

Biogenic amines in wine and cheese

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Biogenic amines in ten samples of wine and five samples of cheese were determined using an HPLC-UV-MS method. Inevitable derivatization was performed with the reagent dansyl chloride, and the derivatives prepared were analysed under optimized chromatographic conditions in reversed phase mode. An octadecylsilicagel column (C18) was used for the separation of ten selected biogenic amines derivatives which were eluted using the mobile phase composed of 5 mM ammonium formate and acetonitrile at a flow-rate 0.6 mL min^{-1} and detected at the wavelength 254 nm. Basic validation of this method was carried out as well. Limits of detection and quantitation, external standard calibration, intra-day repeatability and inter-day reproducibility for spectrophotometric detection were determined. Five various red wines, four white wines and one rosé wine contained very small amounts of biogenic amines. The highest total content was approximately 2.5 mg L^{-1} , which represents a relatively low content in comparison with some results published in literature. Similarly, the content of biogenic amines in cheeses analysed was lower than expected, even in case of fermented cheeses. The highest content was found in one of the typical Czech fermented cheeses called Romadur and being 3.9 mg in 100 g cheese.

Keywords: Biogenic amines; Dansylation; HPLC; Wine; Cheese

Introduction

Biogenic amines (BA) are organic bases with aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) structures that can be found in various foodstuffs [1–3]. Biogenic amines may be of endogenous origin at low concentrations in non-fermented

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foodstuffs, such as fruits, vegetables, meat, milk, and fish. Higher concentrations can be found in fermented food as a result of a contaminating microflora with amino acid decarboxylase activity [4]. Biogenic amines are present in a wide range of fermented foods as, for example, dairy products. Among such foodstuffs, one can consider cheeses, in which enzymatic and microbial activities cause the formation of biogenic amines. In fact, during cheese ripening, degradation of casein resulting in the accumulation of free amino acids that can be converted into biogenic amines by the activity of bacterial decarboxylases [2,5–7].

Wines are not rich in protein, although they contain free amino acids. Some amines, such as putrescine, may already be present in grapes, whereas the others can be formed during the winemaking process, in case of bad sanitary conditions [8,9]. Amines may be formed by yeasts during the alcoholic fermentation, by lactic acid bacteria by malolactic fermentation or maturation of wine [10–12].

Many of biogenic amines have powerful physiological effects, acting as hormones or neurotransmitters, but ingestion of food containing these compounds can lead to several health problems, such as headache, skin irritation, impaired breathing, tachycardia, hypertension, hypotension or nausea [10,11,13]. Histamine is the most widely studied amine due to its ability to produce headache, hypotension, and digestive problems, while tyramine is often associated with migraine and hypertension [14].

The presence of biogenic amines is the subject of attention for two reasons; specifically, at first, that consumption of food containing these amines can have toxicological consequences for consumers, and second, due to their role as possible quality indicators [1,4,14].

Based on these two premises, various analytical techniques including capillary electrophoresis [15], gas chromatography [16] or enzymatic methods and immunoassays [17] have been developed for the determination of these compounds in various foodstuffs. Thanks to its high sensitivity, HPLC coupled to various detection systems is the most extensively used technique [4,18,19]. In practise, the UV/VIS detection or fluorimetric detection are most often used. However HPLC with mass spectrometry is modern method which can be used as a responsive analytical tool for the identification of many food constituents including biogenic amines [20].

One of the most common problems for biogenic amines detection is the lack of important chromophores groups that allow a satisfactory detection by UV/VIS absorption or by fluorescence. Thus, a derivatization process is required improving or even enabling the detection [1,2,14]. The off-line pre-column derivatization is still the most common strategy. Among the most often used derivatization reagents, it is possible to name dansyl chloride (Dns-Cl), *o*-phthalaldehyde (OPA), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) or fluorenylmethylchloroformate (FMOC) [10,14,18,19].

The aim of this work was to develop and validate a simple HPLC method with UV and MS detection which would be applicable for routine analysis of biogenic amines in various foodstuffs and beverages.

Materials and methods

Standards and Reagents

Standard compounds (all of them with more than 95% purity) were obtained from Sigma-Aldrich (Steinheim, Germany) as follows: phenylethylamine, histamine, isoamylamine, cadaverine, putrescine, spermidine, spermine, tryptamine, tyramine, 1,7-diaminoheptane, dansyl chloride, and L-proline.

Another chemicals: perchloric acid ($\geq 70\%$), hydrochloric acid (35 %), sodium hydroxide (p.a.) and sodium carbonate ($\geq 99.5\%$) were obtained from Penta (Prague, Czech Republic). Ammonium formate ($\geq 99\%$) and polyvinylpyrrolidone (average mol wt 10 000), were purchased from Sigma-Aldrich (Steinheim, Germany).

The solvents of choice were acetonitrile and methanol, both of MS grade ($\geq 99.9\%$) and obtained either from Honeywell (Seelze, Germany); the third one then being acetone ($\geq 99.8\%$) purchased from Sigma-Aldrich.

Samples

Ten samples of wine and five samples of cheese were analysed in this work. Specifically, 5 samples of red wine: Dornfelder (2018, CR), Frankovka (2018, CR), Frankovka (2018, Croatia), Rulandské modré (2012, CR) and Port wine (2018, Portugal), 1 sample of pink (rosé) wine Portugieser Weißherbst (2018, Germany) and 4 samples of white wine: Rulandské šedé (2017, CR), Rulandské šedé (2017, “pozdní sběr”, CR), Muscat Alb Demidulce (2019, Moldavia) and Veltlínské zelené (2017, CR).

The samples of cheese were selected as follows: Parmesan cheese (Grana Padano, Italy), fermented cheese (tvarůžky, Germany), camembert cheese with herbs (Kráľ sýrů, provensálské bylinky), camembert cheese (Kráľ sýrů, original), and fermented cheese (Romadur); all representing typical (trademark) products of the Czech Republic.

Preparation Procedures

Standard solutions preparation

The stock solutions of individual biogenic amines of concentration 5 g L^{-1} were prepared by dissolving the relevant amount of each standard in 0.4 M perchloric

acid. From the stock solutions, a mixed working solution of all the standards (concentration: 100 mg L^{-1}) was prepared, together with calibration solutions in the concentration range of $0.05\text{--}2 \text{ mg L}^{-1}$. A solution of dansyl chloride in acetone with concentration 1 g L^{-1} was always prepared fresh just before use.

Sample preparation

Samples of wines were prepared as follows: 0.5 g of polyvinylpyrrolidone (PVP) with purpose to remove the interferences was added to 10 mL wine. This mixture was agitated on magnetic stirrer (Color squid, IKA, Staufen, Germany) and centrifuged (using model Vintrum NF400, Nüve, Ankara, Turkey) at 3600 rpm for 10 min. A supernatant obtained was subjected to the derivatization reaction.

Cheeses were firstly grated or chopped on the small pieces, 5 g of cheese was mixed with 10 mL 0.4M perchloric acid and this mixture vortexed for 2 min. This mixture was centrifuged at 3600 rpm (10 min.) and supernatant used for derivatization.

Derivatization reaction

The derivatization was carried out in this way: in a test tube with a cap, 1 mL of standard (or sample) solution, 50 μL 1,7-diaminoheptane (internal standard, IS), 150 μL 2M sodium hydroxide and 300 μL of saturated solution of sodium carbonate were mixed together and vortexed (model Vortex 1, IKA, Staufen, Germany) for 30 sec. Then, 1 mL of dansyl chloride solution in acetone (1 g L^{-1}) was added, vortexed for 30 sec., and heated in a water bath for 1 hour at $50 \text{ }^\circ\text{C}$ in the dark. Then, the test tubes were left to cool to laboratory temperature and placed in dark for 15 min. after adding of 200 μL L-proline solution (1 g L^{-1}). The final step was filtration using a syringe nylon-membrane filter (pore diameter of $0.45 \text{ }\mu\text{m}$, Labicom, Olomouc, Czech Republic) prior to HPLC analysis.

HPLC Conditions

The dansyl derivatives of biogenic amines were analysed using a liquid chromatograph Agilent 1100 Series LC/MSD trap SL (Agilent Technologies, Santa Clara, CA, USA) equipped with spectrometric and mass spectrometer and a degasser of mobile phase Vacuum Degasser DG 3014 (Ecom, Prague, Czech Republic). The derivatives were separated on Gemini[®] C18 column ($150 \times 3 \text{ mm}$, $3 \text{ }\mu\text{m}$ particle size, Phenomenex, Torrance, CA, USA) operated at $35 \text{ }^\circ\text{C}$ with a flow rate of 0.6 mL min^{-1} . The mobile phase consisted of 5 mM ammonium

formate (A) and acetonitrile (B) with the following gradient elution programme: 0 min: 55 % (B), 5–10 min: 60 % (B), 12 min: 90 % (B). Injected volume was 20 μL , wavelength for UV detection was 254 nm.

The conditions of MS detection were as follows: APCI source in positive ion mode, corona: +4000 V, nebulizer: 60 psi, drying gas (N_2) flow rate 6 mL min^{-1} , drying gas temperature 350 $^\circ\text{C}$, evaporating temperature 450 $^\circ\text{C}$, scan divided to four chronological segments: 1. 0–10 min: 250–400 m/z , target mass 355; 2. 10–15.5 min: 500–900 m/z , target mass 580; 3. 15.5–17 min: 800–900 m/z , target mass 845; 4. 17–20 min: 1000–1300 m/z , target mass 1135.

Results and discussion

Optimization of derivatization reaction

The conditions of derivatization were optimized at the beginning of this study. Czajkowska-Mysiek and Leszczyńska [20] used a solution of 10 g L^{-1} dansyl chloride in acetone but, in our work, it was found out that the solution of this concentration is not possible to prepare because of precipitation and turbidity. Thus, less concentrated solution of 1 g L^{-1} had to be prepared and it could already be successfully used for all further experiments. Then, the same procedure from the work [20] was applied in the same way as described in the chapter “Derivatization reaction”. Further, triplicate and quintuple amounts of sample and of the derivatization agent were tested as well but, in these cases, the derivatization reaction was not as quantitative as in the original experiment. Finally, shorter time of derivatization reaction was tested. A period of 30 min. led to an approximately 80% yield in comparison with that for 60 min. reaction. Based on this, a time period of 1h was chosen and used for all derivatization reactions.

Optimization of chromatographic conditions

Three columns with stationary phase C18 (with various dimensions and particle size) and one column with stationary phase PFP (pentafluoro phenyl) were examined with a purpose to get optimal separation of all dansyl derivatives of biogenic amines. The best separation was achieved using column Gemini[®] C18 (150 \times 3 mm, 3 μm particle size) and therefore this column was selected. Two concentrations (5 mM and 10 mM) of ammonium formate solution as water component and methanol and acetonitrile as organic component of mobile phase were tested. Regarding detection by mass spectrometry, a lower concentration of ammonium formate had led to a lower suppression of the detector response, and therefore, better ionization could be achieved. Regarding organic components, acetonitrile was found to be more suitable than methanol, in which all peaks were

not separated and moreover, tailing peaks were observed in spectrophotometric detection. An optimization of the gradient elution was a next step. Various mobile phase ratios were tested with an objective to separate the peaks of derivatization agent and of all derivatives of amines among each other. Finally, the most suitable flow rate of mobile phase was investigated as follows: 0.4 mL min⁻¹, 0.5 mL min⁻¹, 0.6 mL min⁻¹ and 0.7 mL min⁻¹. Two first mentioned flow rates led to uselessly long separation, whereas the last one gave rise to two coelutions. Therefore, the flow rate 0.6 mL min⁻¹ was evaluated as optimal.

Optimised separation of all dansyl derivatives of biogenic amines is depicted in Fig. 1.

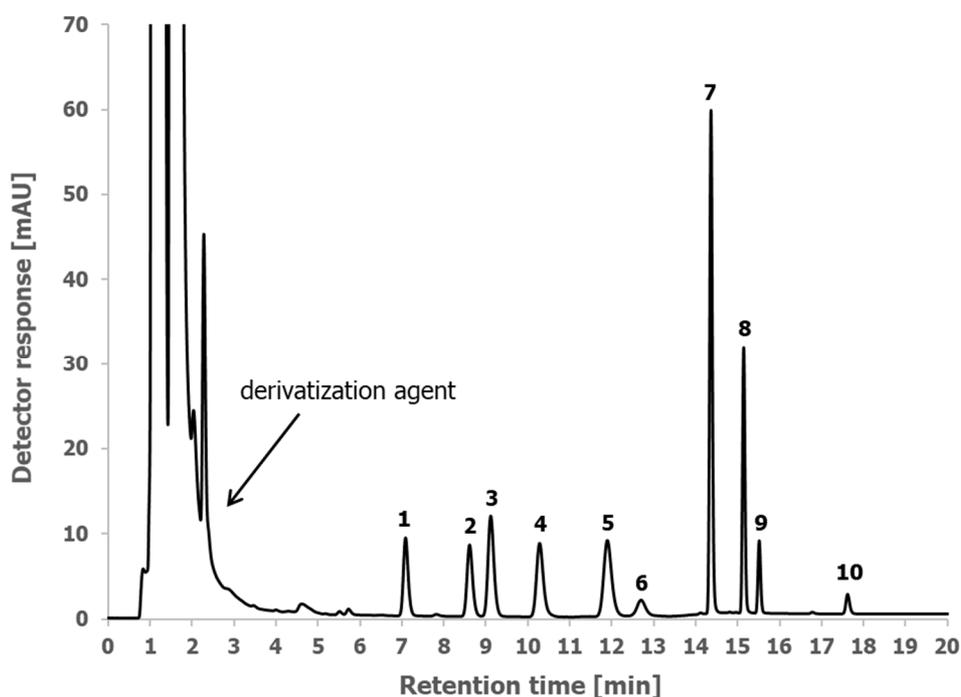


Fig. 1 Separation of biogenic amines derivatives under optimized conditions

Legend: 1: tryptamine, 2: phenylethylamine, 3: isoamylamine, 4: putrescine, 5: cadaverine, 6: histamine, 7: 1,7-diaminoheptane (IS), 8: tyramine, 9: spermidine, 10: spermine.

Conditions: Column Gemini C18 (150 × 3 mm, 3 μm); column temperature: 35 °C; mobile phase A: 5 mM ammonium formate; mobile phase B: acetonitrile; flow-rate: 0.6 mL min⁻¹; gradient programme: 0 min: 55 % (B), 5–10 min: 60 % (B), 12 min: 90 % (B); detection wavelength: 254 nm; injection volume: 20 μL.

Regarding the conditions of both types of detection, the suitable wavelength for spectrophotometric detection of dansyl derivatives of biogenic amines was 254 nm, which corresponded to the previous data [2,13,14]. In case of mass detection, more parameters had to be optimized. At the beginning, both ion modes of APCI source were tested. It was found out that derivatives of amines were not ionized in the negative ion mode and thus the choice of the positive ion mode was

clear. With respect to a relatively high flow rate of the mobile phase, the highest possible flow rate of drying gas was used (6 mL min^{-1}). The drying temperature was used in accordance with the instrument manufacturer's recommendation and the respective flow rate of mobile phase used. For optimal record of the compounds analysed, the analysis was divided into four segments, according to the average values of molecular masses of all the derivatives in the operational range of $m/z = 321\text{--}1135$.

Validation of the method

After selecting the optimum conditions for derivatization reaction and separation of followed compounds, a validation of the method was performed. The limits of detection (LOD) and limits of quantitation (LOQ) were being estimated for the individual dansylated biogenic amines. The LODs were determined using lower concentrations of standards for a S/N of 3:1 ($S/N = 3$). The baseline noise was evaluated by injection of a mobile phase in five replications. Similarly, the LOQs were calculated from a S/N of 10:1. The quantitation of biogenic amines in real samples was then based on external standard calibration for spectrophotometric detection. The regression equations were calculated as seven-point calibration curves plotted as the peak area vs. concentration of biogenic amine.

The limits of detection and quantitation for both types of detection are presented in Table 1 together with regression equations for spectrophotometric detection.

Table 1 Calibration parameters (LOD, LOQ and linearity)

Analyte	LOD [$\mu\text{g L}^{-1}$]		LOQ [$\mu\text{g L}^{-1}$]		Regression equation	R^2
	UV	MS	UV	MS		
Tryptamine	10.6	25.4	35.2	121.3	$46.06 x + 11.41$	0.9813
Phenylethylamine	19.6	31.1	46.5	103.7	$46.33 x + 1.03$	0.9923
Isoamylamine	13.8	21.7	41.5	72.4	$69.72 x + 1.04$	0.9897
Putrescine	16.9	54.2	42.3	108.2	$61.33 x - 0.48$	0.9882
Cadaverine	16.7	37.9	41.6	110.3	$69.66 x + 0.93$	0.9874
Histamine	77.8	68.3	291.0	227.8	$7.85 x + 0.08$	0.9601
Tyramine	17.7	94.5	53.2	315.1	$76.04 x + 0.91$	0.9846
Spermidine	16.6	377.4	54.8	1257.9	$18.84 x - 0.25$	0.9832
Spermine	70.4	359.0	234.7	1196.0	$9.35 x + 1.43$	0.9722

Limits of detection varied from 10.6 to 77.8 $\mu\text{g L}^{-1}$ for spectrophotometric detection and from 21.7 to 377.4 $\mu\text{g L}^{-1}$ for MS detection, respectively. Based on these results, more sensitive spectrophotometric detection was used for quantitation of the analytes. Mass detection was used only for the confirmation of the individual compounds.

The precision of the method was assessed based on intra-day repeatability (one day, $n = 5$) at two concentrations levels and inter-day repeatability (ten consecutive days, $n = 10$) at two concentration levels, too. Relative standard deviations (RSD) were calculated and the respective values are summarized in Table 2.

As can be seen from the table, very low RSDs for both parameters were obtained. Almost all values except inter-day repeatability of tryptamine were below 5%, which means a very good repeatability. As expected, even better values of RSD (mostly under 2%) of both parameters were obtained for the higher concentration level.

Table 2 Intra-day and inter-day repeatability expressed as RSD [%]

Analyte	Intra-day		Inter-day	
	0.1 mg L ⁻¹ ($n = 5$)	1 mg L ⁻¹ ($n = 5$)	0.1 mg L ⁻¹ ($n = 10$)	1 mg L ⁻¹ ($n = 10$)
Tryptamine	3.2	0.2	10.0	7.3
Phenylethylamine	1.0	0.4	3.9	0.8
Isoamylamine	1.9	0.4	2.8	0.6
Putrescine	0.6	0.8	4.6	1.0
Cadaverine	1.4	0.3	3.0	4.0
Histamine	–	1.0	–	2.8
Tyramine	1.3	1.4	1.3	1.7
Spermidine	2.3	1.7	5.8	1.8
Spermine	–	1.7	–	2.2

* The values for histamine and spermine (0.1 mg L⁻¹) are not available, because this concentration level were under their LOQ.

Results of sample analysis

The optimized method was applied to analysis of real samples; namely, ten samples of wine and five samples of cheese. The content of biogenic amines was quantified in both matrixes using external calibration for spectrophotometric detection.

In accordance with literature [10,11,13,21], the wine samples were firstly adjusted by purposely adding of polyvinylpyrrolidone for removal of various interferents. After this treatment, the derivatization was performed with 1 mL of the treated wine and subsequently, the derivate was analysed using HPLC as presented above. Results of biogenic amines content in wines analysed are shown in Table 3. The symbol “+” means that biogenic amines being presented in the wine, but under LOQ.

Table 3 Biogenic amines content in wines [$\mu\text{g L}^{-1}$]

Wine	PHE	ISO	PUT	CAD	HIS	TYR	SPM	SPD
Dornfelder	+		86.1			65.2		
Frankovka (Czech Rep.)	+		+	+	+			
Frankovka (Croatia)	+	+	+			223.6		
Rulandské modré	47.5	+	188.9		+		246.1	199.0
Port wine	+	+	68.2	45.9	+			76.9
Rulandské šedé (Znovín)	146.8	61.7	81.3	252.7	+	1073.0	663.4	262.7
Rulandské šedé (Rajhrad)	+	44.4	78.0		+			
Muscat		42.0	+		+		+	
Veltlínské zelené		+				61.3		
Rosé wine			47.0					

* Putrescine (PUT), cadaverine (CAD), spermine (SPM), spermidine (SPD), tyramine (TYR), phenylethylamine (PHE), histamine (HIS), tryptamine (TRP)

The most abundant biogenic amine in the wines analysed was putrescine, which had been present in nine samples. Phenylethylamine, the second most abundant amine was identified in seven samples, followed by isoamylamine and histamine. On the other hand, tryptamine was not detected in any sample. The highest content of individual amine, which was tyramine, was determined in the sample of white wine Rulandské šedé, namely $1073 \mu\text{g L}^{-1}$. The highest total contents of amines, being more than 2.5 mg L^{-1} , were found in this wine, too.

In comparison with literature [8,10,13], the contents of biogenic amines determined in this work were generally lower. This fact can have more explanations, including different varieties of grapes or their quality, variable geographic and climatic conditions or chemical composition of soil; nevertheless, the most pronounced influence is attributed to a winemaking process, possible microbial contamination, and the period of maturation of wine or storage conditions. Usually, putrescine, histamine, and tyramine were present in higher concentration levels in wine, whereas phenylethylamine, cadaverine and isoamylamine appeared in smaller amounts [8,10,11,13,16].

Significantly higher contents of biogenic amines are published in numerous works, especially for Italian or Greek wines. It is not rare that the total content of biogenic amines is in the range 3–25 mg L⁻¹ in these wines [12,21,22]. Yet higher content was found in Spanish or Chilean wines, up to 50 mg L⁻¹ [8,13,14]. The most similar contents — comparable to those determined in this work — were found in the studies by Polish and Portuguese authors [9,16].

Determination of biogenic amines in cheese is possible to use as a parameter of hygienic quality during the manufacturing process. Consensually, the maturation time of the cheese is a critical factor of accumulation of the biogenic amines. The longer time of maturation the higher content of biogenic amines. Hence, five maturing cheeses were chosen for analysis in this work.

The first step in cheese sample preparation had to be an extraction, the most often performed with the use of a strong acid. In this work, hydrochloric acid and perchloric acid were tested for extraction. Better extraction agent showed to be perchloric acid, because of markedly higher extraction efficiency.

The most abundant amine was putrescine present in all cheeses analysed in a range of 8.8–44.6 µg in 100 g of cheese. Isoamylamine was present in four samples but being quantified only in one of them (8.9 µg in 100 g of cheese Romadur). In this fermented cheese, Romadur, the six following amines were found. Phenylethylamine was present, but at a content under LOQ, the other ones were quantified as follows: putrescine 8.8 µg per 100 g, isoamylamine 8.9 µg / 100 g, cadaverine 275.6 µg / 100 g, tyramine 869.8 µg / 100 g, and histamine in the highest amount in all samples; namely, 2760.1 µg / 100 g. In Romadur, total content of all amines was 3.9 mg / 100 g. Second highest total content was in the sample tvarůžky: 183.6 µg / 100 g, where 20.5 µg represented putrescine and 163.1 µg tyramine. In cheese Parmesan, only cadaverine was quantified (81.3 µg / 100 g), but both isoamylamine and putrescine were also detected. In the two cheeses of the Camembert type, coincidentally, putrescine was quantified (37.1 and 44.6 µg / 100 g) and isoamylamine was detected. Tryptamine, spermidine and spermine were not found in any sample.

Conclusions

The optimization and validation of an RP-HPLC-UV-MS method for the determination of biogenic amines in food samples was performed. A simple way of derivatization of biogenic amines (by 1h reaction with dansyl chloride at 50 °C) was used. Since the spectrophotometric detection was significantly more sensitive than MS detection, the former was used for quantitation of biogenic amines in real samples of wine (red, white, and rosé) and in cheeses. All amounts determined in the samples analysed within this work have shown to be so low that there is no risk of health problems for common consumers.

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