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RESEARCH

Sensitive Ultra-Performance Liquid Chromatography Coupled with Triple Quadruple Mass Spectrometry (UPLC-TQ-MS) for Simultaneous Determination of Biogenic Amines in Montenegrin Red Wines

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Abstract
A highly sensitive ultra-performance liquid chromatography coupled with triple quadruple mass spectrometry (UPLC-TQ-MS) was applied for simultaneous determination of seven biogenic amines (cadaverine, putrescine, histamine, spermine, tyramine, phenyethylamine, and tryptamine) in Montenegrin red Vranac and Kratošija (*Vitis vinifera* L.) wines. The method was validated, confirmed the suitability for determination of biogenic amines in red wines, and it was applied to examine the changes of biogenic amines in three vinification stages: after alcoholic fermentation, malolactic fermentation, and 10 months of bottle aging of the wines. The results showed that the total amine levels were low, ranging from 1.27 to 3.26 mg/L, observing increased concentration in the wines after finished malolactic fermentation. Putrescine, which was constituted as a main amine in wines (range: 0.79 to 2.39 mg/L), was observed in a slightly higher content in Vranac wines compared to Kratošija, followed by the aliphatic cadaverine (range: 0.091 to 0.35 mg/L) and the aromatic phenylethylamine (range: 0.119–0.373 mg/L). Histamine and tyramine which are subject of toxicological interest were present in a very low concentration in wines (on average value: 0.082 and 0.015 mg/L, respectively). Cadaverine and putrescine showed linear relationships with the sum of total biogenic amines, and the reached values were below the toxicity threshold. According to the principal component analysis, wines were clearly separated in three groups according to the three vinification stages.

Keywords Biogenic amines · Alcoholic and malolactic fermentation · Aging · Vranac · Kratošija

Introduction

Biogenic amines (BA) are non-volatile, low molecular weight polar or semi-polar nitrogen organic compounds which possess multiple biological functions, acting as growth regulators, neurotransmitters or inflammatory mediators (Diez-Ozaeta et al. 2022). Wine is a natural product that contains biogenic amines, such as putrescine, cadaverine, spermidine, spermine, phenylethylamine, tyramine, tryptamine and histamine (La Torre et al. 2010, 2023; Tašev et al. 2017; Colombi et al. 2023). These BAs are mainly produced during the wine fermentation (alcoholic and malolactic) and aging processes, as a product of dexarboxylation of the corresponding precursor amino acids in the presence of microorganisms. Thus, the content of biogenic amines in wines depends on the health condition of grapes, content of precursors (usually available free amino acids), presence of microbial population with decarboxylase activity during fermentation), as well as on the biological and physico-chemical conditions (pH, temperature, SO_2) that may allow the growth of potential spoilage microorganisms (Mohammed et al. 2016; Manetta et al. 2016; Diez-Ozaeta et al. 2022). Table 1 contains the classification, structural formulas and precursor amino acids of the main biogenic amines in wine.

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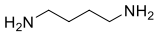
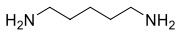
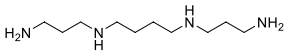
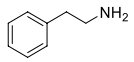
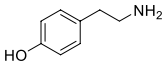
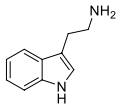
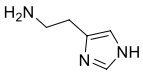
Table 1 Classification, structural formula and precursors of biogenic amines in wine

Classification	Biogenic amine	Structural formula	Precursor (amino acid)
Aliphatic	Putrescine		Arginine, Ornithine
	Cadaverine		Lysine
	Spermine		Arginine
Aromatic	Phenylethylamine		Phenylalanine
	Tyramine		Tyrosine
Heterocyclic	Tryptamine		Tryptophan
	Histamine		Histidine

Excessive consumption of BAs can cause adverse health effects, including headache, nausea, sweating, respiratory distress, heart palpitations and hypo- or hypertension (Shalaby 1996; Frascarelli et al. 2008). High levels of some biogenic amines, such as putrescine and cadaverine, can lower the sensorial quality of food, while tryptamine has toxic effects on humans such as blood pressure increase, causing hypertension (Shalaby 1996). In alcoholic beverages, histamine and tyramine are responsible for the headaches and flushing after ingestion (Stratton et al. 1991). Regarding these potential risks, monitoring of the level of BAs is of high particular importance. To the authors' best of knowledge, there are no regulatory limits established yet concerning the concentration of biogenic amines in wine, only upper limits for histamine content in wine (2–10 mg/L) have been recommended in some European countries, such as 2 mg/L in Germany, 3 mg/L in Netherlands, 5 mg/L in Finland, 5 to 6 mg/L in Belgium, 8 mg/L in France, 10 mg/L in Switzerland, and 10 mg/l in Austria (Lehtonen 1996; Topić Božič et al. 2022; Gutiérrez-Escobar et al. 2024). In fact, histamine is the most toxic biogenic amine, although its effects can be potentiated by the presence of other amines, such as spermine, spermidine, or putrescine (Bauza et al. 1995). Moreover, the International Organization of Vine and Wine (OIV) recommends reduction of BAs content in wine and other vine-based products (Ancín-Azpilicueta et al. 2019).

Analytical methods used for identification and quantification of biogenic amines in wine are mainly separation techniques, such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography (GC) (Sentellas et al. 2016; Zhang et al. 2019). HPLC coupled to UV-Vis, diode array or fluorometric detection, applying pre- or post-column derivatizaiton (Tašev et al. 2016; Shi et al. 2024) is the most frequently used method for BAs analysis in wine due to its universality, versatility, reproducibility and robustness. In the last decade/s, mass spectrometry has been proven to be the most sensitive and suitable technique for analysis of small organic molecules present at low concentration in complex matrices, as biogenic amines are in wine. Moreover, mass spectrometry allows detection of biogenic amines by direct injection of the sample after minimal sample preparation (dilution and filtration), without previous derivatization (Tašev et al. 2017) allowing faster and more sensitive analysis.

Vranac and Kratošija are the most important grape varieties grown in Montenegro. Vranac variety dominates in wine and brandy production (80%) of the country, followed by Kratošija and other local and international varieties. The phenolic composition, as one of the most important compounds influencing the wine quality, has been determined in various red and white Montenegrin wines, important for the country and region as well (Raičević et al. 2015; Pajović-Šćepanović et al. 2016; Raičević et al. 2017; Pajović Šćepanović et al. 2019). To the best of our knowledge, data for the biogenic amines, which are considered as natural contaminants with toxic effects to consumers, are missing for the Montenegrin wines. Therefore, the objectives of this study were to (1) validate and use a suitable, rapid and sophisticated method for fast and accurate determination of biogenic amines in red wines applying ultra-performance liquid chromatography coupled to triple quadruple mass spectrometry (UPLC-TQ-MS), to (2) to identify and quantify the individual biogenic amines in wines, and to (3) compare and examine the changes of their contents in Montenegrin Vranac and Kratošija wines after three vinification stages: alcoholic fermentation, malolactic fermentation and

10 months of wine aging, as key stages of winemaking for biogenic amines formation.

Materials and Methods

Chemical and Reagents

Following standards of biogenic amines, provided by Fluka (Munich, Germany), were used: cadaverine, putrescine, histamine, spermine, tyramine, phenyethylamine, and tryptamine. Methanol, formic acid, ammonium formate, and water (all with HPLC purity) were purchased from Carlo Erba (Cornaredo, Italy). All other chemicals used were of analytical-grade purity.

Winemaking

Wines were produced from Vranac and Kratošija (*Vitis vinifera* L.) grape varieties (vintage 2020). Grapes were cultivated in the Podgorica sub-region in Montenegro and harvested at optimal maturity (parameters are presented in Table 2), in excellent health condition. Harvested grapes were transported to the experimental cellar of the Biotechnical Faculty, Podgorica (Republic of Montenegro) and both varieties (100 kg each) were separated in 2 lots, marked as V for Vranac and K for Kratošija. Additionally, lots of each variety were processed separately using a mechanical inox crusher/destemmer, placed in a fermenter of 100 L volume and treated with sulfur dioxide (5 g/HL) prior to the fermentation. The two lots were undergoing skin spontaneous fermentation (without inoculation with commercial yeast) at 25–28 °C. The alcoholic fermentation and maceration lasted for 8 days, applying punching down manually, three times a day. The alcoholic fermentation was estimated to be finished when reducing sugar's content was below 2.5 g/L, determined with volumetric method based on reduction-oxidation (redox) reaction between sugars and Fehling's solution, according to the Schoorl's method (Ivanova-Petropulos and Mitrev 2014). After the period of maceration and alcoholic fermentation, wines were separated from of the pomace by racking and placed in a tank for stabilization and spontaneous malolactic fermentation, at temperature of 20 to 25 °C, which lasted for 25 days. Dynamics of malolactic fermentation (MLF) and conversion of malic to lactic acid in both wines were followed using paper chromatography. After the MLF finished, wines were stabilized at –4 °C for a period of 6–8 days for tartrate stabilization. The physico-chemical parameters have been determined for both wines and presented in Table 2. The schematic presentation of the winemaking process is shown at Fig. 1.

Wines were bottled and stored in a cellar at 10–15 °C for 10 months. In order to determine the changes of biogenic amines, wine was sampled after finishing the alcoholic fermentation, after malolactic fermentation and after 10 months of bottle aging. The obtained wines were marked as follows: V-1 and K-1–wine after alcoholic fermentation, V-2 and K-2—wine after spontaneous malolactic fermentation and V-3 and K-3—wine after 10 months of aging.

Table 2 Basic physico-chemical parameters determined for Vranac and Kratošija grapes and wines

Varieties/ Parameters	Vranac	Kratošija
Grapes analyses		
Sugar (° Brix)	24	22.4
Total acidity (tartraric acid equivalents)	5.35	6.57
pH	3.45	3.30
Wines analyses		
Alcohol (%)	14	13.1
Total acidity (tartraric acid equivalents)	5.1	6.3
pH	3.50	3.35

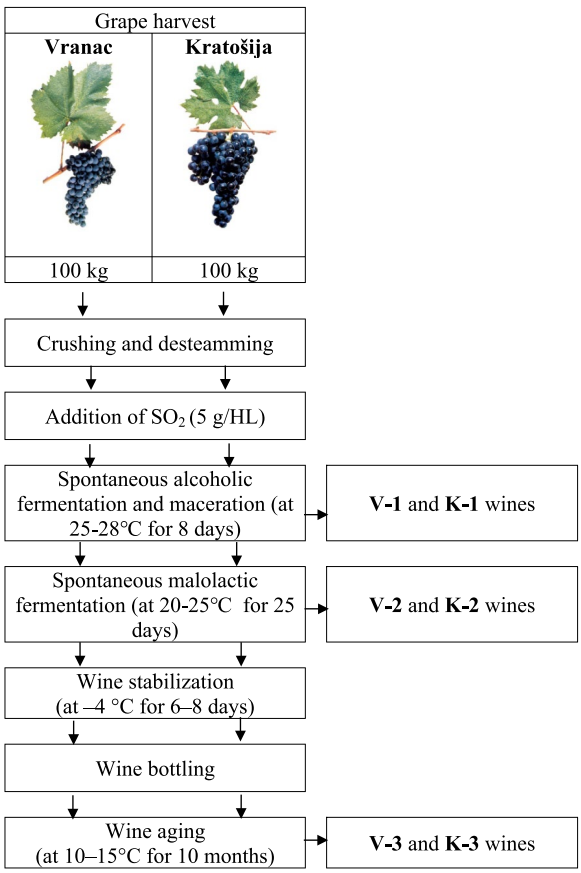


Fig. 1 Schematic presentation of winemaking process of Vranac and Kratošija wines

Wines were diluted with 0.1% (*v/v*) formic acid at a ratio of 1:3, filtered with a 0.45-µm filter (Agilent PTFE) and injected into UPLC-TQ-MS instrument.

UPLC-TQ-MS Analysis

Analysis of biogenic amines in wines was performed with an Agilent UPLC 1290 system consisted of a 1290 Infinity G4220A binary pump, 1290 G4226A autosampler, 1260 G1316A column compartment, and DAD VL Agilent 1260 G1315D detector (Waldbronn, Germany) and Agilent triple quadrupole (TQ) mass spectrometry (MS) detector 6420 (Agilent Technologies, Santa Clara, CA, USA). Separation of the analytes was performed according to a previously published method (Tašev et al. 2017), using a column Agilent Zorbax C18 Plus (dimensions: 100 × 2.1 mm, 1.8-µm particle size). The mobile phase for separation of the components was consisting of ammonium formate with concentration of 5 mmol and 0.1% (*v/v*) formic acid (solvent A), and methanol with 5 mmol ammonium formate and 0.1% (*v/v*) formic acid (solvent B). The gradient elution was performed with a flow rate of 0.2 mL/min at ambient temperature, according to the following conditions for the solvent B: 0–0.05 min, 20%; 3.00–3.50 min, 80%; and 3.75–5 min, 20%. The total run time was less than 4 min, followed by a post-run time of 1 min, allowing fast analysis. The injection volume of the samples was 1 µL.

The detection of the biogenic amines was made with an Agilent 6420 triple quadrupole mass spectrometer. The applied parameters were: capillary temperature 350 °C, gas flow 12 L/min, nebulizer 15 psi, capillary voltage 3500 V in positive ionization mode, peak width 0.07 min, cycle time 500 ms, minimum data point 64, and minimum and maximum dwell time 25.9 and 246.5 ms, respectively (Tašev et al. 2017). Data processing was performed with a Mass Hunter software (v. B.06.00). Optimal quantification and confirmation transitions, their respective fragmentor, collision energies, cell accelerator voltage, are listed in Table 3. The conditions included in Table 2 (framgentor, collision energy, cell accelerator voltage and polarity) are taken directly from the previously optimized, validated and published method and applied on this investigation too.

Validation Parameters

The following validation parameters were determined and confirmed: linearity, accuracy, precision, repeatability, reproducibility, limit of detection (LOD), and limit of quantification (LOQ). The linearity data included slope and the coefficient of determination (R^2, determined from the calibration curves for each biogenic amine). The accuracy and precision of the method were checked using a standard addition method. Precision was determined as repeatability (ten successive injections in one day) and intermediate precision, or reproducibility (five injections during five consecutive days in one week). The LOD was determined as LOD = 3 × SD/slope and the LOQ as LOQ = 10 × SD/slope at the low concentration calibration level.

Since we used previously published method, applying the same analytical conditions for UPLC-TQ-MS analysis of biogenic amines in wines (Tašev et al. 2017) which was optimized and validated four years before the analyses of Vranac and Kratošija wines examined in this study, it was necessary to check the method and therefore we conducted revalidation of the analytical parameters before starting the analyses, in order to be sure that the method and instrument characteristics fulfill the requirements for determination of these small molecules, present in low concentrations.

Calibration Curves

Stock solutions of each biogenic amine (cadaverine, putrescine, histamine, spermine, tyramine, phenyethylamine and tryptamine) were prepared at concentration of 1 mg/mL by dissolving appropriate amounts of amines in 0.1% (*v/v*) formic acid in water. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water. For quantitative analysis of the biogenic amines in

Table 3 UPLC–TQ-MS determination of biogenic amines in analyzed Vranac and Kratošija wines (arranged according to the retention time), identified and quantified by their MS and MS/MS data

Biogenic amines	t_R (min)	Molecular ion (*m/z*)	Fragment ion/s (*m/z*)	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
Cadaverine	1.228	103	86	65	9 & 5	4	positive
Putrescine	1.238	89	72	45	9	4	positive
Histamine	1.260	112	95, 68	80	13 & 21	4	positive
Spermine	1.275	203	129, 112	100	9 & 17	4	positive
Tyramine	1.743	138	121, 77	70	9 & 29	4	positive
Phenylethylamine	2.757	122	105, 77	70	9 & 33	4	positive
Tryptamine	3.038	161	144, 117	70	9 & 25	4	positive

wine samples, six-point calibration curves were constructed in a range from 10 to 1000 µg/L for cadaverine, putrescine, histamine, tyramine, tryptamine and phenyethylamine and for spermine the concentration range was 100–2000 µg/L. Linear regression data for the individual biogenic amines, arranged by retention time, are presented in Table 4.

Statistical Analysis

Obtained results were statistically treated, including calculation of average, sum, standard deviation, relative standard deviation and recovery, performed with Excel, 2013 (Microsoft, Seattle, WA, USA). In order to establish possible significant similarities or differences between the wines for each studied parameter, the Student–Newman–Keuls multiple comparisons test on the mean values was applied to the results of the concentration of biogenic amines, using the statistical package IBM SPSS Statistics 20 (IBM Corporation, New York, USA). In addition, principal component analysis was performed on the results for biogenic amines in both wine varieties produced after alcoholic fermentation, malolactic fermentation, and 10 months of bottle aging, using the XLStat software (Addinsoft, Version 2015.5.01.22537), in order to study possible groupings of wines as an influence of the winemaking stages and variety.

Results and Discussion

UPLC-TQ-MS Identification of Biogenic Amines

A highly sensitive ultra-performance liquid chromatography coupled with triple quadruple mass spectrometry (UPLC-TQ-MS) using MRM (multiple reaction monitoring) mode was applied for wine analysis in order to identify and quantify seven individual biogenic amines. Identification was performed by comparing the retention times of analytes and standards as well as by comparing the MS/MS data obtained for the standards and those found in the literature (Table 3) (Sagratini et al. 2012; Tašev et al. 2017). For the separation of the biogenic amines, we used a mobile phase compatible with the MS detection, consisted of aqueous ammonium formate, formic acid, and methanol. The total ion chromatograms of the individual biogenic amines in standard solution and one Vranac wine sample (V-1) are presented at Fig. 2a. Figure 2b and c contains the ion extracted peaks of the individual biogenic amines in standard solution and wine (V-1), respectively, according to their molecular ions.

Table 4 Linear regression data

Biogenic amins	Concentration range (µg/L)	Slope	R^2	LOD* (µg/L)	LOQ* (µg/L)
Cadaverine	10–1000	83.4	0.9962	3	9
Putrescine	10–1000	87.5	0.9922	4	12
Histamine	10–1000	452	0.9988	0.5	1.5
Tyramine	10–1000	301	0.9968	1	3
Spermine	100–2000	247	0.9992	30	90
Phenylethilamine	10–1000	562	0.9991	1	3
Tryptamine	10–1000	428	0.9983	0.5	1.5

Validation Parameters of the Method

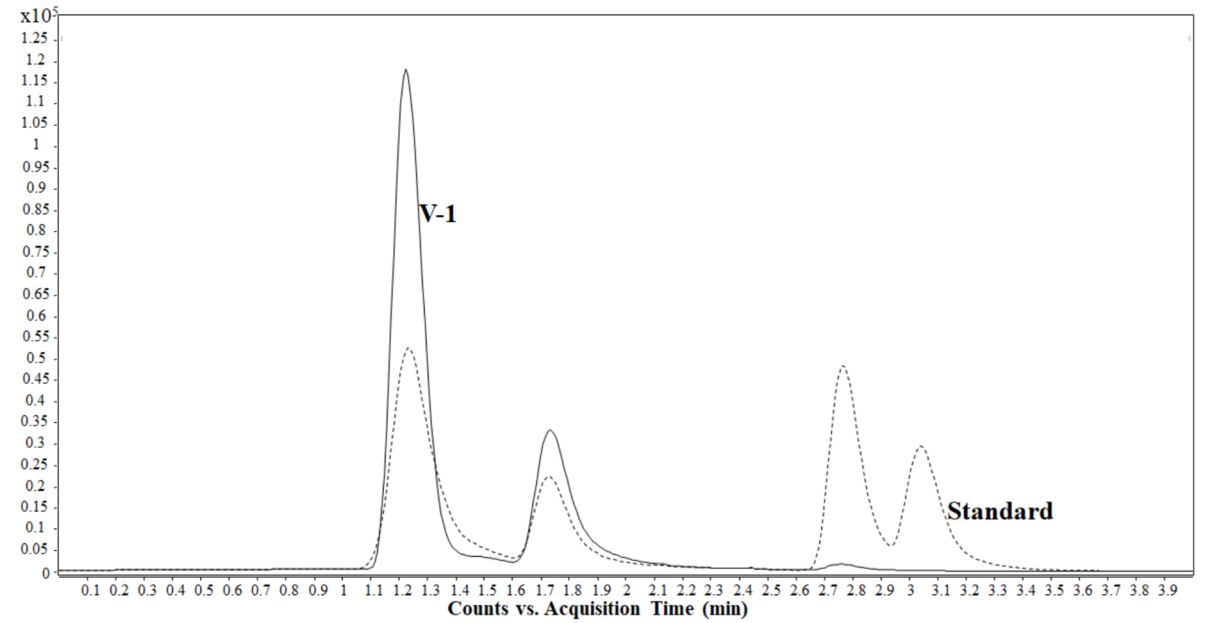
The main parameters of the proposed UPLC-TQ-MS method were thoroughly evaluated. The validation parameters, including linearity of concentration curve, accuracy, precision, LOD, LOQ, recovery, repeatability, and reproducibility, were determined (Table 4).

Linearity was tested in 3 days at six concentration levels, covering the expected concentration ranges for each biogenic amine in the wine samples (Table 3). The calibration, plotting the extracted ion peak area versus the concentration of the test biogenic amines, was linear for all amines investigated, with the coefficients of determination $R^2 > 0.99$, ranging from 0.9922 for cadaverine to 0.9992 for tyramine. *Limit of detection* (LOD) and *limit of quantification* (LOQ) were determined for each biogenic amine, ranging from 0.50–30.0 µg/L and 1.50–90.0 µg/L, respectively.

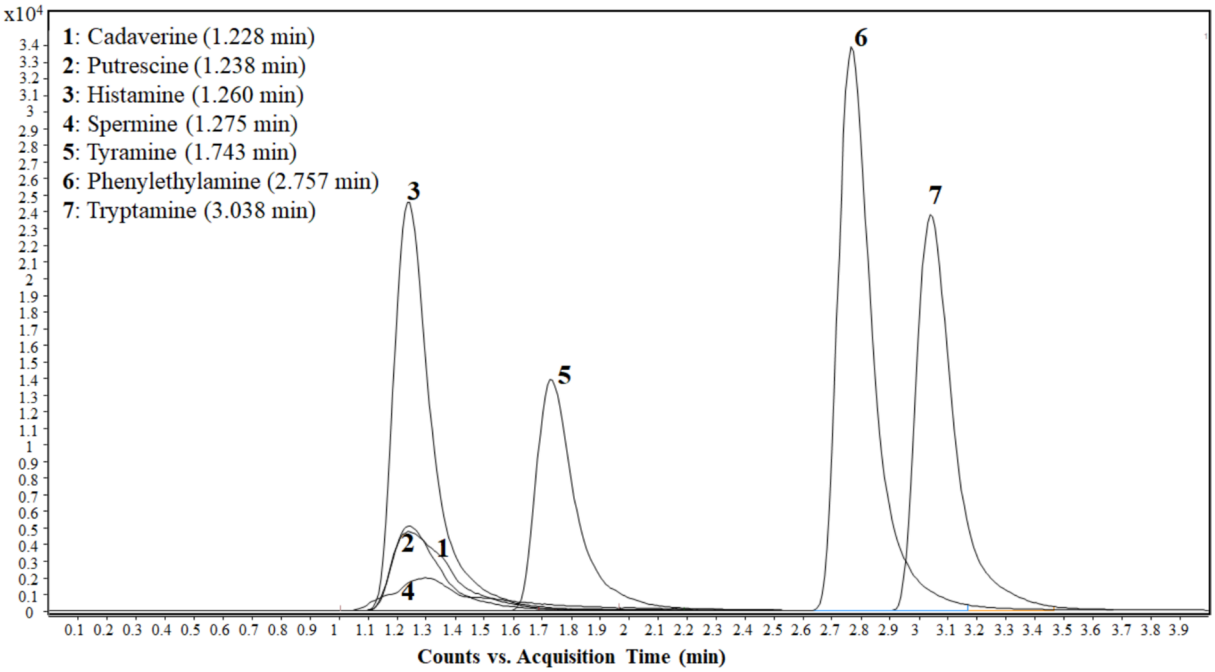
The *accuracy* of the procedure was estimated using the standard addition method, expressed with the standard deviation and recovery of the experimentally found concentration compared with the determined (nominal) value in the wine sample. For this purpose, one wine sample (V1) was spiked with a mixture of the biogenic amines (cadaverine, putrescine, histamine, spermine, tyramine, phenyethylamine, and tryptamine) at appropriate each concentration. Table 5 presents the results for the recovery studies of amines, showing the recovery ranging from 96.56% for tyramine to 103.01% for histamine, confirming the accuracy and suitability of the method for analysis of biogenic amines in red wine. In addition, Table 6 contains results for the *repeatability* and *reproducibility* of the applied method which were studied with one wine sample (V-1). The obtained relative standard deviations (% RSD) data for the intra-day repeatability were satisfactory, ranged from 1.36 to 4.46% for all biogenic amine concentrations, while the inter-day reproducibility provided good RSD values between 0.57 and 4.88%.

Chracterization of Biogenic Amines in Vranac and Kratošija Wines

The validated UPLC-TQ-MS method was applied for characterization of biogenic amines profile of Vranac and Kratošija wines in three stages: after finishing of the alcoholic fermentation (determined by measuring the reducing sugars



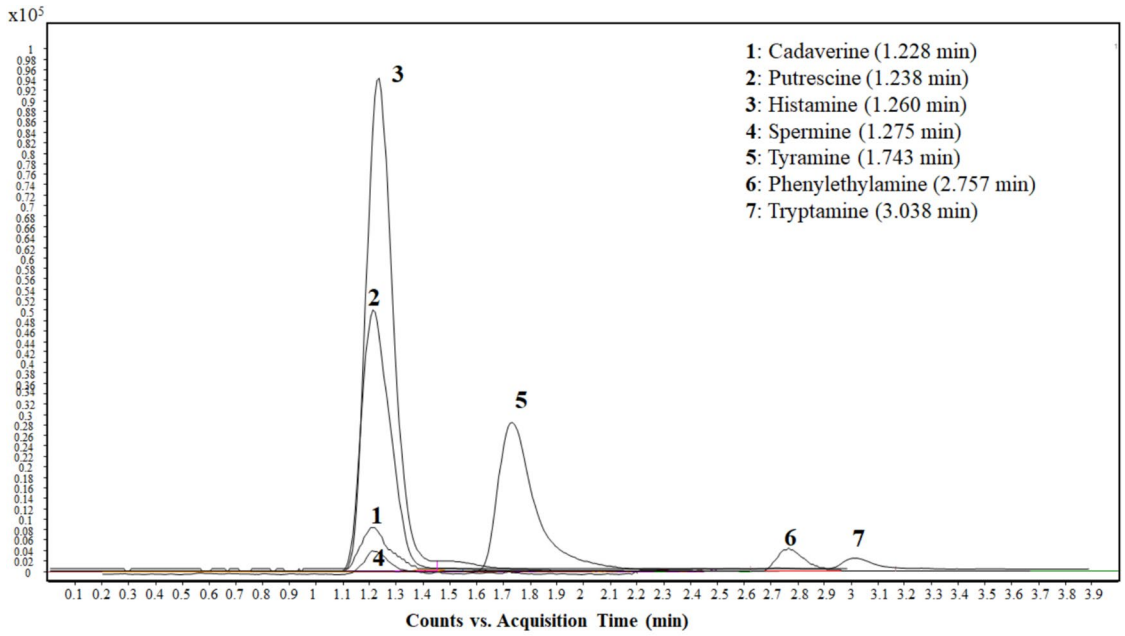
a



b

Fig. 2 a. Total ion chromatogram of standard solution (-----) and Vranac wine (V-1) (——) obtained with UPLC-TQ-MS under MRM (multiple reaction monitoring). **b**. Ion extracted peak chromatogram of biogenic amines (200 µg/L) in standard solution. **c**. Ion extracted peak chromatogram of biogenic amines in Vranac wine (V-1)

content), after finishing of the malolactic fermentation and after 10 months of wine bottle aging in order to follow and examine their changes during these important and key vinification stages when changes of biogenic amines mainly occur.



c

Fig. 2 (continued)

Table 5 Standard additions for checking the accuracy of the UPLC-TQ-MS method for determination of biogenic amines in wine ($n = 3$)

Biogenic amine	Wine (V1) (μg/L)	Added (μg/L)	Calculated (μg/L)	Experimentally found (μg/L)	SD	Recovery (%)
Cadaverine	91.4	100	191	190	0.85	99.5
Putrescine	1179	100	1279	1266	9.19	99.0
Histamine	79.2	100	179	184	3.82	103
Tyramine	54.0	100	154	148	3.75	96.1
Spermine	114	100	214	210	2.83	98.1
Phenylethilamine	373	100	473	470	2.12	99.4
Tryptamine	10.3	100	110	108	1.13	98.2

Table 6 Intra-day repeatability and inter-day reproducibility measurements for the biogenic amines content in one Vranac wine sample (V-1)

Biogenic amine	Intra-day repeatability (10 replicates × 1 day)		Inter-day reproducibility (5 replicates × 5 days)	
	Mean concentration (μg/L)	RSD (%)	Mean concentration (μg/L)	RSD (%)
Cadaverine	90.4	1.52	90.2	0.57
Putrescine	794	1.36	801	0.73
Histamine	51.8	2.06	52.6	1.75
Tyramine	47.3	2.36	47.5	2.31
Spermine	114	2.59	110	3.66
Phenylethilamine	163	2.34	161	2.18
Tryptamine	9.12	3.67	9.23	4.88

Quantitative results of the seven, the most important biogenic amines in wines are shown in Table 7. Spermidine was identified in the wines, but its content was below the LOQ, so quantitative interpretation of the results for this biogenic amine was not possible. In general, the total biogenic amine content was slightly higher in Vranac wines (2.79 mg/L on average), compared to Kratošija wines (2.23 mg/L on average). Compared to the results for Italian wines of Abruzzo (Martuscelli et al. 2013), where the average total content of biogenic amines was 19.3 mg/L in red wines, 7.67 mg/L in white wines and 9.20 mg/L in rose wines, the amounts of biogenic amines in Vranac and Kratošija wines in this study were significantly lower, but similar to those determined in Sicilian wines (0.89 to 2.35 mg/L) (La Torre et al. 2010). In addition, the determined average total content of biogenic amines in Macedonian red wines (5.79 mg/L) (Tašev et al.

Table 7 Concentration of biogenic amines (μg/L) in Vranac and Kratošija wines determined after alcoholic fermentation, after malolactic fermentation and after 10 months of bottle aging

Wines	Cadaverine	Putrescine	Histamine	Tyramine	Spermine	Phenylethylamine	Tryptamine	Σ
Vranac								
V-1	91.4 ± 2.1	1179 ± 83.4	79.2 ± 3.0	54.0 ± 3.4[a]	114 ± 6.1[a]	373 ± 8.8	10.3 ± 1.0	**1901 ± 109**
V-2	350 ± 21.7[a]	2398 ± 123[a]	89.6 ± 5.3	31.2 ± 3.2[a]	115 ± 13.1[a]	219 ± 12.5	15.4 ± 0.9[a]	**3218 ± 180**
V-3	342 ± 17.8[a]	2371 ± 74.4[a]	97.2 ± 5.2	55.6 ± 5.3	115 ± 15.6[a]	254 ± 20.8	25.2 ± 1.4	**3260 ± 141**
Kratošija								
K-1	90.3 ± 3.3	795 ± 29.8	52.1 ± 2.1	47.1 ± 1.4	111 ± 15.1[a]	161 ± 10.6	9.2 ± 0.2	**1266 ± 62.5**
K-2	263 ± 14.1	1989 ± 47.9[b]	94.9 ± 4.0	37.8 ± 1.5	125 ± 13.1	181 ± 15.1	15.4 ± 1.6[a]	**2706 ± 97.3**
K-3	234 ± 18.3	2100 ± 71.4[b]	80.1 ± 1.6	64.1 ± 1.8	114 ± 9.0[a]	119 ± 8.8	18.8 ± 0.5	**2730 ± 111**

Results are presented as average value of three repetitions (μg/L) ± standard deviation (SD). Values with same letter(s) within a column are not significantly different at $p>0.05$, applying Student–Newman–Keuls multiple comparisons test on the mean values of each biogenic amine. V-Vranac, K-Kratošija, 1—wine after spontaneous alcoholic fermentation, 2—wine after spontaneous malolactic fermentation, 3—wine after 10 months of bottle aging

2017) as well as the contents in wines from other published studies (Gloria et al. 1998; Soufleros et al. 2007; Hajos et al. 2000; Arrieta and Prats-Moya 2012), where higher compared to the biogenic amine content of Vranac and Kratošija wines.

Figure 3 represents a linear regression showing a relationship between the total biogenic amine content and cadaverine or putrescine content. In addition, the total biogenic amine content showed linear relationships to cadaverine ($R^2=0.92$) and putrescine ($R^2=0.98$), while no other linear relationships were observed with the other amines in both wine varieties (Fig. 3).

In this study, putrescine was the dominant compound in all wines studied, regardless the variety. All wines contained putrescine which content ranged from 795 to 2398 μg/L, reaching values up to 2.39 mg/L in Vranac wines and

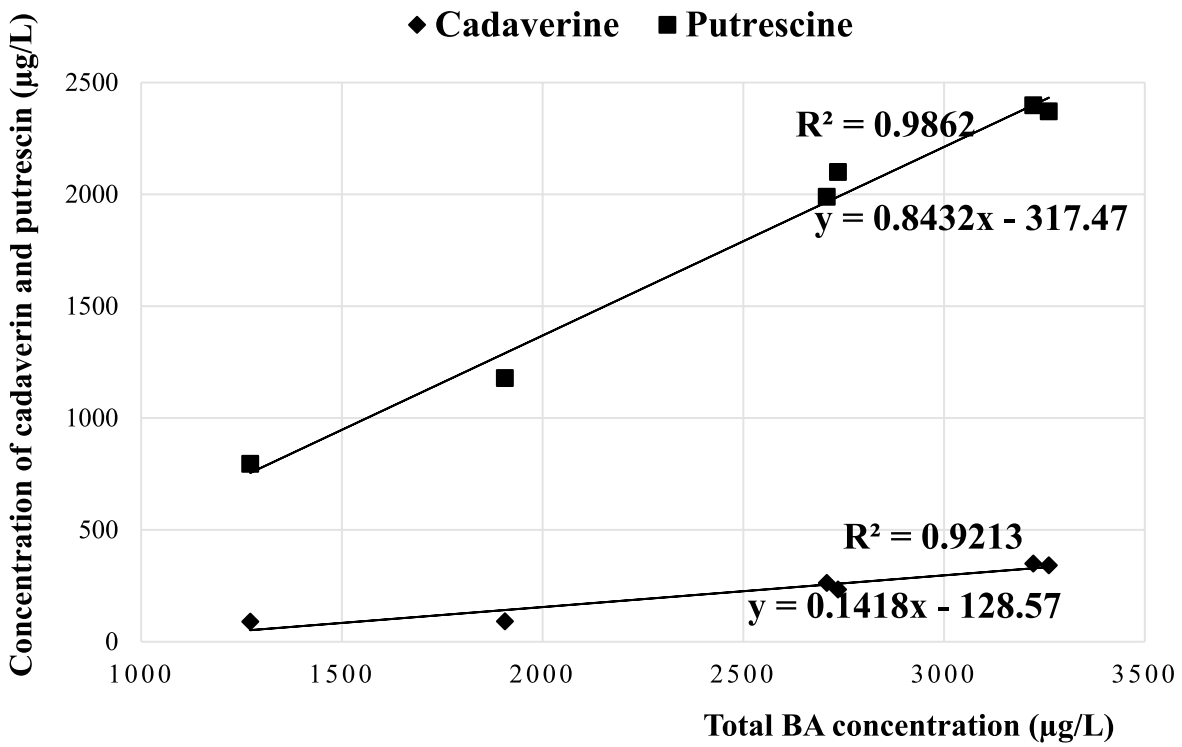


Fig. 3 Scatterplot of total biogenic amines (BA) against putrescine and cadaverine showing linear relationships and coefficients of determination (R^2) of each single amine (putrescine and cadaverine) against total aines found in Vranac and Kratošija wines

2.10 mg/L in Kratošija wines, which were much lower compared with previously published results (Martuscelli et al. 2013; Bover-Cid et al. 2006; Landete et al. 2005). The accumulation of putrescine into grapes can be associated with potassium deficiency in the soil (Adams et al. 1990; Vaz de Arruda Silveira et al. 2001) (K content in soil was not investigated), leading to increased content of this amine. Moreover, putrescine was detected in higher concentrations in Vranac wines (1.98 mg/L on average) than in Kratošija wines (1.62 mg/L on average), probably due to the grape variety differences, which is in accordance with previous studies (Gutiérrez-Escobar et al. 2024; Del Prete et al. 2009). In fact, the grape variety and genetic factor are related to the presence of biogenic amines in wines, but also climatic conditions could affect the accumulation of these compounds in grapes. In our study, Vranac and Kratošija grapes were grown at same soil and climatic conditions, so the observed differences in biogenic amines content between the varieties can be attributed to the varietal/genetic characteristics. In addition, the biogenic amines formation results from the decarboxylation of the corresponding amino acids (Marques et al. 2008), and the amino acid composition (which was not determined in this study) is probably different in both grape varieties, leading to different levels of biogenic amines in wines.

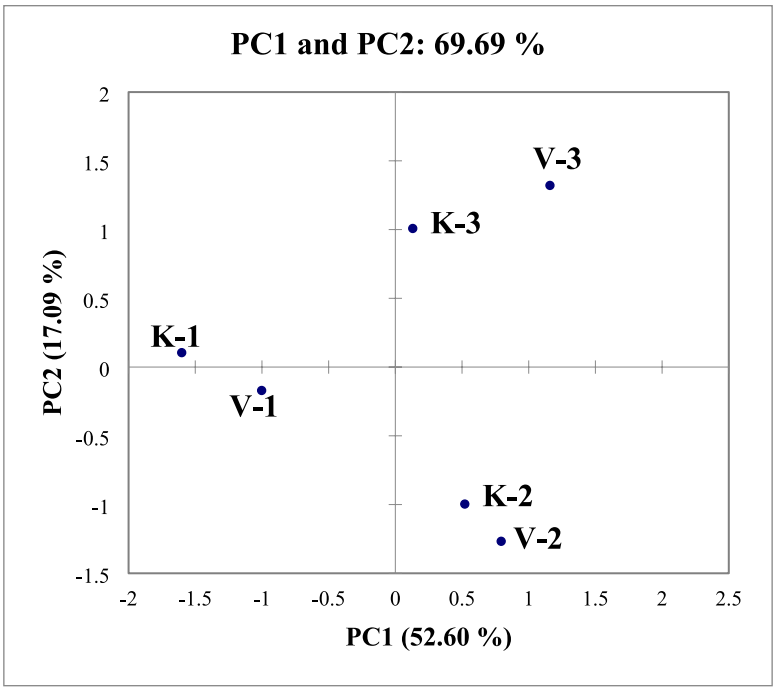
During the alcoholic fermentation of red wines in presence of grape skins and seeds, putrescine is released from the grape skins, which concentration increases frequently during the maceration, in accordance to previously published results (Izquierdo Cañasa et al. 2008). Moreover, increased concentration of putrescine from must to the wine could be a result of microbial decarboxylation of amino acid ornithine or can be produced from the metabolism of arginine via agmatine (Beneduce et al. 2010). Thus, the concentration of putrescine after spontaneous alcoholic fermentation was 1.17 mg/L in Vranac and 0.79 mg/L in Kratošija wine, followed by almost double increased content after spontaneous malolactic fermentation (2.39 mg/L in Vranac and 1.98 mg/L in Kratošija). Results were in accordance to previously published study in which the content of putrescine significantly increased double from 4.12 to 8.52 mg/L after spontaneous malolactic fermentation (Izquierdo Cañasa et al. 2008). Concerning the wine aging of 10 months, no significant change of the concentration of amines was observed between the wines.

The diamine cadaverine was determined in the Montenegrin Vranac and Kratošija wines in a very small amount (<0.4 mg/L). In fact, the content of cadaverine was very similar between both varieties after finishing the spontaneous alcoholic fermentation, followed with significantly increased content after spontaneous MLF. The increased content of cadaverine was significantly higher in Vranac wine, which confirms that formation of this amine is attributed to the action of bacteria involved in MLF. Even the content of cadaverine was very low, it is important to use selected starter cultures with low or without aminogenic activity in order to have inoculated and controlled wine fermentation. After 10 months of bottle aging, the content of cadaverine was slightly decreased. Usually, putrescine and cadaverine are associated with poor sanitary conditions of the grapes and quality of the vinification process (Leitão et al. 2005), but in this study, the content of both amines was very low in the studied wines, meaning that the sanitary conditions of the grapes (we used healthy grapes), as well as conditions in the experimental winery were in a very good and satisfactory stage.

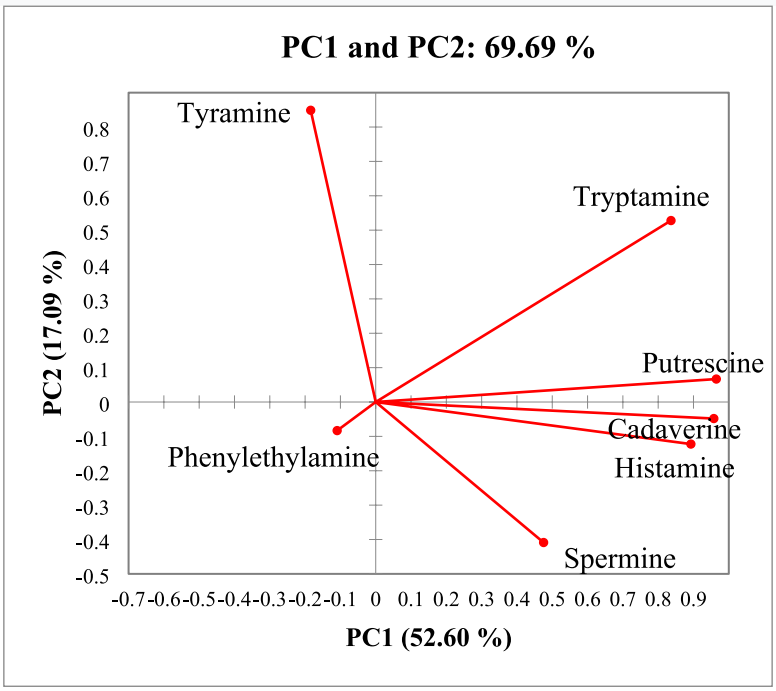
Levels of the aromatic phenylethylamine and tyramine were quite low in all wine samples, although Vranac wines (on average: 0.28 mg/L phenylethylamine) also contained significantly higher contents then Kratošija wines (on average: 0.15 mg/L phenylethylamine) due to the variety differences. These values were lower than those found in the consulted literature (Izquierdo Cañasa et al. 2008; Tašev et al. 2016, 2017; Bordiga et al. 2020). Considering the influence of spontaneous MLF, the contents of phenylethylamine and tyramine have been changed similarly and non-uniformly. Thus, highest concentration of both amines, phenylethylamine and tyramine, was observed in Vranac wine after alcoholic fermentation (0.37 mg/L and 0.054 mg/L, respectively) followed with decrease of the content to 0.219 mg/L and 0.031 mg/L, respectively, after MLF, and then increasing to 0.254 mg/L and 0.055 mg/L, respectively, after 10 months of storage. Opposite, in Kratošija wine, the content of phenylethylamine increased after MLF, followed with slight decrease after 10 months of the storage. Even excessive intake of phenylethylamine is considered to be deleterious for human health due to its vasoactive and psychoactive properties, the wines were safe for consumption since the content of phenylethylamine was very low in the wines, as well as the content of tyramine, which is considered harmful at concentration of 40 mg/L (Bordiga et al. 2020).

The content of spermine was also very low in the studied wines (0.153 mg/L on average) and there were no significant differences observed regarding the influence of alcoholic fermentation, malolactic fermentation as well as wine storage, which means that the formation of this amine was not associated with lactic acid bacteria and wine storage.

Histamine is often associated with intoxication of food as well as of wine too. Its concentration increased after the MLF in both varieties (from 79.2 to 89.6 µg/L in Vranac and from 52.1 to 94.9 µg/L in Kratošija), probably due to the decarboxylation of the corresponding amino acid histidine. In general, concentrations of histamine were much lower than the lowest recommended value in Germany, 2 mg/L, as well as much lower than 20 mg/L, a content that is considered harmful (Bordiga et al. 2020) confirming the safety of the studied wines for consumption.



(a)



(b)

Fig. 4 Principal component score plot (**a**) and correlation scatterplots (**b**) of the variables with PC1 and PC2 based on biogenic amines in Vranac and Kratošija wines determined at three vinification stages: after alcoholic fermentation (V-1 and K-1), after malolactic fermentation (V-2 and K-2) and after 10 months of bottle aging (V-3 and K-3)

The content of heterocyclic tryptamine was very low, ranging from 9.2 to 25.2 µg/L in all wines. The concentration of tryptamine slightly increased after the MLF and after storage of 10 months, probably due to the decarboxylation of the amino acid tryptophan. However, its content, which was very low in the analyzed wines, cannot be considered as dangerous and harmful.

Generally, the total level of biogenic amines in wine considered to be safe for consumers is 10 mg/L, either from healthy or from a legal point of view (Marques et al. 2008). Moreover, concentrations higher than 20 mg/L for histamine, 40 mg/L for tyramine and 3 mg/L for phenylethylamine are considered harmful and toxic (Bordiga et al. 2020). In this study, all values for the seven individual biogenic amines appeared much below these limits, also in respect of legal limits of different countries concluding that Vranac and Kratošija wines, regarding the content of biogenic amines, can be considered as safe for consumption for healthy individuals. Moreover, increase of biogenic amines was observed after spontaneous malolactic fermentation, which content stayed almost stable after 10 months of wine aging.

Principal Component Analysis (PCA)

Principal component analysis supported and confirmed our findings obtained from the UPLC-TQ-MS analysis of biogenic amines in wines. Thus, PCA was performed on the data for biogenic amines determined in Vranac and Kratošija wines at three different vinification stages: after alcoholic fermentation, malolactic fermentation and 10 months of bottle aging in order to explore possible groupings among the analyzed wines. The first two principal components, PC1 and PC2, accounted for 69.69% of the total variance (52.6% for PC1 and 17.09% for PC2). Projection of the wines on the first two principal components showed a separation mainly according to the vinification stages (Fig. 4a).

Thus, the wines from both varieties, analyzed after the alcoholic fermentation (V-1 and K-1) were located at the negative part of PC1, and were clearly separated from the wines analyzed after malolactic fermentation (V-2 and K-2) as well as from the wines after 10 months of bottle aging (V-3 and K-3). Wines V-2 and K-2, and V-3 and K-3 were located in the positive part of PC1, which were additionally separated from each other: V-2 and K-2 were located in the negative part of PC2, while V-3 and K-3 were placed in the positive part of PC2.

As can be seen from the correlation scatterplot in Fig. 3b, only tyramine and phenylethylamine contributed negatively to PC1, while all other biogenic amines (cadaverine, putrescine, histamine, spermine, and tryptamine) contributed positively to PC1, which means that grouping of the samples was performed according to the tyramine and phenylethylamine in correlation with the first vinification stage, the alcoholic fermentation.

Conclusions

The proposed UPLC-TQ-MS method is suitable, fast, and accurate for simultaneous determination of the following seven biogenic amines: cadaverine, putrescine, histamine, spermine, tyramine, phenyethylamine and tryptamine in red wine. The method confirmed satisfactory analytical performances, including good linearity, accuracy, precision, repeatability, reproducibility, LOD (limit of detection) and LOQ (limit of quantification) and it was applied for analysis of Montenegrin red wines Vranac and Kratošija for the first time. Therefore, these results are very important to support the Montenegrin, as well as the regional Balkan winemaking sector, which at the same time are a confirmation of the quality and safety of the Balkan wines. In fact, all wines presented low levels of biogenic amines then maximal allowed. Putrescine was the dominant amine in wines, followed by cadaverine and phenylethylamine. Histamine and tyramine which are considered as toxic amines, were present in a very low concentration. Moreover, the content of individual biogenic amines was determined in the wines after alcoholic fermentation without yeast inoculation, after spontaneous malolactic fermentation and after ten months of bottle aging, concluding that malolactic fermentation led to slightly increased values of biogenic amines, while aging did not influence their content. In general, all wines presented very low levels of individual and total biogenic amines in all vinification stages, confirming that the wines are safe for consumption from health point of view and in accordance to the formal regulations and recommendations for biogenic amines. PCA presented clear grouping of the wine according to the three vinification stages. Since this is a first study focused and limited on determination of biogenic amines in Vranac and Kratošija wines produced in Montenegro, additional extended research focused on other varieties as well as different maceration and winemaking practices is necessary to be performed in order to better understand the Montenegrin wines.

Author Contribution Conceptualization, D.R. and V.I.-P.; investigation, D.R., R.P-Š. and T.P.; methodology, D.R., R.P-Š., T.P. and K.T.; formal analysis, K.T.; writing—original draft, V.I.-P.; writing—review and editing, V.I.-P. All authors have read and agreed to the published version of the manuscript.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Ethics Approval This article does not contain any studies with animals or human participants.

Consent to Participate The authors agreed to participate in this work.

Consent for Publication The authors agreed to publish this work.

Competing Interests The authors declare no competing interests.

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