In memory of prof. dr. Simion Gocan

NOVEL APPROACHES FOR THE DETERMINATION OF BIOGENIC AMINES IN FOOD SAMPLES

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ABSTRACT. Wine is a fermented beverage that could be affected by high concentrations of biogenic amines which alter organoleptic properties.

In this work, new analytical approaches for determination of biogenic amines in wines were developed.

For the first time, we studied the derivatization of BAs in wines with naphthalene-2,3-dicarboxaldehyde (NDA) and with dabsyl chloride (DBS) and analysis of derivatized BAs by HPLC coupled to fluorescence (HPLC-NDA-FL) and thermal lens spectrometry (HPLC-DBS-TLS) detectors. The sensitivity of the two methods (LODs HPLC-NDA-FL in the range 27-73 μg/L; LODs HPLC-DBS-TLS in the range 3.4-11 μg/L) was higher than that of the official method for biogenic amines in wines, OIV-MA-AS315-18 (60-77 μg/L). For its best performances, the HPLC-DBS-TLS technique was applied to the analysis of putrescine, cadaverine, histamine and tyramine in two white wine samples. Additionally, exploiting the Berthelot reaction, the TLS fast screening of biogenic amines in wines, following the release of ammonia by transglutaminase, was also proposed. This approach allowed us to determine total biogenic amount content in concentrations below 0.1 mg/L, expressed as equivalents of histamine.

Keywords: biogenic amines, NDA, liquid chromatography, TLS, fluorescence, wine.

INTRODUCTION

Biogenic amines (BAs) are low molecular weight compounds which could be frequently found in food and beverage matrices [1-2]. They are

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divided in three main classes, aliphatic (e.g. putrescine, cadaverine, spermine, spermidine), aromatic (e.g. tyramine, phenylethylamine) and heterocyclic (e.g. histamine, tryptamine). They mostly derive from four enzymatic reactions of bacteria (decarboxylation, transamination, reductive amination and degradation) performed on precursor amino compounds, such as amino acids.

Highest concentrations of biogenic amines are found in fermented food, where different species of bacteria, in particular lactic acid bacteria (LAB), are responsible for decarboxylase activity, in different degrees depending on the bacteria strain [3]. Lower concentrations are also found in non-fermented foods, as an endogenous origin [4].

Biogenic amines play essential roles in the development, metabolism and physiological functions of humans; however, several studies show that, biogenic amines at concentrations higher than 500 ppm can induce cancerogenic alteration of DNA [5-8]. At lower concentrations, BAs can cause many toxicological effects, such as headache and hypertension besides originating organoleptic alterations of the affected foods. Recently, several European Union Countries have set regulations for limits of histamine (one of the most active amine) in food matrices. As an example, EU Directive 1441/2007 regulates histamine in fish (the mean value must not exceed the value of 100 mg/kg) [9]. Local legislation of several countries regulates the concentration of histamine in wines also (i.e. France 8 mg/L, Germany 2 mg/L, Switzerland 10 mg/L).

Among the fermented beverages that could be affected by relevant concentrations of BAs, wines are intensively studied matrices. In fact, in these matrices, BAs are produced, along many stages of winemaking and ageing, by LAB bacteria, in concentrations which depend on regional variability, as well as on agricultural and production techniques [10-11]. Histamine, tyramine, putrescine, and cadaverine are among the biogenic amines mainly present in wines [3].

Various analytical methods have been developed for rapid and simultaneous determinations of biological amines in wine, such as liquid chromatography (LC), thin-layer chromatography (TLC) [12], gas chromatography (GC) [13] and capillary electrophoretic methods (CE) [14]. Among these, GC is not so often applied, whereas CE methods are not sensitive to low BA concentrations and have poor reproducibility [15]. Conversely, LC is the most applied technique for the determination of biogenic amines. MS detection is frequently reported, even if its routine application can be limited by the costs and by analyst's expertise required. More frequently, ion [16] and reversed-phase chromatography coupled with UV-Vis and fluorescence (FL) [17] detections are proposed for the analysis

of BAs. These approaches require a derivatization procedure. O-pthalaldehyde (OPA) [18], dansyl-chloride (DNS-Cl) [19], dabsyl-chloride (DBS-Cl)[20] and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [21] are some of the derivatizing agents used for fluorescence detection. Despite the good sensitivity achieved, the above mentioned derivatizing agents have peculiar disadvantages. More in detail, DNS-Cl is characterized by slow kinetics. OPA can derivatize primary amine groups also at low concentrations (ng/L) but at strictly controlled conditions of pH [22]. Recently [23], naphthalene-2,3-dicarboxaldehyde (NDA) was used to form derivatives of amines with fluorescent and electroactive properties. If compared with the products formed by derivatization with OPA, the NDA derivatives exhibit improved chemical stability, excitation maxima in the visible region (at 420 and 440 nm) and enhanced fluorescence quantum efficiency [24].

These properties, particularly absorption maxima in the visible range, open the possibility of applying novel techniques complementary to FL, such as highly sensitive thermal lens spectrometry (TLS). TLS is however based on radiationless de-excitation processes and is therefore hindered by higher fluorescence quantum yields [25-26]. For nonfluorescing analytes TLS offers detection of absorbances as low as 10^{-7}, and was shown to actually enable detection at single molecule level when performed in its microscopic configuration (Thermal lens microscopy – TLM) adapted for lab-on-chip detection [27]. Even thought TLS enables sensitive detection also for fluorescing analytes such as cyanobacterial pigments [28], non-fluorescing derivatives such as dabsylated BAs, which have the primary absorbance maximum of BAs shifted into the visible spectral range, appear advantageous over the fluorescing derivatives. Besides an easier matching of absorbance maxima with the available laser emission lines, the longer wavelength further reduces the possibility of interference from other compounds. Due to high sensitivity, fast signal response and capability of probing sub-μL volumes, TLS and TLM have recently found many applications also in vanguard analytical methods for fast screening of environmental and food samples. Such applications include determination of organophosphorous pesticides, microcystin [28], Cr(VI) [29], and ammonia [30]. As reported, ammonia is also produced from biogenic amines by the action of transglutaminase (TG), which hence can be used for determination of BAs [31].

In this work, for the first time, we compared NDA and dabsyl chloride as derivatizing agents for BAs determination in wines by HPLC coupled to fluorescence (HPLC-FL) and thermal lens spectrometry (HPLC-TLS) detectors. Additionally, exploiting the Berthelot reaction, leading to the

formation of indophenol, we propose for the first time the fast screening of BAs in wines by TLS, following the release of ammonia by TG.

In detail, after a preliminary optimization of chromatographic conditions, the effects of the main derivatization parameters on BA signals were assessed, so optimizing derivatization conditions.

The figures of merit for HPLC-NDA-FL and HPLC-DBS-TLS methods (linearity, limits of detection, limits of quantification) were evaluated and compared. Since the best performances were obtained by the HPLC-DBS-TLS method, this method was successfully applied for the determination of putrescine, cadaverine, histamine and tyramine in two white wine samples, comparing results with those obtained by using the official method for BAs determination in wines, which is based on the HPLC-OPA-FL procedure.

RESULTS AND DISCUSSION

DERIVATIZATION: OPTIMIZATION OF PARAMETERS

NDA derivatization

To optimize the derivatization of BAs in wine matrix, the effects of reaction time and initial NDA concentration on the derivatization performance were studied for ethanolamine, chosen as a model compound, in synthetic wine matrix.

Effect of reaction time.

A solution of 50 mg/L ethanolamine was derivatized with NDA at six reaction times ranging from 15 to 40 min and subsequently injected into the HPLC-FL system, measuring the peak area obtained for each trial. As shown in Figure 1, the response increases with reaction time up to 25 min, then reaching a plateau for increased reaction times. In the absence of other literature studies performed on wines, this reaction time was compared with other studies using NDA for BAs derivatization, observing that about 16 h are required to complete derivatization e.g. in human immunoglobulin samples [17]. This study, tests were performed at reaction times as long as16 h, highlighted a 30% decrease of the responses, probably due to degradation of the derivatizing products. Further studies were performed for max. 40 min, choosing 25 min as optimal reaction time.
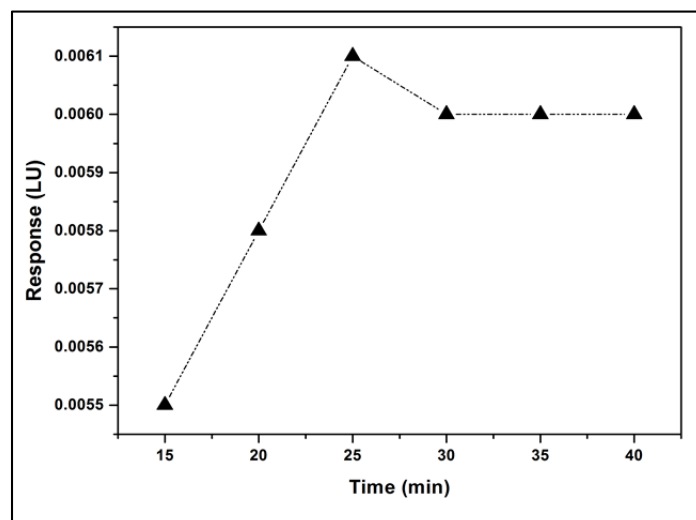
Figure 1. Effect of reaction time in the derivatization of 50 mg/L ethanolamine in synthetic wine. Response is referred to peak area in HPLC-FL analysis of derivatized amine.

Effect of initial NDA concentration.

Once identified the optimal reaction time, concentration of NDA necessary to obtain the highest derivatization yields (signal responses) was studied, applying the same detection procedure as detailed before. Studies involving BAs derivatization with NDA in biological human samples point out that concentration of $8·10^{-5}$ M NDA was used to derivatize up to 0.15 mg/L (corresponding to $8·10^{-7}$ M) histamine dihydrochloride [17]. Concentration of BAs in wines can achieve tens of mg/L. Our study was performed under precautionary conditions, considering concentrations of BAs in the range 50-500 mg/L in synthetic wine, which cause perceivable alterations of wine such as the reduction in overall wine aroma, observed with the formation of metallic, meaty or putrid aromas in wine. According to the BAs concentration levels studied, the tested NDA concentrations were 1.34 and 13.4 mM. Lower NDA concentrations are not advisable, especially for real samples, since derivatization of BAs could not be complete if more than one BA is present (see Figure 2).

Finally, the possibility to increase the derivatization efficiency of biogenic amines was investigated by evaluating the effect of the solvent in which NDA is dissolved. Previous published works reported that NDA could be dissolved both in acetonitrile or methanol, without detailing if a contribution of the solvent in the derivatizing reaction is present [17, 32].

Our study demonstrated that no statistically significant difference is observed when derivatizing with NDA dissolved in methanol or acetonitrile.
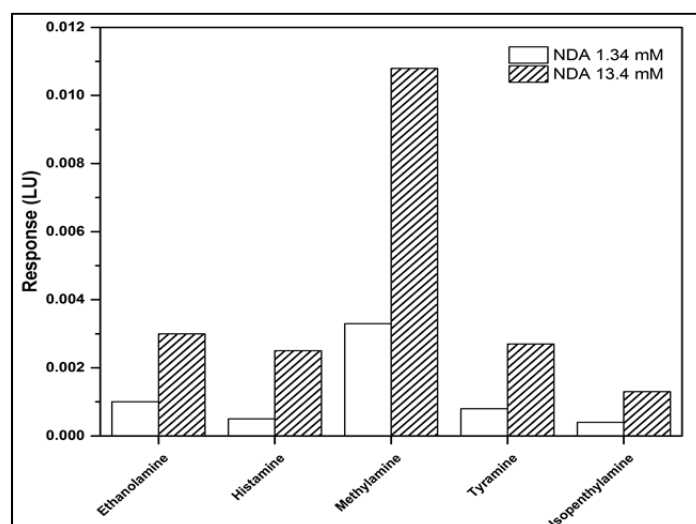

Figure 2. Response area for BAs in synthetic wine derivatized with two different concentration levels of NDA (1.34 and 13.4 mM). Analysis conditions are detailed in Experimental section.

Dabsyl derivatization

To optimize the derivatization of BAs, the effects of reaction time, temperature and DBS concentration on the derivatization performance were studied for 5.5 mg/L putrescine, as a model compound.

Effect of reaction temperature.

The DBS-putrescine peak area was monitored after heating for 21 min at temperatures ranging from 25 to 70 °C. It must be underlined that, at lower temperatures, dabsyl chloride remains undissolved. The highest reaction efficiency was obtained at 50 °C as shown in Table 1. Nevertheless, the response obtained at higher temperatures remains within the uncertainty of the experimental error. It should be remarked that at temperature of 50 °C, the efficiency of dabsylation decreases for concentrations of putrescine below 0.5 mg/L. For this reason, derivatization at 70 °C was chosen for further optimization steps.

Table 1. Effect of temperature on derivatization of putrescine with DBS.

T [°C]	Response [peak area]
25	290 ± 20
40	715 ± 50
50	830 ± 50
60	720 ± 50
70	760 ± 50

Effect of reaction time.

As shown in Table 2, the maximum efficiency for dabsylation reaction was achieved already after five minutes; longer derivatization times led to decreased efficiency (however within the limits of experimental error), which can be associated with degradation of the derivatization product.

Table 2. Effect of reaction time on derivatization of putrescine with DBS.

time [min]	Response [peak area]
5	970 ± 40
10	960 ± 40
21	900 ± 40

Effect of DBS concentration.

The effect of dabsyl chloride concentration on putrescine derivative peak area was studied for 400- and 3300-fold excess with respect to amine concentration, suggesting that higher excess of the reagent resulted in a 7.1-fold increase in dabsylation efficiency.

INDIRECT DETERMINATION OF BAs THROUGH ENZYMATIC RELEASE OF AMMONIA AND INDOPHENOL BLUE REACTION

During the TG enzymatic reaction with substrate N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) and biogenic amines, ammonia is released and detected by exploiting indophenol blue formation in the Berthelot reaction. Detection of structurally different biogenic amines (putrescine, cadaverine, histamine and tyramine) was investigated. Previously optimized Berthelot reaction [30] was used as colorimetric method for detection of biogenic amines based on indophenol blue formation.

Effect of buffers on TG reaction.

Optimum pH for TG is between 6 and 7, as specified by the producer. On the other side, recommended pH for indophenol formation is between 8-11.5 [33]. For this reason, a study on buffer influence on indophenol blue reaction in pH range 6-7 was necessary. Experiments were monitored at 650 nm by spetroctrophotometry, with phosphate- (pH 7), acetate- (pH 6) and Tris- (pH 7) buffers; results have shown that highest sensitivity of biogenic amine determination by TG and indophenol reaction is obtained in phosphate buffer at pH 7 (Figure 3). It should be remarked that TRIS buffer, usually suggested for TG enzymatic activity test protocols, was revealed incompatible with indophenol reaction.
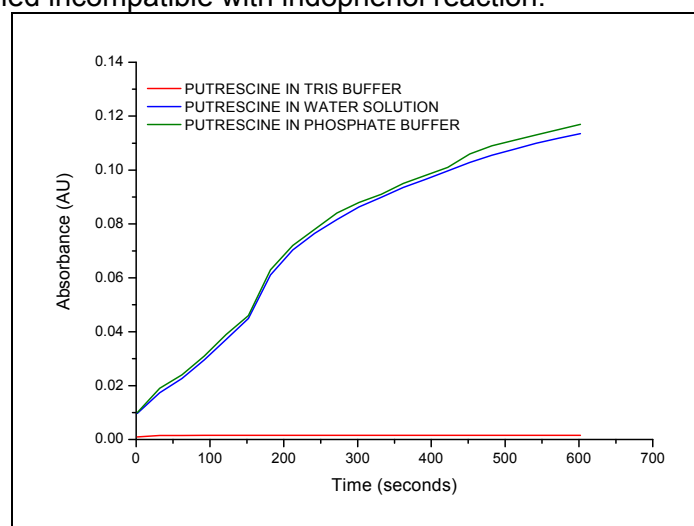

Figure 3. Effect of buffers on kinetics of indophenol formation using 2 mM putrescine as representative biogenic amine. Spectrophotometric detection was performed at 650 nm.

VALIDATION OF HPLC-NDA-FL AND HPLC-DBS-TLS METHODS AND COMPARISON WITH HPLC-NDA-FL AND HPLC-DBS-DAD

Validation of the novel methods was performed as suggested by European Union (EUR-FA Guide, Annex I) and IUPAC guidelines [34], evaluating the following performance parameters: linearity, limits of detection (LODs) and limits of quantification (LOQs) by analysing standard solutions of putrescine, cadaverine, histamine and tyramine in synthetic wine matrix. LODs and LOQs (see Table 3) were evaluated as LOD = 3 × SD_{xy}/b and LOQ = 10 × SD_{xy}/b (where SD_{xy} is the standard deviation of the response and b is the slope of the calibration curve) [35].

Table 3. Limits of Detections (LODs) and Limits of Quantitation (LOQs) for BAs obtained by HPLC-NDA-FL, HPLC-DBS-TLS and HPLC-DBS-DAD.

BAs	LOD (µg/L)			LOQ (µg/L)		
	NDA-FL	DBS-TLS	DBS-DAD	NDA-FL	DBS-TLS	DBS-DAD
PUT	73	3	15	219	11	50
CAD	55	4	23	165	14	77
HIS	27	11	85	81	40	282
TYR	66	10	86	198	34	288

The linearity of the methods was verified from LOQs to 1.2 mg/L, providing R^2 ranging from 0.985 to 0.997.

It is interesting to note that the detection limits obtained by the newly developed methods are about 10-fold (HPLC-NDA-FL) and 50-fold (HPLC-DBS-TLS) lower if compared with those obtained by CE with indirect UV detection [36] and almost equal (HPLC-NDA-FL) or lower (HPLC-DBS-TLS) to those obtained using direct analysis by CE coupled with mass spectrometric detection [37]. The LODs of the newly proposed methods were lower than those obtained by the official OIV method [38] based on OPA derivatization (i.e. LOD_{OPA} histamine= 60 µg/L; LOD_{OPA} tyramine= 77 µg/L).

VALIDATION OF ENZYMATIC METHOD

The performance of the enzymatic method coupled with TLS detection was initially verified by analyzing aqueous solutions with different concentrations levels of single biogenic amines. Results demonstrate acceptable recoveries (118-100 % for putrescine, 70 -133 % for cadaverine, 93-112 % for histamine, and 88-108 % for tyramine) and very low limits of detection. The linearity range is rather narrow (from LOQs to $2.5 \cdot 10^{-7}$ M) due to a TLS signal saturation effect observed for investigated solutions.

When compared to a previously reported method for BA determination by using TG [31], the LODs achieved in this work for standard solutions represent over 450-fold improvement. In addition to the TLS enhancement of sensitivity by 30 times for the 200 mW excitation power, such improvement in LOD is attributed also to 13.5-times higher extinction coefficient of indophenol at 650 nm compared to the extinction coefficient of NADH absorption maximum at 340 nm, which was related to concentration of BA in the method developed by Punakivi et al. [31]. However, matrix effects hinder the LODs in real samples, which were therefore found up to 3 times higher in the analyzed wines, as reported below (Table 4). Besides the significant improvement of the LODs, it should

be pointed out that for the previously reported TG method, two hours (reaction time) are required to achieve the indicated LODs, while the newly developed method here described requires just 10 min of reaction time, thus reducing the total time of analysis by 12 times.

Table 4. LODs and LOQs for batch mode determination of BAs in wine with TG and indophenol reaction on TLS detection unit.

BAs	LOD [µg/L]	LOQ [µg/L]
PUT	8	24
CAD	10	29
HIS	23	68
TYR	15	44

REAL SAMPLE ANALYSIS

Real samples (Slovenian white wines of Rebula variety) were analyzed to ascertain the presence of biogenic amines using HPLC-DBS-TLS since it resulted the best performing method developed according to what previously discussed. Samples were analyzed by applying the standard addition calibration, comparing the obtained concentrations with those obtained by the International method for analysis of biogenic amines in wine [29], i.e. OIV-MA-AS315-18, performed in our laboratory (Table 5). Typical chromatograms obtained for analysis of wine samples by HPLC-DBS-TLS are shown in Figure 4.

Table 5. Concentrations (mg/L) of BAs in wines determined by HPLC-DBS-TLS and HPLC-OPA-FL (official OIV method).

		PUT	CAD	HIS	TYR
Wine A	DBS-TLS	0.64±0.02	0.20±0.03	0.08±0.01	< LOD
	OPA-FL	0.59±0.13	0.20±0.11	< LOD	< LOD
Wine B	DBS-TLS	1.14±0.03	0.25±0.03	0.09±0.01	< LOD
	OPA-FL	1.10±0.23	0.15±0.08	< LOD	< LOD

For putrescine and cadaverine, the results obtained by the two HPLC approaches were in good accordance for both wine samples. Due to its higher sensitivity, the HPLC-DBS-TLS method enabled the determination of histamine as well, which could not be determined by the OIV method due to inadequate LODs.
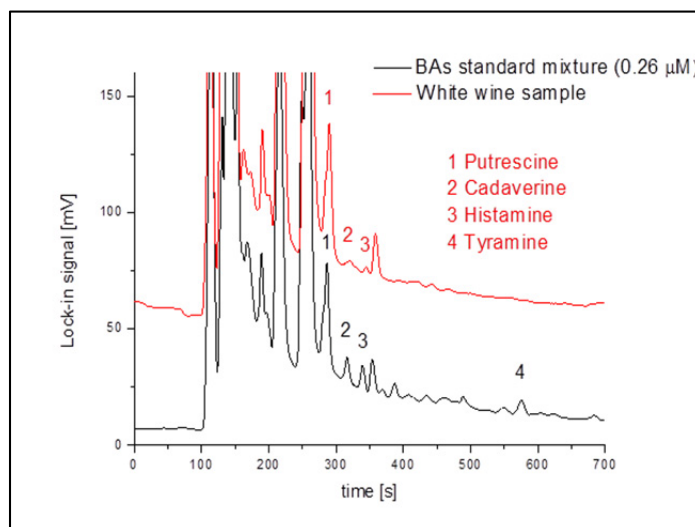
Figure 4. Typical chromatogram obtained for analysis of wine sample by HPLC-DBS-TLS

Rebula wines (one home made and one commercial) were additionally analyzed by the newly developed method based on indophenol reaction, through the standard addition procedure to confirm the applicability of the method. Synthetic wine was used as blank.
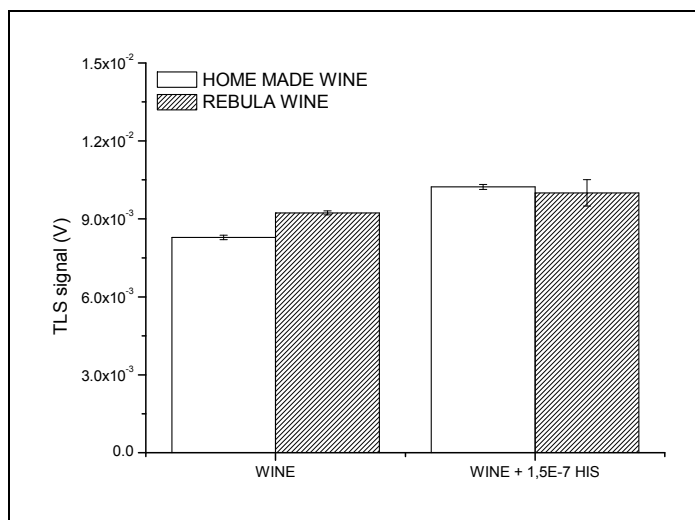

Figure 5. Signals of home made wine and Rebula wine witk TLS detection unit.

Since the assay is not specific for identification of single biogenic amines, it allows the determination of the total biogenic amines present. As shown in Figure 5, the presence of BAs in samples can be confirmed. Higher signal in case of Rebula wine (commercial), is due to higher concentration of biogenic amines.

The quantity of biogenic amines present in wines was expressed as equivalents of histamine, the only BA for which the maximum contamination level in wine is regulated.

Based on the standard addition of histamine ($1.5 \cdot 10^{-7}$ M) and on the difference of TLS signals from spiked and original wine samples, a concentration of BAs corresponding to $(6.4 \pm 0.3) \cdot 10^{-7}$ M which corresponds to 0.069 ± 0.003 mg/L equivalents of histamine were estimated for home-made wine, and $(1.8 \pm 0.9) \cdot 10^{-6}$ M concentration which corresponds to 0.2 ± 0.1 mg/L equivalents of histamine for commercial Rebula wine. These results indicate that the concentrations of BAs are in good agreement with values found by HPLC-OPA-FL, and also comparable (on the lower range) to the previously reported values (recalculated as histamine equivalents) in Greek wines [33]. Due to the high sensitivity of TLS method, even concentrations below 0.1mg/L can still be reliably detected.

CONCLUSIONS

In this work, naphthalene-2,3-dicarboxaldehyde (NDA) was tested for the first time for the analysis of BAs in wine samples by HPLC with fluorimetric detection. Optimal derivatizing and chromatographic conditions were successfully derived.

The method was validated in terms of linearity, limits of detection and quantification in a synthetic wine matrix. If compared to HPLC-OPA-FL method, the results showed that, despite slightly longer derivatization time (reaction time 25 min), the sensitivity of the NDA-FL method is greatly enhanced. It is worth to mention that the analysis time using NDA is about one fourth of the time needed with OPA.

The LODs were importantly decreased by HPLC-TLS using dabsyl chloride (DBS) as derivatizing non-fluorescing agent which provides up to 8.4-fold improvement compared to the HPLC-DAD applying the same chromatographic conditions. The newly developed method was tested on the analysis of BAs in white wine samples, finding good accordance with the results obtained by the standard OIV method for determination of BAs in wines. The newly developed HPLC-TLS method is therefore appropriate for determination of BAs in wine samples.

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High sensitivity of TLS also enables fast detection of BAs by a novel vanguard screening method developed in this work, which is based on enzymatic transformation of BAs into ammonia and further to indophenol which can be sensitively detected by TLS. In real wine samples, BAs can be detected at concentration levels of 10^{-7} M with over 10 times improved sample throughput compared to previously reported enzymatic methods.

EXPERIMENTAL SECTION

Chemicals

Biogenic amine standards studied were: putrescine·2HCl (PUT), cadaverine (CAD), bought from Sigma Aldrich (Seelze, Germany), histamine (HIS), from Panreac Applichem (Darmstadt, Germany), and tyramine (TYR), from Alfa Aesar (Karlsruhe, Germany). Dabsyl-chloride was from Alfa Aesar. All other standards and reagents were bought from Sigma Aldrich and were of analytical grade.

High-purity water (18.2 MΩ·cm resistivity at 25 °C), produced by a Thermo Scientific Barnstead NANOpure Water Purification Systems (Barnstead, Germany) was used for standard solution and eluent preparation.

Microbial transglutaminase (TG) (E.C. 2.3.2.13, 1670 nkat/g) was purchased from Ajinomoto Co. INC., Japan (Europe Sales GMBH, Hamburg, Germany) and N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) was purchased from Sigma-Aldrich.

For the Berthelot reaction, sodium salicylate (99.5 %) purchased from Sigma-Aldrich, sodium hydroxide from Riedel-de-Haën (Seelze, Germany), solution of sodium hypochlorite (Pejo, Slovenia), potassium sodium tartrate tetrahydrate (99 %) and manganese sulfate monohydrate (98 %) from Fluka (Seelze, Germany) were used.

Preparation of solutions

Derivatizing solutions

3 mM o-pthalaldehyde (OPA) solution was prepared by dissolving 20 mg OPA in 50 mL methanol (the solution must be stored in dark conditions and should be prepared daily). 13.4 mM naphthalene-2,3-dicarboxaldehyde (NDA) solution was prepared by dissolving 12.3 g NDA in 5 mL acetonitrile. Both derivatization reactions should occur at controlled basic pH, therefore a 0.1 M borate buffer (pH 10.5) was used. In case of NDA, borate buffer contains 0.09M KCN. 1.3 mM NDA was prepared by 10-fold dilution of 13.4 mM solution in 0.1 M borate buffer.

The dabsyl chloride (DBS) solution was prepared by diluting 40 mg of dabsyl chloride in 10 mL of acetone, followed by 15 min ultrasonic treatment (Iskra PIO, Sonis GT, Slovenia), to obtain the final 12.4 mM concentration.

Solutions for enzymatic reaction

Standard solutions of BAs were prepared in water and stored in glass vials at 4° C. Donor substrate (2 mM) N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) was prepared in water. The TG enzyme was dissolved in cold water to obtain enzyme activity 16.7 nkat/mL (1 U/mL). Solutions for indophenol reaction. Two mixtures of reagents were used for indophenol blue reaction [39]. Mixture A: 10 mL of sodium salicylate (1.5 M), 10 mL of sodium potassium tartrate tetrahydrate (30 g/L) and 2 mL of manganese sulphate monohydrate (2.5×10^{-3} M). Mixture B: 10 mL of sodium hydroxide (0.5 M) and 10 mL of commercial bleach (source of hypochlorite). Each mixture was freshly prepared daily.

Synthetic wine (HPLC-NDA-FL)

This sample (1 L) was prepared by dissolving 3.5 g tartaric acid in 120 mL ethanol and acidifying with HCl to pH 3.5. Stock solutions of biogenic amines were prepared in synthetic wine for NDA derivatization; their concentrations were 50.6 mg/L (0.83 mM) ethanolamine, 487 mg/L (2.64 mM) histamine·2HCl, 114 mg/L (1.70 mM) methylamine·HCl, 158 mg/L (0.90 mM) tyramine·HCl, 150 mg/L (1.70 mM) isopenthylamine, 118 mg/L (1.62 mM) butylamine.

BAs standards (HPLC-DBS-TLS

Standards were prepared as 2.5 mM solutions by diluting the hydrochloride salts in 0.1 M HCl, containing 0.2 % (w/v) 3,3'-thiodipropionic acid (TDPA, Merck, Germany). An appropriate volume of the stock solution (or 1 mL of white wine) was afterwards transferred in a 10 mL-beaker and the pH was adjusted to 8.2 with Na_2CO_3 buffer.

Derivatizing reactions

OPA: the reaction was performed according to the indications reported within the Compendium of International Methods of Analysis [38]. Derivatization of BAs is performed according to a two-steps reaction. In detail, in a flask, 500 µL of OPA solution are mixed with 500 µL of borate

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buffer and 150 µL of 2-mercaptoethanol. In another vial, 500 µL of the sample or synthetic wine solution containing biogenic amines at proper concentration are mixed with 500 µL of methanol; then 150 µL of this latter solution are spiked into solution in the first flask and, after a reaction time of 1 min, the solution is injected in the HPLC system.

NDA: 500 µL of the sample or synthetic wine solution containing biogenic amines are mixed with 200 µL of methanol, 50 µL of borate buffer containing potassium cyanide and 200 µL of NDA solution. The optimized reaction time was 20 min (see Results and Discussion section).

DBS-Cl: 500 µL of the BA (wine) buffer was transferred into an amber glass vial and 220 µL of the dabsyl chloride was added. The reaction mixture was heated in water bath at 70 °C for 5 min. Afterwards, 610 µL of the dilution solution, containing acetonitrile, eluent A and ethanol in the ratio 2:1:1 (v/v/v), was added to dissolve the remaining dabsyl chloride in the vial and heated at 70 °C for further ≈5 min.

HPLC mobile phases were prepared from the following solutions A: 0.05 M sodium acetate: tetrahydrofuran (96:4 v/v) and B: methanol, by gradient mixing as detailed in Table 6.

Table 6. Gradient conditions used for the separation of biogenic amines derivatized with NDA and OPA

NDA derivatization			OPA derivatization		
Time [min]	%A	%B	Time [min]	%A	%B
0	30	70	0	80	20
11	30	70	15	70	30
25	25	75	23	60	40
40	10	90	42	50	50
50	10	90	55	35	65
60	30	70	60	35	65
			70	80	20
			95	80	20

Experiments indicated that the presence of thetrahydrofuran (THF) in the mobile phase has two effects on the elution of BAs derivatized with NDA: it decreases the total time of the analysis and it increases the peak response (from 13 to 25% depending on BAs). The optimized total time of the elution for HPLC-NDA-FL is 25 min, about one fourth of the time needed to analyse BAs with OPA derivatization.

For dabsylation: The eluent was prepared as solvent A - solvent B composition of 35:65 % v/v. Solution A: $4.0 \cdot 10^{-2}$ M sodium acetate with 10 % (v/v) dimethylformamide and 0.23 % (v/v) triethylamine, adjusted to pH 5.0 with diluted acetic acid); solution B: acetonitrile, *t*-butylmethyl ether and water (87.5:10:2.5, v/v/v).

Enzymatic and Berthelot reaction

For determination of BAs, 1 mL of CBZ-Gln-Gly, 1 mL of enzyme solution and 1 mL of BA of interest were introduced into a 10 mL-flask. Afterwards, 1 mL of each mixture (A and B) was added to the enzymatic mixture and diluted with buffer and acetonitrile (total 25% v/v) to obtain the final volume of 10 mL. After 10 minutes, 2 mL of the solution was transferred into a quartz cuvette (batch mode measurement) and introduced in a TLS cuvette holder.

White wine samples were tested for the presence of studied BAs, all experiments were done with phosphate-diluted wine samples to reach basic pH.

Instrumentation

HPLC-FL system

An Agilent 1100 chromatograph (Agilent, Santa Clara, CA, USA) equipped with a Kinetex EVO (250 mm×4.6 mm I.D., 5 µm particle size) with a 4 mm×3 mm I.D. guard column (Phenomenex), a 10µL-injection loop and a fluorescence detector was used. Eluent flow rate was set at 1.0 mL/min, while fluorescence detector was set at 356 nm (absorbance wavelength) and 445 nm (emission wavelength). Chromatographic data were handled by OriginPro 8.5 software, without any data correction or smoothing.

HPLC-TLS system

The dual-beam thermal lens spectrometer used in this work is schematically presented in Figure 6. It consists of a Kripton laser (Innova 300C, Coherent, USA) operating at 413 nm (35 mW–150 mW), which provides the pump beam, and a He-Ne probe laser (Model 1103P, Uniphase, USA) of 2 mW output power (632.8 nm). The pump beam was modulated with a mechanical chopper (300CD, Scitec Instruments, UK) at 28 Hz and focused onto the sample cell with a 100 mm focal length lens (L1). The probe beam was focused using two lenses with focal lengths of 100 mm (L2) and 60 mm (L3). To obtain a collinear propagation of pump

and probe beams, a dichroic mirror (T:633 nm, R: 410 nm) was used. Thermal lens signal was detected using an amplified photodiode detector (Thorlabs, USA) equipped with a 633 nm interference filter (Melles Griot, USA) and connected to a lock-in amplifier (SR830 DSP, Standford Research, USA, 1 s time constant), which was further connected to a PC for data treatment and storage. The chromatographic system consisted of a HPLC pump (Smartline pump 1000, Knauer, Germany), a metal free manual injection valve (Knauer, Germany) for liquid chromatography equipped with 10 µL injection loop. The column was the same as for HPLC-FL system. Detection was performed in an 8 µL flow-through cell (Hellma, UK) with 10 mm optical path length.

TLS detection unit for indophenol blue measurements

The TLS detection unit was identical to the one described for HPLC-TLS system, with the only difference that the Kr-laser was operating at 647 nm (200mW) and the probe beam source was a He-Ne laser operating at 543.5 nm (5mW, MellesGriot, Carlsbad, California, USA). The dichroic mirror and the interference filter were replaced accordingly to enable transmission at 543.5 nm and reflection of the pump beam. The flow-through cell is replaced with a 10 mm quartz cuvette.
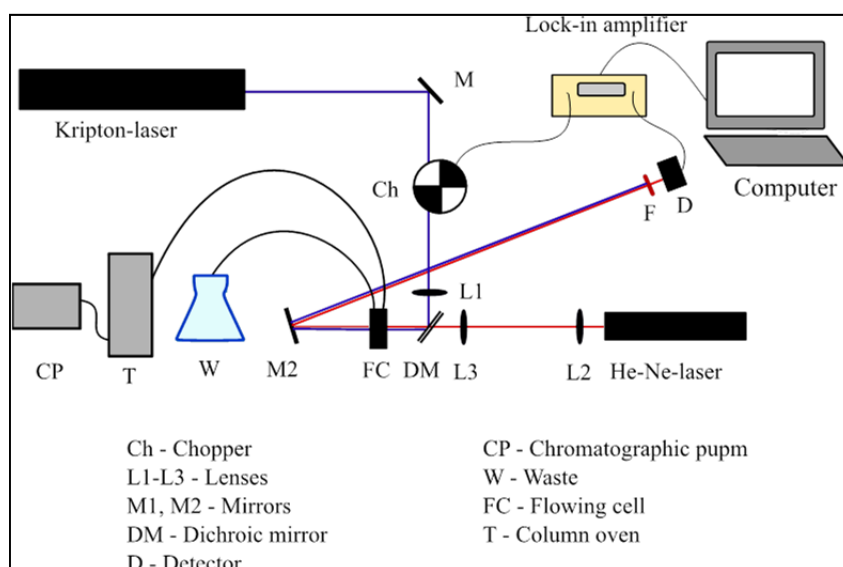

Figure 6. Schematic presentation of a HPLC-TLS system

Wine samples

Wine samples of Rebula variety were from producers in the Obalno-Kraška wine growing region in Slovenia. All wines were produced using the classic procedures of crushing, pressing, fermentation, filtration and bottling.

Samples were stored in 0.75 stoppered dark glass bottles, and kept at 4 deg. C until analysis. For the analysis, 2 mL of the sample was filtered through a 0.45 nylon syringe filter to remove suspended solids. 500 μL aliquot of the filtrate was then derivatized with OPA and DBS as described above, and injected for HPLC analysis.

ACKNOWLEDGEMENTS

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