

# Ultra-Performance Liquid Chromatography-Triple Quadruple Mass Spectrometry (UPLC-TQ/MS) for Evaluation of Biogenic Amines in Wine

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**Abstract** In this study, a fast, simple, and sensitive analytical method for direct determination of biogenic amines tryptamine, putrescine, histamine, phenylethylamine, tyramine, cadaverine, spermine, and spermidine in wine has been developed and validated. Detection of analytes was performed with ultra-performance liquid chromatography (UPLC) coupled to triple quadruple mass spectrometer (TQ/MS). The calibration curves of all amines were linear with correlation coefficients ( $R^2$ ) ranging from 0.9906 for putrescine to 0.9998 for histamine and 2-phenylethylamine. The accuracy of the method was checked with a standard addition method, showing good accuracy, repeatability, and reproducibility ( $RSD < 10\%$ ). The limit of detection (LOD) and limit of quantification (LOQ) ranged from 0.50 to 30  $\mu\text{g/L}$  and 1.50 to 90  $\mu\text{g/L}$ , respectively, for all amines. The validated method was applied to detect and quantify biogenic amines in Macedonian red and white wines. Higher concentration of amines was observed in red wines (5797  $\mu\text{g/L}$ , on average) compared to the white wines (1485  $\mu\text{g/L}$ , on average).

**Keywords** Wine · Biogenic amines · Validation · UPLC-TQ/MS

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## Introduction

Biogenic amines (BAs) are naturally occurring low molecular weight organic bases. In wine, they are formed during the must fermentation by yeasts and lactic bacteria (Manetta et al. 2016). The amount of biogenic amines in wine is related with climatic and geological factors of the wine regions as well as to grape variety and vinification practices (Guo et al. 2015; Anli and Bayram 2009; Martuscelli et al. 2013). It has been reported that biogenic amines are formed by decarboxylation of the corresponding amino acids by microorganisms through substrate-specific decarboxylase enzymes (Preti et al. 2016) and by amination and/or transamination of aldehydes and ketones (Jastrzębska et al. 2016; Goñi and Azpilicueta 2001). Many technological and environmental factors influence the occurrence of biogenic amines in wines, such as skin maceration, contact with lees, treatments with yeast mannoproteins or proteolytic enzymes, and addition of clarification substances such as bentonite or polyvinylpyrrolidone (PVPP) (Alcaide-Hidalgo et al. 2007; Hernández-Orte et al. 2008; Marques et al. 2008; Pérez-Serradilla and Luque de Castro 2008; Martín-Álvarez et al. 2006; García-Marino et al. 2010; Preti and Vinci 2016).

Histamine (HIST), tyramine (TYR), and cadaverine (CAD) are the most representative biogenic amines of the wine; other amines such as putrescine (PUT), 2-phenylethylamine (PEA), spermine (SPM), and spermidine (SPD) are also present in the grape must and wine (Rios and Valcarel 1998; Lonvaud-Funel 2001; Loukou and Zotou 2003; Moreno-Arribas and Polo 2005; Landete et al. 2007; Onal 2007). At high concentrations, they may be responsible for undesirable toxicological effects, such as headache, respiratory distress, heart palpitation, hypotension, hypertension, nausea, and dizziness (EFSA 2011). Tyramine alone, or with 2-phenylethylamine, causes headache due to their vasoconstrictive properties, while

histamine, except headaches, causes low blood pressure, heart palpitations, edema, vomiting, and diarrhea (Littlewood et al. 1988; Smit et al. 2008). These effects depend on the individual sensitivity and simultaneous presence of co-factors such as ethanol, drugs, or other biogenic amines (Lonvaud-Funel 2001; Marcobal et al. 2006).

Monitoring of biogenic amine levels in wines can be an important marketing advantage. Actually, for the wine industry, there are no regulation limits of BAs established, only a recommended upper limit for histamine in red and white wine, 2 mg/L (Maintz and Novak 2007; Spano et al. 2010). The only country that has established an official maximum limit for the presence of histamine in wines (10 mg/L) was Switzerland, but this country has removed it in imported wines (Martuscelli et al. 2013). Generally, the toxic dose in alcoholic beverages is considered to be between 8 and 20 mg/L for histamine and 25 and 40 mg/L for tyramine, whereas lower values than 3 mg/L of phenylethylamine can cause negative physiological effects (Soufleros et al. 1998).

Determination of biogenic amines is a complex and challenging analysis because of their low concentrations in wine, wine matrix complexity, their structure, strong polarity, and presence of potentially interfering structurally similar substances. The most frequently reported technique for BA analysis in wine, as well as in other food samples, is liquid chromatography (LC) coupled to UV-Vis or fluorimetric detection (Sentellas et al. 2016), with pre- or post-column chemical derivatization using different derivatization reagents: dansyl chloride (DnsCl) (Tašev et al. 2016; Manetta et al. 2016), *o*-phthalaldehyde (OPA) (Arrieta and Prats-Moya 2012; Kelly et al. 2010; Vidal-Carou et al. 2003), dansyl chloride (Dabs-Cl) (Romero et al. 2000), benzoyl chloride (Bnz-Cl) (Ozdestan and Üren 2009), fluorenylmethylchloroformate (FMOC) (Bauza et al. 1995), 1,2-naphthoquinone-4-sulphonate (NQS) (Hlabangana et al. 2006), and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Hernández-Orte et al. 2006). In addition, biogenic amines can be determined by capillary electrophoresis (CE) (Herrero et al. 2014), gas chromatography (GC) (Fernandes and Ferreira 2000), and enzymatic methods (Lange and Wittmann 2002). Moreover, determination of biogenic amines in wine can be performed either with conventional HPLC or ultra-high-performance liquid chromatography (UHPLC) coupled with MS detector (Sagratini et al. 2012; Daniel et al. 2015), which is the most powerful analytical tool for organic compound analysis allowing direct injection of the wine sample without derivatization processes prior to analysis. In fact, application of MS detector has advantages due to the ability to confirm the presence of biogenic amines in very difficult matrixes, as wine is. Recently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and ultra-high-performance liquid chromatography coupled to a quadrupole time of flight mass spectrometry (UPLC/Q-TOF-MS) techniques have been

introduced as new method approaches for analysis of biogenic amines in wines (García-Villar et al. 2009).

Limited data are available for wines from south-east Europe and the Balkans, and to the best of our knowledge, only a preliminary study on the biogenic amine composition of Macedonian wines has been performed with pre-column chemical derivatization using dansyl chloride followed by HPLC-DAD determination (Tašev et al. 2016). In order to extend that study and provide more detailed information about BAs in red and white wines, faster and more accurate method without derivatization was developed, optimized, and validated using ultra-performance liquid chromatography coupled to a triple quadrupole MS detector (UPLC-TQ/MS). The method was optimized at two product ions ( $m/z$ ) for identification and quantification of the amines that give higher confidence of the results especially in a complex matrix such as wine. The novelty of the proposed method compared to the conventional HPLC method with pre-column chemical derivatization is the approach for analysis of the biogenic amines, in which we avoided the derivatization time-consuming step, difficult sample preparation, and long run time for analysis. As a consequence of this approach, the main benefits are very fast, accurate, and short analysis of the amines in wine samples. The developed and validated method was applied for determination of BAs in various Macedonian red and white wines giving an overview on the nature and content of biogenic amines in wines from this region.

## Materials and Methods

### Chemicals and Reagents

Standards of biogenic amines used in the study were tryptamine (TRP), PUT, HIST, PEA, TYR, CAD, SPM, and SPD, all provided by Sigma-Aldrich (Munich, Germany), and 2-phenylethylamine provided by Fluka (Munich, Germany). Water, methanol, ammonium formate, and formic acid (purity for LC/MS) were from Carlo Erba (Cornaredo, Italy). All other chemicals used were of analytical grade.

### Wine Samples

In total, 34 wines, including 15 white, 17 red, and 2 rose wines produced in the Tikveš wine region (latitude 41° 25' 57" N and longitude 22° 05' 11" E) (Republic of Macedonia), vintage 2015, were analyzed. Wine sample abbreviations were as follows for the white wines: W1 (Grenache Blank), W2 (Belan & Chardonnay), W3 (Temjanika), W4 (Temjanika), W5 (Belan & Chardonnay), W6 (Muscat Otonel), W7 (Riesling), W8 (Smederevka), W9 (Sauvignon Blank), W10 (Italian Riesling), W11 (Traminec), W12 (Žilavka), W13 (Rkaciteli), W14 (Chardonnay), and W15 (Chardonnay & Riesling); the

red wines were marked as R1 (Plavec), R2 (Vranec), R3 (Kadarka), R4 (Kratošija & Merlot), R5 (Pinot Noir), R6 (Merlot & Vranec), R7 (Vranec), R8 (Vranec & Cabernet Sauvignon), R9 (Cabernet Sauvignon), R10 (Vranec, sweet wine), R11 (Kadarka), R12 (Vranec & Plavec), R13 (Kratošija), R14 (Vranec & Kratošija), R15 (Merlot), R16 (Sirah), and R17 (Vranec & Merlot & Cabernet Sauvignon); and rose wines as were marked as Rose 1 (Pinot Noir & Merlot & Cabernet Sauvignon) and Rose 2 (Kratošija). All wines were kept at 4 °C before analysis.

Before the UPLC-TQ-MS analysis, wines were diluted with 0.1% (v/v) formic acid in ratio 1:3 and filtrated with 0.45- $\mu$ m filter (Agilent PTFE).

### UPLC-TQ/MS Analysis

The instrumentation used in this study was an Agilent UPLC 1290 system consisted of a binary pump 1290 Infinity G4220A, autosampler 1290 G4226A, column compartment 1260 G1316A, and detector DAD VL Agilent 1260 G1315D (Waldbronn, Germany), coupled with Agilent triple quadrupole LC/MS detector 6420 (Agilent Technologies, Santa Clara, CA, USA). Separation of the biogenic amines was achieved on Agilent Zorbax C18 Plus column (100  $\times$  2.1 mm, 1.8- $\mu$ m particle size), dedicated for rapid and high-pressure analysis up to 1200 bar. The mobile phase used consisted of solvent A, 5 mmol ammonium formate and 0.1% (v/v) formic acid, and solvent B, methanol with 5 mmol ammonium formate and 0.1% (v/v) formic acid, at flow rate of 0.2 mL/min. The gradient elution program was as follows: 0–0.05 min, 20%; 3.00–3.50 min, 80%; and 3.75–5 min, 20%. Total run time was 5 min and post-run time was 1 min. The injection volume was 1  $\mu$ L.

In addition, an Agilent HILIC Plus column (100  $\times$  2.1 mm, 1.8- $\mu$ m particle size) (Hydrophilic Interaction Liquid

Chromatography) column was tested for separation of biogenic amines. Mobile phase for separation of biogenic amines on this column composed of solvent A: 0.5 mmol ammonium formate and 0.1% (v/v) formic acid and solvent B: acetonitrile with 0.1% (v/v) formic acid. The gradient program was as follows: 0–0.05 min, 90%; 6.00–7.00 min, 20% B; and 7.50–10 min, 90% B with a flow rate of 0.4 mL/min.

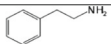

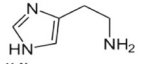
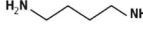
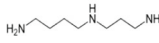
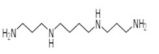
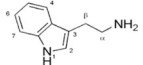
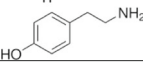
The tandem MS/MS conditions for determination of biogenic amines were optimized on the Agilent 6420 triple quadrupole mass spectrometer. Mass Hunter software (v. B.06.00) was used for instrument control, data acquisition, and evaluation. The applied parameters were capillary temperature 350 °C, gas flow 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.

The optimal quantification and confirmation transitions, their respective fragmentor, collision energies, cell accelerator voltage, polarity, and retention time with delta retention time windows are listed in Table 1. Mass Hunter Optimization Software was used for the Multiple Reaction Monitoring (MRM) optimization purposes. Automatically generated Dynamic Multiple Reaction Monitoring (DMRM) mode was used for precursor and product ion retention windows.

### Calibration Curves

Stock solutions (1 mg/mL) of each biogenic amine (tryptamine, 2-phenylethylamine, putrescine, cadaverine, spermine, spermidine, tyramine, and histamine) were prepared in 0.1% (v/v) formic acid in water. For quantification, seven-point calibration curves were constructed in a range from 10 to 1000  $\mu$ g/L for all amines, except for spermine (concentration range 100–2000  $\mu$ g/L) (Table 2). For evaluation of the effect of wine matrix, the same series of standard

**Table 1** Multiple Reaction Monitoring (MRM) conditions optimized for Agilent Triple Quad MS 6420 analysis for biogenic amines

Biogenic amine	Structural formula	Precursor ion [M+H <sup>+</sup> ]	Product Ion 1 (m/z)	Product Ion 2 (m/z)	t <sub>R</sub> (min)	Delta t <sub>R</sub> (min)	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
PEA		122	105	77	2.78	1.78	70	9 & 33	4	Positive
CAD		103	86	/	1.24	3.49	65	9 & 5	4	Positive
HIST		112	95	68	1.24	1.81	80	13 & 21	4	Positive
PUT		89	72	/	1.25	2.67	45	9	4	Positive
SPD		146	103	72	1.19	2.53	75	13 & 13	4	Positive
SPM		203	112	129	1.26	2.47	100	9 & 17	4	Positive
TRP		161	144	117	3.05	1.90	70	9 & 25	4	Positive
TYR		138	121	77	1.75	1.97	70	9 & 29	4	Positive

PEA phenylethylamine, CAD cadaverine, HIST histamine, PUT putrescine, SPM spermine, SPD spermidine, TRP tryptamine, TYR tyramine

**Table 2** Range of determination, coefficients of the regression curves (slope), correlation coefficient ( $R^2$ ), coefficients of the regression curves in matrix, correlation coefficient ( $R^2$ ) in matrix, LOD, LOQ, and results for repeatability and reproducibility of biogenic amines ( $\mu\text{g/L}$ ) in red wine (R7)

Biogenic amines	Calibration range ( $\mu\text{g/L}$ )	Slope	$R^2$	LOD ( $\mu\text{g/L}$ )	LOQ ( $\mu\text{g/L}$ )	Matrix slope	Matrix $R^2$	SSE (%)
SPD	10–1000	203	0.9969	3.00	9.00	192	0.9975	95.1
HIST	10–1000	466	0.9998	0.50	1.50	422	0.9949	90.6
CAD	10–1000	85.8	0.9957	3.00	9.00	112	0.9953	131
PUT	10–1000	85.2	0.9906	4.00	12.0	96.8	0.9958	114
SPM	100–2000	258	0.9997	30.0	90.0	102	0.9906	39.2
TYR	10–1000	292	0.9979	1.00	3.00	342	0.9998	116
PEA	10–1000	572	0.9998	1.00	3.00	575	0.9997	100
TRP	10–1000	410	0.9979	0.50	1.50	450	0.9988	109

SSE signal suppression enhancement, LOD limit of detection, LOQ limit of quantification

solutions was prepared using dilution in half volume of red wine (0.5 mL red wine in 1 mL standard solution).

### Statistical Analysis

Statistical treatment, including calculation of mean, relative standard deviation, standard error, and one-way ANOVA, were performed with STATISTICA 6.0 software (Stat Soft Inc., USA). Principal component analysis (PCA) was employed to evaluate the possible grouping of the wines, using XLSTAT Software, version 7.5.2, Addinsoft (Paris, France).

## Results and Discussion

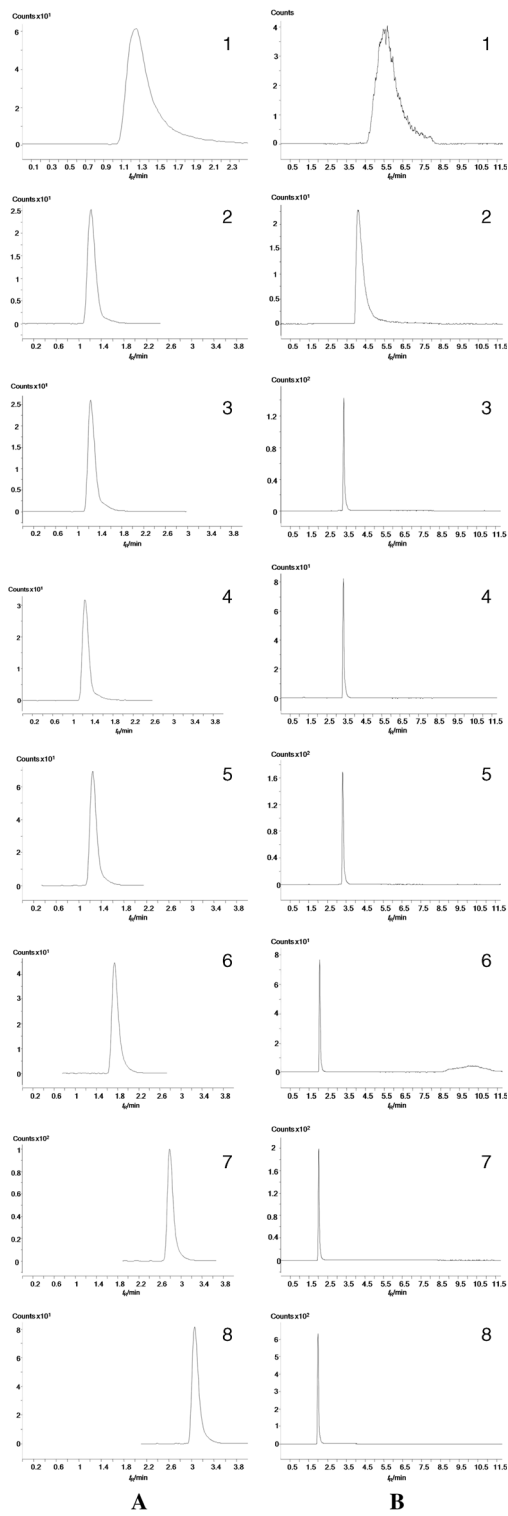
### UPLC-Triple Quadruple MS Identification of Biogenic Amines

The UPLC-TQ/MS technique was used for separation and detection of the following biogenic amines: PEA, CAD, HIST, PUT, SPM, SPD, TRP, and TYR (Table 1). The assignment of the individual compounds in the wines was carried out by comparing with the MS/MS data obtained for the standards, analyzed under the same experimental conditions, and also those found in literature (Sagrati et al. 2012; Daniel et al. 2015). Characterization of the mass spectra was performed by injection of a standard solution of each biogenic amine, with concentration of 1000  $\mu\text{g/L}$ . All biogenic amines had similar fragmentation pattern, obtaining  $m/z$  signal that corresponds to the protonated molecule  $[\text{M} + \text{H}]^+$  or precursor ion  $m/z$ , as follows: for 2-phenylethylamine at  $m/z$  122 (fragment ions at  $m/z$  105 and 77), cadaverine at  $m/z$  103 (fragment ion at  $m/z$  86), histamine at  $m/z$  112 (fragment ions at  $m/z$  95 and 68), putrescine at  $m/z$  89 (fragment ion at  $m/z$  72), spermidine at  $m/z$  146 (fragment ions at  $m/z$  103 and 72), spermine at  $m/z$  203 (fragment ions at  $m/z$  112 and 129), tryptamine at  $m/z$  161 (fragment ions at  $m/z$  144 and 177), and

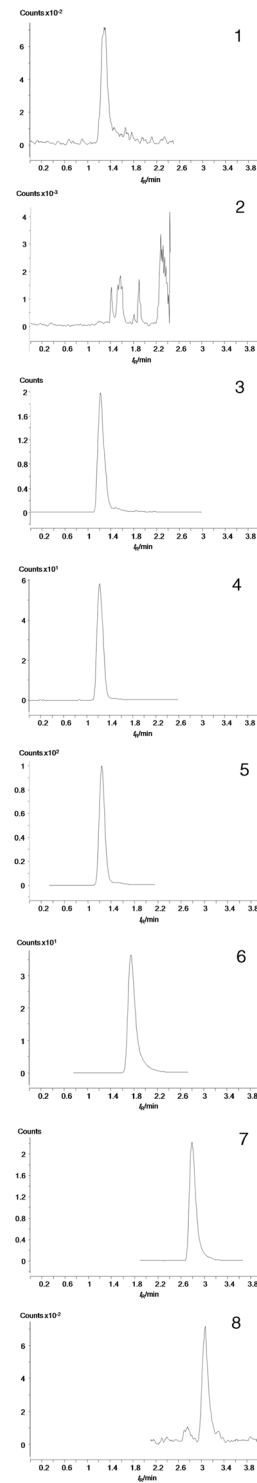
TYR at  $m/z$  138 (fragment ions at  $m/z$  121 and 77). For the six of eight amines, i.e., cadaverine, histamine, 2-phenylethylamine, putrescine, tyramine, and tryptamine, the main product ion used for quantification (the “quantifier”) was generated by loss of a neutral ammonia molecule with  $m/z$  17  $[\text{M} + \text{H}-\text{NH}_3]^+$  (Sagrati et al. 2012). The product ion of spermidine was giving a signal at  $m/z$  103, while the quantifier ion for spermine at  $m/z$  112 corresponded to the loss of a larger fragment explained as  $[\text{M} + \text{H}-(\text{CH}_2)_3\text{N}_2\text{H}_4-\text{NH}_3]^+$  (Sagrati et al. 2012). Putrescine and cadaverine produced only one product ion at  $m/z$  72 and 86, respectively, and additional optimization did not result in obtaining another ions.

### Optimization of the Chromatographic Conditions for Separation of Biogenic Amines

For separation of the biogenic amines, two different columns were tested in the preliminary experiments: Agilent Zorbax C18 Plus (100  $\times$  2.1 mm, 1.8- $\mu\text{m}$  particle size) and Agilent Zorbax HILIC Plus (100  $\times$  2.1 mm, 1.8- $\mu\text{m}$  particle size), both dedicated for rapid and high-pressure analysis. The difference between these columns was the stationary phase; Zorbax contains stationary phase with octadecyl chains (C18)-bonded silica (Fig. 1a), while HILIC contains a normal phase stationary phase using mobile phase conditions same as for reversed phase columns (Fig. 1b). Even both columns exhibited satisfactory peak shapes of the analytes, the peak of spermine (1) had more intensive tailing peak shape on the HILIC column compared to the Zorbax column. Also, the total run time was twofold shorter when Zorbax column (5 min) was used for separation of the biogenic amines, compared to HILIC column (10 min). Therefore, Zorbax column, presenting good peak shapes, satisfactory resolution, and short analysis time (5 min), was selected for further analyses, including optimization of method parameters, validation, and application on real wine samples. Figure 2 presents the ion extracted chromatograms of biogenic amines in Vranec wine (R2).



**Fig. 1** UPLC-TQ/MS ion extracted chromatogram biogenic amines in a standard solution obtained on *two columns*. **a** Zorbax C18 Plus column (run time 5 min). **b** HILIC Plus column (run time 10 min). Peak assignment spermine (1), spermidine (2), cadaverin (3), putrescine (4), histamine (5), tyramine (6), 2-phenylethylamine (7), tryptamine (8)



**Fig. 2** UPLC-TQ/MS ion extracted chromatogram biogenic amines in wine (Vranec) on Zorbax C18 Plus column. Peak assignment spermine (1), spermidine (2), cadaverin (3), putrescine (4), histamine (5), tyramine (6), 2-phenylethylamine (7), tryptamine (8)

In order to study the effect of ionization efficiency, different concentrations of ammonium formate (0.5, 1, 1.5, 2, and 2.5 mmol/L) and formic acid (0.05, 0.1, 1, 1.5, 2, 2.5, 3% (v/v)) added in the mobile phase were tested. Increasing the concentration of ammonium formate in the mobile phase led to a reduction of the peak areas, especially evident for the spermine (data not shown). Therefore, a concentration of 0.5 mmol/L of ammonium formate in the mobile phase was chosen for the analysis. The influence of the mobile phase acidity on biogenic amines was also checked. Lower peak intensities were obtained with 0.05% formic acid in the mobile phase presumably due to the insufficient ionization of the molecules under these conditions. Using 0.1% formic acid in the mobile phase allowed best molecule ionization. From these data, 0.5 mmol/L of ammonium formate and 0.1%

formic acid in the mobile phase were the best compromise for simultaneous analysis of biogenic amines in wine.

The effect of the wine matrix on the ionization and thus sensitivity for quantification of the tested biogenic amines was also studied using Vranec wine sample (R2). For this purpose, calibration standards were prepared and measured in the wine matrix (R2) with standard additions of all investigated biogenic amines. Slopes and correlation coefficients were then calculated for these calibration curves and compared to the ones prepared in 0.1% formic acid in water, without the wine matrix (Table 2). To evaluate the matrix effect, the signal suppression enhancement (SSE) was calculated as the ratio of the slope of the standards in wine matrix and the slope of the standards prepared without matrix for each biogenic amine studied (Sagrati et al. 2012).

$$\text{SSE} = (\text{slope matrix matched calibration} / \text{slope standard calibration in solvent}) \times 100$$

From the results presented in Table 2, it can be seen that wine matrix has diverse influence on each biogenic amine causing enhancing or suppression of the process of ionization that is expressed by the signal suppression enhancement calculated in percent. Values higher than 100% mean higher slopes with matrix that are due to signal enhancement and more efficient ionization, whereas  $\text{SSE} < 100\%$  is due to ionization suppression in the presence of the matrix. A negative influence and ionization suppression causing decrease of the signals was observed for spermidine (SSE 95.1) and histamine (SSE 90.6), whereas increased signals were observed for cadaverine (SSE 131), putrescine (SSE 114), tyramine (SSE 116), and tryptamine (SSE 109), and no influence was observed for 2-phenylethylamine (SSE 100). The wine matrix caused most severe suppression of the signal of spermine where suppression was greater than 60% (SSE 39). So, the proposed method can be successfully used for all biogenic amines, with standard calibration curves in aqueous solvent, except for spermine where the negative matrix effect, i.e., ionization suppression, has to be taken into account and the measurements should be made using calibration standards prepared in wine matrix.

In fact, the main advantage of the proposed method is analysis of the biogenic amines in wine without derivatization. In that way, the analysis is simpler and faster (only dilution of wine is performed) compared to the HPLC-DAD method, whereas derivatization step is necessary to be performed prior the HPLC analysis (Tašev et al. 2016).

### Validation of the Method

Linearity was tested in 3 days at seven concentration levels. The *linearity data*, including slope, intercept, and correlation

coefficient ( $R^2$ ), were calculated, and they are presented in Table 2. As it can be seen from the table, the linearity is satisfactory in all cases with correlation coefficients ( $R^2 > 0.99$ ), ranging from 0.9957 for cadaverine to 0.9998 for histamine and 2-phenylethylamine.

*Limit of detection* (LOD) was determined as a concentration of the analyte that gives a signal equal to the average background ( $S_{\text{blank}}$ ) plus three times the standard deviation of the blank ( $S_{\text{blank}}$ )  $\text{LOD} = S_{\text{blank}} + 3 \times (S_{\text{blank}})$ , and *limit of quantification* (LOQ) was determined as  $\text{LOQ} = 3 \times \text{LOD}$ . The obtained values for LOD and LOQ ranged from 0.50 to 30.0  $\mu\text{g/L}$  and 1.50 to 90.0  $\mu\text{g/L}$ , respectively, for all amines. The lowest limits of detection were noticed for histamine and tryptamine with 0.50  $\mu\text{g/L}$ , followed by tyramine and 2-phenylethylamine with LOD 1.00  $\mu\text{g/L}$ , then spermidine and cadaverine with 3.00  $\mu\text{g/L}$ , and putrescine with 4.00  $\mu\text{g/L}$ , and the highest LOD was found for spermine with 30  $\mu\text{g/L}$  (Table 2).

### Accuracy and Precision

The intra-day and inter-day accuracy and precision were determined by injection of standard solutions with low (40  $\mu\text{g/L}$ ), medium (100  $\mu\text{g/L}$ ), and high concentrations (500  $\mu\text{g/L}$ ) of all tested biogenic amines: tryptamine, 2-phenylethylamine, putrescine, cadaverine, spermine, spermidine, tyramine, and histamine. For determination of intra-day accuracy and precision, freshly prepared solutions were used, analyzed immediately, in three repetitions during 1 day. Inter-day accuracy and precision were determined during three consecutive days with three repeated analyses. The results are presented in Table 3.

**Table 3** Inter- and intra-day accuracy and precision and standard addition method for checking the accuracy on real samples

Standard additions		STD I (40 µg/L)				STD II (100 µg/L)				STD III (500 µg/L)			
Biogenic amines	Concentration in wine (µg/L)	<x>	SD	RSD (%)	Recovery (%)	<x>	SD	RSD (%)	Recovery (%)	<x>	SD	RSD (%)	Recovery (%)
Inter-day accuracy and precision (three replicates in 1 day) and recovery													
SPD	68.5	107	3.27	3.05	101	181	7.25	3.99	93.1	507	23.2	4.58	112
CAD	76.8	121	0.71	0.58	96.5	182	2.23	1.22	97.1	576	5.64	0.98	100
PUT	199	236	10.0	0.56	101	334	8.77	0.48	89.5	738	14.1	0.78	94.7
HIST	51.5	92.2	0.98	1.07	99.2	164	2.94	1.79	92.4	635	10.9	1.71	86.9
SPM	<LOQ	<LOQ	–	–	–	103	0.41	0.40	103	522	4.66	0.89	104
TYR	37.9	79.7	2.92	5.72	97.7	147	2.54	1.72	93.8	593	9.60	1.62	90.7
PEA	40.9	84.5	0.97	1.14	95.7	151	2.43	1.61	93.3	600	3.51	0.58	90.2
TRP	<LOQ	37.8	0.00	0.00	94.5	98.5	6.74	1.35	98.5	501	6.74	1.35	100
Intra-day accuracy and precision (three replicates in three different days)													
SPD	68.5	108	3.28	3.05	100	183	7.28	3.99	92.1	509	23.3	4.58	112
CAD	76.8	122	0.71	0.58	95.7	183	2.24	1.22	96.6	579	5.66	0.98	100
PUT	199	238	10.1	0.56	100	334	8.81	0.48	89.5	740	14.1	0.78	94.5
HIST	51.5	92.7	0.99	1.07	98.7	164	2.95	1.79	92.4	638	10.9	1.71	86.4
SPM	<LOQ	<LOQ	–	–	–	103	0.41	0.40	103	525	2.7	4.69	105
TYR	37.9	80.1	2.94	3.67	97.3	148	2.56	1.72	93.2	596	9.65	1.72	90.3
PEA	40.9	84.9	0.97	1.14	95.3	152	2.44	1.61	92.7	603	3.53	0.58	89.7
TRP	<LOQ	38.0	0.00	0.00	95.0	99.0	4.18	4.22	99	503	6.77	1.35	101

STD standard addition, <x> average value, SD standard deviation, RSD relative standard deviation, LOQ limit of quantification

The accuracy was expressed with the recovery of the determined concentration compared with the true (nominal) value. It was checked using the standard addition method on real wine sample (Vranec). Red wine sample was spiked at three concentration levels with mixed standard solution of biogenic amines with low (40 µg/L), medium (100 µg/L), and high concentrations (500 µg/L). The analysis of these spiked samples led to calculated recoveries ranging between 90.2 and 112% (Table 3), which confirmed the accuracy of the method and its suitability for analysis of biogenic amines in wine samples.

Precision was expressed as a relative standard deviation (RSD). Obtained values for RSD were lower than 5% for all amines, except for tyramine (RSD was 5.72% for the low standard addition with concentration of 40 µg/L), which confirmed that the proposed method is precise.

#### Repeatability and Reproducibility

Repeatability was checked with six repetitions in 1 day, while reproducibility was checked with three repetitions in five consecutive days, both performed on a real red wine sample. Values for the relative standard deviation were very low, ranging from 0.43 to 7.89% for repeatability and 0.36 to 7.56% for reproducibility, the highest ones obtained for spermine, whereas all others were below 3%, confirming that the method

is reproducible and can be applied for determination of biogenic amines in white and red wines (data not shown).

#### Biogenic Amines Content in Red and White Wines

The optimized and validated UPLC-TQ/MS method was applied for analysis of biogenic amines in Macedonian white and red wines from various varieties. The content of the determined biogenic amines in the wines is presented in Table 4.

Biogenic amines were detected in all analyzed wines since they are naturally present in wine, and it is very difficult, or even impossible, to obtain a wine that does not contain any biogenic amines. Among all biogenic amines, histamine was the most abundant in all wines, ranging from 14.9 to 67.4 µg/L in white wines and from 42.5 to 5131 µg/L in red wines. Four wines (R6 Merlot & Vranec, R7 Vranec, R9 Cabernet Sauvignon, and R17 Vranec & Merlot & Cabernet Sauvignon) contained histamine in a higher concentration (4.1, 5.1, 2.7, and 4.5 g/L, respectively) than the recommended values of 2 mg/L (Maintz and Novak 2007; Spano et al. 2010). The other biogenic amines (tyramine, 2-phenylethylamine, and tryptamine) in white wines were in the range of 12.0–22.4 µg/L for tyramine, 16.9–138 µg/L for 2-phenylethylamine, and 3.69–5.68 µg/L for tryptamine, while in red wines, they ranged from 183 to 6538 µg/L for tyramine, 59.4–1001 µg/L for 2-phenylethylamine, and 2.27–

**Table 4** Content of biogenic amines in Macedonian wines given in  $\mu\text{g/L} \pm$  standard deviation ( $n = 3$ )

Wines	SPD	CAD	PUT	HIST	SPM	TYR	PEA	TRP	$\Sigma$
White wines									
W1	56.6 $\pm$ 0.31 a	117 $\pm$ 3.21	1,444 $\pm$ 10.54	42.3 $\pm$ 1.79 a	<LOQ	16.4 $\pm$ 0.07 a	16.9 $\pm$ 0.11	5.46 $\pm$ 0.63 a	1,706 a
W2	56.0 $\pm$ 0.31 a	309 $\pm$ 8.48 a	893 $\pm$ 6.52	46.2 $\pm$ 1.96 a	<LOQ	14.4 $\pm$ 0.06 b	80.4 $\pm$ 0.53 a	4.38 $\pm$ 0.50 b	1,414
W3	55.7 $\pm$ 0.30 a	388 $\pm$ 10.6 c	1,007 $\pm$ 7.35	67.4 $\pm$ 2.86	<LOQ	13.0 $\pm$ 0.05 b	70.2 $\pm$ 0.46	3.94 $\pm$ 0.45 c	1,622 d
W4	505 $\pm$ 2.75	416 $\pm$ 11.4	669 $\pm$ 4.88 b	50.8 $\pm$ 2.15 b	<LOQ	19.0 $\pm$ 0.08 c	80.7 $\pm$ 0.53 a	4.39 $\pm$ 0.50 b	1,762 a
W5	711 $\pm$ 3.88	193 $\pm$ 5.29	741 $\pm$ 5.41	56.7 $\pm$ 2.41 b	<LOQ	18.5 $\pm$ 0.08 c	77.5 $\pm$ 0.51 a	5.43 $\pm$ 0.62 a	1,813
W6	143 $\pm$ 0.78	152 $\pm$ 4.16	753 $\pm$ 5.31	45.4 $\pm$ 1.93 a	<LOQ	18.1 $\pm$ 0.08 c	25.8 $\pm$ 0.17 b	5.68 $\pm$ 0.65 a	1,137 b
W7	60.0 $\pm$ 0.33 a,b	724 $\pm$ 19.8	762 $\pm$ 5.56	50.6 $\pm$ 2.15 b	<LOQ	15.1 $\pm$ 0.06 a	92.0 $\pm$ 0.61	5.55 $\pm$ 0.64 a	1,728
W8	57.0 $\pm$ 0.31 a	290 $\pm$ 7.96 a	820 $\pm$ 5.99 a	38.1 $\pm$ 1.61 a	<LOQ	15.9 $\pm$ 0.07 a	133 $\pm$ 0.88	5.08 $\pm$ 0.58 a	1,368 c
W9	113 $\pm$ 0.62	175 $\pm$ 4.81 d	815 $\pm$ 5.95 a	28.3 $\pm$ 1.20	<LOQ	22.4 $\pm$ 0.09	35.4 $\pm$ 0.21	4.74 $\pm$ 0.54 b	1,200 b
W10	59.2 $\pm$ 0.32 a	650 $\pm$ 17.8	786 $\pm$ 5.74 a	52.3 $\pm$ 2.22 b	<LOQ	14.1 $\pm$ 0.06 b	63.3 $\pm$ 0.42 c	4.51 $\pm$ 0.52 b	1,644 d
W11	133 $\pm$ 0.73	457 $\pm$ 12.5 b	782 $\pm$ 5.71 a	55.0 $\pm$ 2.33 b	<LOQ	15.3 $\pm$ 0.06 a	85.3 $\pm$ 0.56 d	4.17 $\pm$ 0.48 c	1,544
W12	59.7 $\pm$ 0.33 a	330 $\pm$ 9.03 c	1,121 $\pm$ 8.18	27.8 $\pm$ 1.18	<LOQ	13.2 $\pm$ 0.06 b	138 $\pm$ 0.91	4.56 $\pm$ 0.52 b	1,705 a
W13	65.3 $\pm$ 0.36 b	173 $\pm$ 4.73 d	556 $\pm$ 4.06	14.9 $\pm$ 0.63	<LOQ	18.6 $\pm$ 0.08 c	27.1 $\pm$ 0.18 b	5.45 $\pm$ 0.63 a	866
W14	113 $\pm$ 0.62	484 $\pm$ 13.3 b	635 $\pm$ 4.64 b	38.3 $\pm$ 1.62 a	<LOQ	12.0 $\pm$ 0.05	86.6 $\pm$ 0.57 d	4.00 $\pm$ 0.46 c	1,385 c
W15	61.5 $\pm$ 0.34 b	313 $\pm$ 8.59 a	885 $\pm$ 6.46	40.6 $\pm$ 1.72 a	<LOQ	13.7 $\pm$ 0.06 b	63.3 $\pm$ 0.42 c	3.69 $\pm$ 0.42	1,391 c
Average	150	345	844	43.7	<LOQ	16.0	71.7	4.7	1,485
Range	55.7–712	117–724	556–1,444	14.9–67.4	<LOQ	12.0–22.4	16.9–138	3.67–5.68	866–1,813
Red wines									
R1	59.5 $\pm$ 0.90 a	654 $\pm$ 9.85 a	1,476 $\pm$ 22.2	138 $\pm$ 2.08 a	<LOQ	6,538 $\pm$ 98.4	228 $\pm$ 3.43 a	6.48 $\pm$ 0.10	9,111
R2	65.9 $\pm$ 0.99 b	383 $\pm$ 5.77	2,255 $\pm$ 34.0 a	521 $\pm$ 7.85	<LOQ	2,704 $\pm$ 40.7 a	157 $\pm$ 2.37 b	3.63 $\pm$ 0.05 a	6,097 a
R3	55.7 $\pm$ 0.84 a	639 $\pm$ 9.63 a	2,116 $\pm$ 31.9	74.5 $\pm$ 1.12 b	<LOQ	2,642 $\pm$ 39.8 a	283 $\pm$ 4.26 a	5.30 $\pm$ 0.08 c	5,827
R4	55.9 $\pm$ 0.84 a	619 $\pm$ 9.32	1,867 $\pm$ 28.1 b	44.4 $\pm$ 0.67 c	<LOQ	756 $\pm$ 11.4 b	97.1 $\pm$ 1.46	4.49 $\pm$ 0.07 b	3,452
R5	55.7 $\pm$ 0.84 a	480 $\pm$ 7.23 b	1,192 $\pm$ 18.0	142.4 $\pm$ 2.14	<LOQ	802 $\pm$ 12.1	86.4 $\pm$ 1.30 b	4.94 $\pm$ 0.07 b	2,770 d
R6	174 $\pm$ 2.63	495 $\pm$ 7.46 b	1,704 $\pm$ 25.7 b	4,163 $\pm$ 62.7 d	<LOQ	5,687 $\pm$ 85.6	165 $\pm$ 2.48 c	3.98 $\pm$ 0.06 a	12,410 b
R7	63.7 $\pm$ 0.96 b	585 $\pm$ 8.81	1,923 $\pm$ 29.0 b	5,131 $\pm$ 77.3	<LOQ	4,364 $\pm$ 65.7	103 $\pm$ 1.56	4.36 $\pm$ 0.07 b	12,191 b
R8	56.5 $\pm$ 0.85 a	203 $\pm$ 3.05	210 $\pm$ 3.21	67.4 $\pm$ 1.01 b	<LOQ	912 $\pm$ 13.7	84.1 $\pm$ 1.27 b	2.27 $\pm$ 0.03	1,542
R9	100 $\pm$ 1.51	488 $\pm$ 7.35 b	2,397 $\pm$ 36.1 a	2,731 $\pm$ 41.1	<LOQ	2,916 $\pm$ 43.9 a	1,001 $\pm$ 15.1	15.9 $\pm$ 0.24	9,654
R10	58.6 $\pm$ 0.88 a	837 $\pm$ 12.6	2,152 $\pm$ 32.4 a	384 $\pm$ 5.79	<LOQ	715 $\pm$ 10.8 b	318 $\pm$ 4.79	5.93 $\pm$ 0.09 c	4,481 c
R11	57.2 $\pm$ 0.86 a	454 $\pm$ 6.84 b	2,363 $\pm$ 35.6 a	57.2 $\pm$ 0.86	<LOQ	183 $\pm$ 2.75	59.4 $\pm$ 0.89	4.12 $\pm$ 0.06 b	3,187
R12	57.2 $\pm$ 0.86 a	595 $\pm$ 8.96	1,961 $\pm$ 29.5	348 $\pm$ 5.25	<LOQ	3,025 $\pm$ 45.5	149 $\pm$ 2.25 c	4.66 $\pm$ 0.07 b	6,151 a
R13	56.2 $\pm$ 0.85 a	677 $\pm$ 10.0 a	1,848 $\pm$ 27.8 b	125 $\pm$ 1.89 a	<LOQ	2,975 $\pm$ 44.8 a	348 $\pm$ 5.24	8.43 $\pm$ 0.13	6,046 a
R14	57.0 $\pm$ 0.86 a	439 $\pm$ 6.60 b	1,619 $\pm$ 24.4 c	107 $\pm$ 1.62	<LOQ	1,548 $\pm$ 23.3	193 $\pm$ 2.91	3.18 $\pm$ 0.05	3,970
R15	55.5 $\pm$ 0.84 a	574 $\pm$ 8.65 c	1,663 $\pm$ 25.0 c	42.5 $\pm$ 0.64 c	<LOQ	262 $\pm$ 3.94	86.4 $\pm$ 1.30 b	3.73 $\pm$ 0.06 a	2,698 d
R16	56.0 $\pm$ 0.84 a	670 $\pm$ 10.1 a	2,780 $\pm$ 41.9	239 $\pm$ 3.60	<LOQ	212 $\pm$ 3.19	109 $\pm$ 1.64	4.56 $\pm$ 0.07	4,081 c
R17	55.7 $\pm$ 0.84 a	547 $\pm$ 8.24 c	2,400 $\pm$ 36.1 a	4,494 $\pm$ 67.7 d	<LOQ	757 $\pm$ 11.4	209 $\pm$ 3.15	3.26 $\pm$ 0.05	8,489
Average	67.7	554	1,879	1,210	<LOQ	1,850	219	5.3	5,797
Range	55.5–174	203–837	210–2,780	42.5–5,131	<LOQ	183–6,538	59.4–1,001	2.27–15.9	1,542–12,410
Rose wines									
Rose 1	64.2 $\pm$ 0.97 a	344 $\pm$ 5.18	961 $\pm$ 14.5	47.1 $\pm$ 0.71 a	<LOQ	50.9 $\pm$ 0.77	60.5 $\pm$ 0.91	2.43 $\pm$ 0.04	1,538 a
Rose 2	65.8 $\pm$ 0.99 a	187 $\pm$ 2.82	865 $\pm$ 13.0	40.0 $\pm$ 0.60 a	<LOQ	31.3 $\pm$ 0.47	32.1 $\pm$ 0.48	1.74 $\pm$ 0.03	1,231 a
Average	65.0	266	913	43.5	<LOQ	41.1	46.3	2.1	1,385
Range	64.2–65.8	187–344	865–961	40.0–47.1	<LOQ	31.3–50.9	32.1–60.5	1.74–2.43	1,231–1,538

Same letters in the column indicate the values that are not significantly different ( $p > 0.05$ ), analyzed by the ANOVA Tukey-Kramer multiple comparison test

<LOQ value lower than the limit of quantification

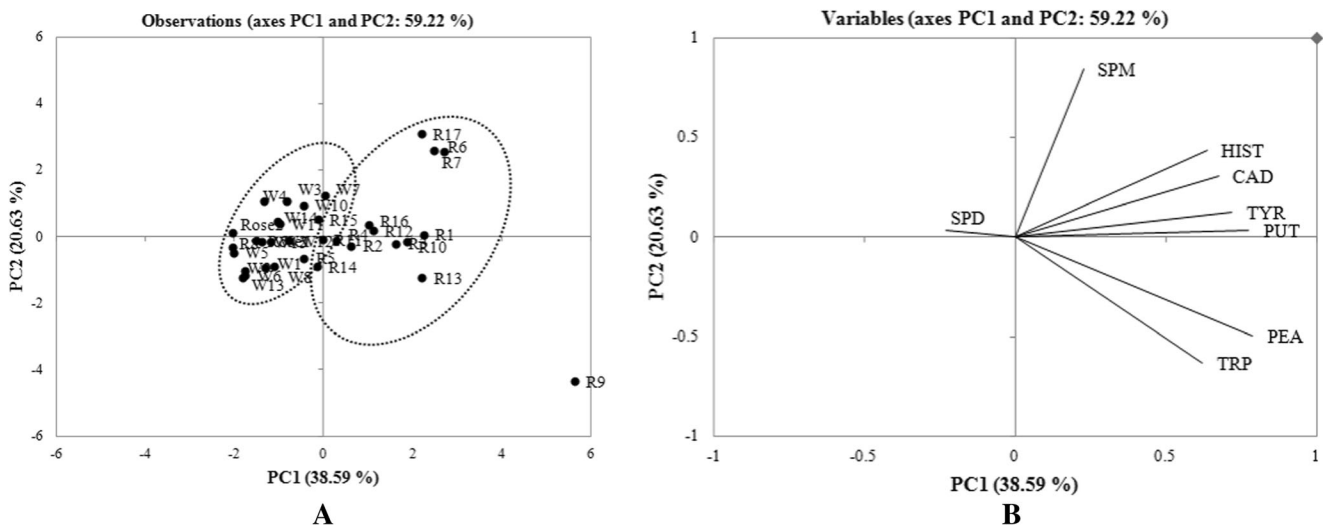
W1 (Grenache Blank), W2 (Belan&Chardonnay), W3 (Temjanika), W4 (Temjanika), W5 (Belan&Chardonnay), W6 (Muscat Otonel), W7 (Risling), W8 (Smederevka), W9 (Sauvignon Blank), W10 (Italian Risling), W11 (Traminac), W12 (Žilavka), W13 (Rkaciteli), W14 (Chardonnay), W15 (Chardonnay&Risling)

R1 (Plavec), R2 (Vranec), R3 (Kadarka), R4 (Kratošija&Merlto), R5 (Pinot Noir), R6 (Merlot&Vranec), R7 (Vranec), R8 (Vranec&Cabernet Sauvignon), R9 (Cabernet Sauvignon), R10 (Vranec, sweet wine), R11 (Kadarka), R12 (Vranec&Plavec), R13 (Kratošija), R14 (Vranec&Kratošija), R15 (Merlot), R16 (Syrah), R17 (Vranec&Merlot&Cabernet Sauvignon), Rose 1 (Pinot Noir&Merlot&Cabernet Sauvignon), Rose 2 (Kratošija)

15.9  $\mu\text{g/L}$  for tryptamine. Putrescine and cadaverine were also found; putrescine was in the range from 210 to 2780  $\mu\text{g/L}$  for red wines and 556 to 1444  $\mu\text{g/L}$  for white wines, and cadaverine 203 to 837  $\mu\text{g/L}$  and 117 to 724  $\mu\text{g/L}$  for the red and white wines, respectively. Spermidine was present in a low range in both red (55.5–174  $\mu\text{g/L}$ ) and white wines (55.7 to 711  $\mu\text{g/L}$ ), while spermine was below the LOQ for all wines.

These results corresponded to other reported results for biogenic amines in wines (Daniel et al. 2015). In general, the sum of the concentration of all determined biogenic amines in red wines was significantly higher (5797  $\mu\text{g/L}$ , on average) compared to the white wines (1485  $\mu\text{g/L}$ , on average). The variability in the BA content between red and white wines could be associated to different vinification techniques, such as the





**Fig. 3** **a** Correlation scatter plot of wine classification in function of PC1 and PC2, for all the analyzed variables. **b** PCA loadings of all analysed

compounds. Labels of the wines correspond to the ones in Table 4. Abbreviations of compounds are explained in Table 1

must contact time with grape skins that is applied for red winemaking or amino acids content at the initial and final phases of alcoholic fermentation.

In general, analyzed Macedonian wines contained low amount of biogenic amines and their concentrations would not have negative effect on consumers' health, which means that both red and white wines were safe for consumption with regards to the biogenic amines (Soufleros et al. 1998; Martuscelli et al. 2013).

### Principal Component Analysis

Principal component analysis (PCA) was applied using the dataset of individual biogenic amines obtained from the UPLC-TQ/MS analysis. PCA was used to explore the effect of grape variable (red vs. white) based on the biogenic amines profile of the analyzed wines. The first two principal components, PC1 and PC2, accounted for 59.22% of the total variance (38.59% for PC1 and 20.63% for PC2), thus explaining a significant information in the dataset. The projection of the wines on the first two principal components showed a clear separation of the samples into two groups, according to the wine type (white vs. red) (Fig. 2a); red wines (R) were clearly separated from the white (W) and rose wines. Red wines were mainly located in the positive part of PC1, while white wines were located in the negative part of PC1.

The principal components responsible for the differences in the BA composition of the wines produced were determined and presented in the scatter plot in Fig. 2b. The responsible components for the separation of red wines were CAD, PUT, HIST, TYR, PEA, and TRP, which were present in a higher concentration in red wines, prevailed in the positive part of the first principal component, while only SPD was characteristic

for the white wines. So, the separation of the wines was observed according to the wine type (red vs. white; Fig. 3).

### Conclusion

The proposed UPLC-TQ/MS method is suitable for fast, accurate, and simultaneous determination of the following biogenic amines: TRP, PUT, HIST, PEA, TYR, CAD, SPD, and SPM in red and white wines without previous derivatization. The developed method was validated showing satisfactory analytical performance without significant effect of the wine matrix on ionization efficiency (except for spermine). The method was then applied for analysis of real samples, Macedonian red and white wines, observing that red wines presented higher content of amines compared to the white wines. All wines contained biogenic amines in concentration levels lower than the maximal allowed. For the first time, biogenic amine content was determined in local varieties grown in Republic of Macedonia such as white wines Žilavka, Temjanika, and Smederevka and red wines Kratošija and Kadarka.

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### Compliance with Ethical Standards

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**Conflict of Interest** Krste Tašev declares that he has no conflict of interest. Violeta Ivanova-Petropulos declares that she has no conflict of interest. Marina Stefova declares that she has no conflict of interest.

**Ethical Approval** This article does not contain any studies with animals.

**Informed Consent** It was obtained from all individual participants included in the study.

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