. According to available literature, there are only few data concerning biogenic amines content in traditional dry fermented sausages from Serbia.

The aim of this paper was to determine the formation of nine biogenic amines in Petrovská klobása during drying and ripening period, with regard to raw material (hot deboned meat or cold meat) and processing conditions (traditional room or industrial ripening chamber).

2. Materials and Methods

2.1 Sausage Samples

Three groups of sausages were examined in this study (Table 1). Traditionally Petrovská klobása is made from hot deboned meat, but as in recent years, food safety is an increasingly important public health issue, as well as because of some technological disadvantages of hot deboned meat processing, producers have to slightly modify the production conditions to meet the requirements.

All groups of sausages were produced in traditional manner. Minced lean pork meat 80% and pork fat 20% were mixed with red hot paprika powder (2.50%), salt (1.80%), crushed garlic (0.20%), caraway (0.20%) and sugar (0.15%). All ingredients were mixed approximately 10 min using traditional technique. The mixture was stuffed into natural casings—pig intestine (rectum) around 40 cm long and near 5 cm in diameter. Traditionally processed sausages were smoked using cool procedure for 10 d with pauses, while sausages processed in industrial conditions were smoked for 6 h (12 × 30 min), during 3 d.

According to Serbian legislation, moisture content for dry fermented sausages have to be less than 35%. Sausages from A1 and B1 group dried in traditional room (temperature from 2.6 °C to 12.4 °C; relative humidity from 43.3% to 93.0%) needed 90 d to reach required moisture content, while sausages from B3 group dried in industrial ripening chamber (temperature

<table>
<thead>
<tr>
<th>Group of sausages</th>
<th>Raw material</th>
<th>Processing conditions</th>
<th>Days of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Hot deboned meat (2 h post mortem)</td>
<td>Traditional room</td>
<td>0, 2, 6, 9, 12, 15, 30, 60, 90, 120</td>
</tr>
<tr>
<td>B1</td>
<td>Cold meat (24 h post mortem)</td>
<td>Traditional room</td>
<td>0, 2, 6, 9, 12, 15, 30, 60, 90, 120</td>
</tr>
<tr>
<td>B3</td>
<td>Cold meat (24 h post mortem)</td>
<td>Industrial ripening chamber</td>
<td>0, 2, 6, 9, 12, 15, 30, 45, 120</td>
</tr>
</tbody>
</table>
of 10 °C; relative humidity ~75%) reached this value after 45 d, what determined the sausage sampling.

At the end of drying period, the 90th day for A1 and B1, and 45th day for B3, sausages from all groups were vacuum packed and ripened at 10 °C and 75% relative humidity further on until the 120th day, when it achieves the optimum quality [15, 17].

Samples of 250 g were homogenized on Waring 8010 ES Blender, USA with capacity 1 L and speed 18,000 rpm around 15 s with temperature after homogenization < 10 °C. Homogenized samples were stored at -20 °C until analyzed.

All determinations were performed on three samples from each batch in duplicate.

2.2 Biogenic Amines Determination

Tryptamine, phenylethylamine, putrescine, cadaverine, histamine, serotonin, tyramin, spermidine and spermine were determined following the high-performance liquid chromatography (HPLC). Sample extraction and derivatization were done according to Eerola et al. [21]. Briefly, 2 g of each sample were weight and put into test tube. Appropriate amount of internal standard was added and sample was homogenized with 10 mL 0.4 M perchloric acid using Ultraturrax blender. The homogenate was centrifuged at 3,000 rpm for 10 min, and supernatant was filtered into 25 mL bottle. Extraction was repeated with 10 mL 0.4 M perchloric acid solution, mixed with Vortex and centrifuged as before. Supernatants were combined and adjusted to 25 mL with 0.4 M perchloric acid. After, 1 mL of sample extract was made alkaline by adding 200 µL 2 M NaOH and buffered with 300 µL saturated NaHCO3. Then, 2 mL of dansyl chloride solution was added and the reaction mixture was incubated at 40 °C for 45 min. Residual dansyl chloride was removed by adding 100 µL ammonia. After 30 min, mixture was adjusted to 5 mL with acetonitrile, filtered (0.45 µm, PTFE, MS Springer filter) and analyzed.

HPLC analysis was performed by using a liquid chromatography (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a binary pump, an online vacuum degasser, an auto sampler and a thermostated column compartment, on an Agilent, Eclipse XDB-C18, 1.8 µm, 4.6 mm × 50 mm column.

Solvent gradient was performed by varying the proportion of solvent A (acetonitrile) to solvent B (water) as follows: initial 50% B, linear gradient to 10% B in 7.6 min, 10% B to 10 min, linear gradient to 50% B in 2 min. System was equilibrated 3 min before next analysis. Flow rate was 1.5 mL/min, column temperature was 40 °C and 5 µL of sample was injected [22]. Detection limits of the amines were determined to be 0.1 µg/g for putrescine and spermidine, 0.167 µg/g for cadaverine and tyramin, 0.25 µg/g for tryptamine, phenylethylamine and histamine, and 0.5 µg/g for serotonin and spermine, respectively.

2.3 Statistical Analysis

Statistical analysis in biogenic amines content between groups of sausages (A1, B1 and B3) was done at the end of drying and at the end of ripening period. One way ANOVA post-hoc (Duncan test) was performed. Differences were considered significant at $P < 0.05$. Correlation coefficients among biogenic amines were also calculated. The software package Statistica 9.1 for Windows, Stat Soft, Tulsa, Oklahoma, USA, 2009 was used.

3. Results and Discussion

Formation of biogenic amines in traditional dry fermented sausage Petrovská klobása was determined using HPLC method for analysis. Chromatographic separation of nine biogenic amines was completed in 8 min.

3.1 Formation of Biogenic Amines

Formation of tryptamine, phenylethylamine, putrescine, cadaverine and tyramine are shown in
Fig. 1. Tryptamine content in samples detected varied from 0 mg/kg to 133 mg/kg. The concentrations ranged from 14.7 mg/kg (A1) to 38.1 mg/kg (B1) at the end of drying, and from 39.8 mg/kg (B3) to 133 mg/kg (A1) at the end of ripening when tryptamine was the prevailing biogenic amine in all groups of sausages. Tryptamine concentrations were significantly different ($P < 0.05$) between all groups at the end of drying, as well as at the end of ripening period. At the end of ripening, concentrations of tryptamine in sausages dried in traditional manner were much more closer (133 mg/kg and 121 mg/kg, for A1 and B1, respectively) and higher than tryptamine concentration for sausages dried in industrial chamber (39.8 mg/kg for B3).

The content of phenylethylamine, aromatic amine, ranged from 0 mg/kg (B3) to 51.6 mg/kg (A1) at the end of drying, and from 16.6 mg/kg (B3) to 43.1 mg/kg (B1) at the end of ripening. The differences between all groups at the end of drying, as well as at the end of ripening, were significant ($P < 0.05$). Unlike B3, where phenylethylamine was found only
on the 120th day, in A1 and B1 this amine was found in all samples analyzed at the mentioned days. At the end of drying period, putrescine content in A1 group was 5.54 mg/kg. On the contrary to A1, in B1 and B3 groups putrescine was not detected during whole drying period, while at the end of ripening period putrescine was found in B3 group, with values of 19.2 mg/kg and 4.6 mg/kg for A1 and B3, respectively. Formation of cadaverine was similar. This amine was not detected in B1 and B3 groups during whole drying period, while in A1 group it was detected until the 60th day.

Unlike the putrecin, cadaverin was found in all groups at the end of ripening period in the range from 6.69 mg/kg (B3) to 19.4 mg/kg (A1). These differences could be explained by the larger amount of diamines, especially cadaverine in the raw material (hot deboned meat) of weaker microbiological quality, with a relatively higher number of Enterobacteriaceae, as it was refered by Bover-Cid et al. [23].

Histamine and serotonin were not detected in any of the analyzed samples. Since histamine is well known for its toxity, the absence of this biogenic amine is very important from toxicology point of view. Histamine was not found in examined sausages because of low temperatures applied during smoking, drying and ripening. Both drying models used in this study were slow drying models with the maximum temperatures up to 12.4 °C, which are not adequate conditions for histamine formation [22].

Tyramine, reported as the most abundant amine in fermented sausages [2, 8, 18, 24, 25], was determined in the highest concentration in sausages dried and ripened in industrial chamber, while in sausages produced from hot deboned meat dried and ripened in traditional room, this amine was not determined at both end of drying and ripening period. Observed values between all groups were significantly different \( (P < 0.05) \).

The great variety in type and content of biogenic amines was determined in traditional dry fermented sausages. Tyramine and putrescine were the most abundant biogenic amines found in different types of dry fermented sausages produced in Finland, while in Danish sausages, cadaverine was found in high amounts. In French sausages, putrescine, cadaverine, tyramine and histamine were detected. And in sausages produced in Southern Italy, tyramine was the most important biogenic amine, followed by putrescine and cadaverine, while 2-phenylethylamine was found at low levels [4].

### 3.2 Formation of Polyamines

In terms of polyamines content (Table 2), spermidine was not found in any sample, while spermine is the only biogenic amine which was found in all groups during whole time of drying and ripening period in the range from 21.5 mg/kg to 101 mg/kg. Quantity and trend of spermine changes during dry sausage production were nearly the same in A1 and B3 group, while in B1 group, the increase was observed after the 30th day. Thus, at the end of drying period, content of spermine in B1 group was more than 100% higher than this content in A1 and B3.

It is well known that spermine originate from meat as the main raw material, and its level mainly remains stable with slight increase or decrease depending on different technological factors during production [26]. On the contrary to other biogenic amines, polyamine synthesis is a more complex process. Although the first few steps also include a decarboxylation reaction, for spermine syntheses, additional enzymes are required [26]. The polyamines spermine and spermidine always occur in fresh meat, and their relevant presence in sausages has been related to drying process involved in the manufacture of this product [24]. Nevertheless, enterobacteria and some lactic acid bacteria isolated from sausages [27], and enterococci from cheese [28] were able to produce these polyamines. Obviously, in B1 group, such conditions were made. Obtained value for spermine is lower than this value found in different dry fermented
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Table 2  Content of spermidine and spermin in examined sausage groups during drying and ripening.

<table>
<thead>
<tr>
<th>Drying and ripening period (d)</th>
<th>Polyamines content (mg/kg)</th>
<th>Spermidin</th>
<th>Spermin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A1</td>
<td>B1</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>90</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>120</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are expressed as means ± standard deviations. ND: not detected.
*a-g* Means with different letters in the same raw are significantly different (*P* < 0.05).

Sausages after ripening, except in Saucisson (industrial) where spermine content ranged from 59 mg/kg to 119 mg/kg [2].

### 3.3 Total Biogenic Amine Content

The total level of biogenic amines in all investigated sausages did not exceed the value of 174 mg/kg at the end of drying period and 238 mg/kg at the end of ripening period, respectively (Fig. 2). At the end of drying, significant differences (*P* < 0.05) were observed between all groups of sausages, while at the end of ripening, significant differences were obtained between groups of sausages produced in traditional room (A1 and B1) and in industrial ripening chamber (B3).

The difference in content of biogenic amines was observed between sausage mixtures produced from hot deboned (A) and cold meat (B) (Fig. 2). Higher content in B mixture could be explained by longer activity of endogenous peptidases (24 h), which is mainly active during this stage [29, 30].

In the later stages of drying and ripening, when egzopeptidases [31, 32] generate amino acids, application of slow fermented drying model in sausage production results in similar biogenic amines formation, especially in groups B1 and B3. Decreases in total biogenic amines content at the 60th day in A1 group was obtained possibly due to their transformation into compounds of the aroma and taste holder. Moreover, aminogenesis and accumulation of biogenic amines are higher and faster at higher processing temperatures and relative humidity [14].

### 3.4 Correlations between Biogenic Amines Contents

Correlation coefficients between individual and total biogenic amines are presented in Table 3. There are very significant correlations between total biogenic amines content and tryptamine and cadaverine concentrations in all examined groups, while very significant correlations between total biogenic amines content and phenylethylamine, putrescine and tyramine concentrations were observed only for sausages ripened in industrial chamber. Consequently, certain biogenic amines, such as tryptamine and cadaverine may be used as indicators of total biogenic amines for sausages produced in traditional and industrial conditions from hot deboned and cold meat.

Based on results obtained in this study, regarding biogenic amines as food safety parameter, it is obvious that both hot deboned meat and cold meat could be used for sausage production. If we consider the
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Fig. 2 Total biogenic amine content in examined sausage groups during drying and ripening.

Table 3 Correlations between biogenic amines contents in three examined groups.

<table>
<thead>
<tr>
<th>Biogenic amines</th>
<th>A1</th>
<th>B1</th>
<th>B3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRY-PHE</td>
<td>0.41**</td>
<td>0.74**</td>
<td>0.96**</td>
</tr>
<tr>
<td>TRY-PUT</td>
<td>0.65**</td>
<td>0.56**</td>
<td>0.96**</td>
</tr>
<tr>
<td>TRY-CAD</td>
<td>0.56**</td>
<td>0.93**</td>
<td>0.97**</td>
</tr>
<tr>
<td>TRY-TYR</td>
<td>0.46**</td>
<td>0.59**</td>
<td>0.98**</td>
</tr>
<tr>
<td>TRY-Total</td>
<td>0.71**</td>
<td>0.77**</td>
<td>0.71**</td>
</tr>
<tr>
<td>PHE-PUT</td>
<td>0.90**</td>
<td>0.96**</td>
<td>0.98**</td>
</tr>
<tr>
<td>PHE-CAD</td>
<td>0.13\text{NS}</td>
<td>0.90**</td>
<td>0.99**</td>
</tr>
<tr>
<td>PHE-TYR</td>
<td>0.90**</td>
<td>0.95**</td>
<td>0.96**</td>
</tr>
<tr>
<td>PHE-Total</td>
<td>0.03\text{NS}</td>
<td>0.24\text{NS}</td>
<td>0.57**</td>
</tr>
<tr>
<td>PUT-CAD</td>
<td>0.58**</td>
<td>0.00\text{NS}</td>
<td>1.00**</td>
</tr>
<tr>
<td>PUT-TYR</td>
<td>0.95**</td>
<td>0.97**</td>
<td>0.97**</td>
</tr>
<tr>
<td>PUT-Total</td>
<td>0.10\text{NS}</td>
<td>0.02\text{NS}</td>
<td>0.50**</td>
</tr>
<tr>
<td>CAD-TYR</td>
<td>0.62**</td>
<td>0.18\text{NS}</td>
<td>0.97**</td>
</tr>
<tr>
<td>CAD-Total</td>
<td>0.77**</td>
<td>0.73**</td>
<td>0.54**</td>
</tr>
<tr>
<td>TYR-Total</td>
<td>-0.07\text{NS}</td>
<td>0.12\text{NS}</td>
<td>0.69**</td>
</tr>
</tbody>
</table>

TRY: Tryptamine, PHE: phenylethylamine, PUT: putrescine, CAD: cadaverine.

Correlation coefficient values marked with NS indicate no statistically significant correlation, values marked with * indicate significant correlation \((P < 0.05)\) and values marked with ** indicate very significant correlation \((P < 0.01)\).

The fact that biogenic amines contribute to the aroma of sausage [33], it is better to produce this traditional sausage from cold meat using slow processes of drying and ripening.

Accumulation of free amino acids as well as formation of specific sausage properties is consequence of proteolysis during ripening.

Availability of precursors (i.e., amino acids) and the presence of amino acid decarboxylases producing microorganisms and favourable conditions for their growth and activity, are necessary preconditions for biogenic amines formation [1, 2, 4]. Formation and changes in the type and content of biogenic amines can be different, even when the final product has been
produced from the same raw material. The fact is that amine formation is influenced by numerous factors, and their complex interaction is also confirmed in this study. Among these factors, the raw materials used in this experiment (hot deboned and cold meat) and the use of an appropriate drying model, certainly took an important role and had impact on the dynamics of proteolysis.

4. Conclusions

Histamine, the most important amine from food safety point of view, was not found in any of the analyzed samples. Total level of biogenic amines in all investigated sausages did not exceed 174 mg/kg at the end of drying and 238 mg/kg at the end of ripening period. Tryptamine and cadaverine may be used as indicators of total biogenic amines for sausages produced in traditional and industrial conditions from hot deboned and cold meat.

Amines formation is influenced by numerous factors and their complex interaction is conformed in this study. Among these factors, raw material and production conditions had an important role and influenced the dynamics of proteolysis.

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