Journal of Chromatography B, 904 (2012) 135–139

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Determination of selected biogenic amines in red wines by automated on-line combination of capillary isotachophoresis–capillary zone electrophoresis

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ARTICLE INFO

Article history:
Received 16 November 2011
Accepted 16 July 2012
Available online 25 July 2012

Keywords:
Capillary isotachophoresis
Capillary zone electrophoresis
Histamine
Tyramine
2-Phenylethylamine
Red wine

ABSTRACT

A screening analytical method based on an automated on-line combination of capillary isotachophoresis–capillary zone electrophoresis (cITP–CZE) in hydrodynamically closed separation system, equipped with photometric detection at 280 nm, was developed for a routine determination of the selected biogenic amines, namely histamine, 2-phenylethylamine and tyramine, in red wines. The evaluated limits of detection (LODs) were 0.35 mg L^{-1} for histamine, 0.33 mg L^{-1} for 2-phenylethylamine and 0.37 mg L^{-1} for tyramine. The repeatability of the migration time and peak area for histamine were 1.1% and 2.6%, respectively, for 2-phenylethylamine 0.7% and 2.0%, respectively, and for tyramine 0.8% and 2.1%, respectively. The method recoveries were 92.1% for histamine, 96.4% for 2-phenylethylamine and 95.5% for tyramine. The developed automated cITP–CZE–UV method was applied for the determination of histamine, 2-phenylethylamine and tyramine in seven red wine samples originating from Czech Republic.

1. Introduction

Biogenic amines (BAs), namely histamine, 2-phenylethylamine and tyramine (chemical structures are shown in Fig. 1), are produced in wine mainly during alcoholic and malolactic fermentation of amino acids such as histidine, phenylalanine and tyrosine, respectively [1,2]. Their concentrations in wine also depend on the concentrations of amino acids in the must, the degree of maturation of the grape and the fermentation conditions [3]. Wine samples containing high concentration levels of BAs (typically several tens and hundreds of mg kg^{-1}) can cause health problems. Toxic levels of BAs depend on the tolerance of the individual human being [4]. Histamine, 2-phenylethylamine and tyramine are known to be associated with several diseases, e.g., headache, eczema, hot flash, nausea and hypertension. The content of selected BAs in food can be monitored for two reasons – the first one is their potential toxicity and the second one is the fact that the quantity of BAs can be used as the food quality marker [5–7].

Up to date, several analytical methods were used for the determination of histamine, 2-phenylethylamine and tyramine in wine. For example capillary electrophoresis [8,9], capillary isotachophoresis [10], gas chromatography [11,12] and thin layer chromatography [13]. The mostly used separation method was liquid chromatography with spectrophotometric [7] or fluorimetric detection [14]. In the case of fluorescence detection, derivatization of the analytes must be performed and the derivatization step can be time and labor consuming [14,15].

To increase the sensitivity of the final analytical method usually the off-line preconcentration of the analytes has to be performed before the separation. The preconcentration of histamine, 2-phenylethylamine and tyramine from the food matrices was carried out, for example, by solid phase extraction [16] and liquid–liquid extraction [17]. The using of off-line preconcentration step can be time consuming and, moreover, there is a high risk of the analyte(s) loss [15].

Capillary zone electrophoresis (CZE) is a powerful analytical separation technique suitable for the separation of charged analytes from various types of matrices. The main advantages of CZE are high separation efficiency, fast analysis and low solvent, electrolyte and sample consumption [18]. On the other hand, the disadvantages of CZE are the low concentration sensitivity of spectrophotometric detection, which is the mostly used detection technique in CZE and the lower reproducibility of migration times in the comparison with liquid chromatography [19]. The sensitivity of spectrophotometric detection can be improved, for example, by on-line sample preconcentration directly in the separation capillary [20] and/or by on-line coupling of CZE with capillary isotachophoresis (cITP).

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http://dx.doi.org/10.1016/j.jchromb.2012.07.018

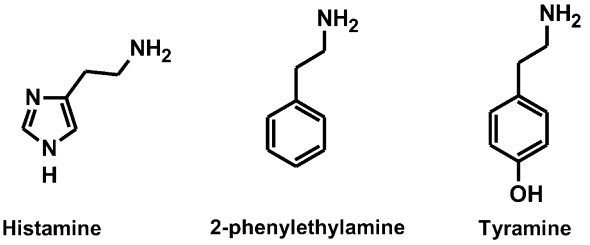

Fig. 1. Chemical structures of histamine, 2-phenylethylamine and tyramine.

The main advantage of cITP is an on-line preconcentration of selected analytes and the possibility to clean up the sample before the CZE step when column-coupling configuration of separation system is used [21,22].

Column-coupling system, developed by Everaerts et al. [21], is using hydrodynamically closed system with two columns, which are filled independently. This configuration was proved to be robust without losing analytes during its transportation process from the first to the second column in both manually operated CE analyzer [23] as well as in automated CE analyzer [24]. The proper combination of the ITP and CZE electrolyte systems and time of switching of the direction of the driving current in the bifurcation block are necessary to achieve ITP clean up the sample before CZE separation [25–27].

The poor reproducibility of migration times in CZE can be caused by the electroosmotic flow (EOF), which flow through the separation capillary and/or by the hydrodynamic flow [27]. The EOF can be suppressed either by using the separation capillary made of the inert material and/or by adding a suitable additive into the separation electrolyte. The hydrodynamic flow can be suppressed by using a separation system hydrodynamically closed, e.g., by semi-permeable cellophane membrane, which can separate the electrolyte vessels from the electrolytes inside the separation capillary [21].

The aim of this work was to develop analytical method based on an on-line coupling of cITP with CZE and UV detection (cITP–CZE–UV) for the separation and determination of selected biogenic amines in red wine samples. The main advantages of the proposed cITP–CZE–UV combination are an on-line preconcentration of selected analytes in the ITP step, the high separation efficiency of the CZE separation and the fact that the selected BAs, i.e., histamine, 2-phenylethylethylamine and tyramine, can be detected by selective photometric detection without the derivatization step [28,29].

2. Materials and methods

2.1. Apparatus

The separations were carried out by using an automated electrophoretic analyzer EA 202 A (Villa Labeco, Spišská Nová Ves, Slovak Republic) equipped with a column-coupling separation system. The ITP column (800 μm I.D., 140 mm of a total length) for an on-line preconcentration of analytes was provided with a contactless conductivity detector and served for an on-line preconcentration of analytes. A CZE column (300 μm I.D., 160 mm of a total length) was provided with a contactless conductivity detector and a photometric detection cell coupled to the photometric detector K-2000 (Knauer, Berlin, Germany) via the optical fibers. Both columns were made of fluorinated ethylene-propylene copolymer. The driving currents were 200 μA in the ITP column and 80 μA in the CZE column, respectively. The samples were injected using the Triathlon autosampler (Spark Holland, Emmen, Netherlands) into a 30 μL internal sample loop of the injection valve of the electrophoretic analyzer. To control the separation process, to acquire data from the detectors and to evaluate and process experimental data, WinACES, version 1.5 (D. Kaniansky Consulting, Bratislava, Slovak Republic) program was used.

Table 1
The composition of electrolyte systems used for on-line cITP–CZE–UV separation.

	Leading electrolyte	Terminating electrolyte	Background electrolyte
Cation	K^+	$EACA^+$	$GABA^+$
Concentration (mmol L^{-1})	10	10	25
Counter ion	MES^-	$Acetate^-$	$Acetate^-$
Concentration (mmol L^{-1})	20	20	50
EOF suppressor	HEC	HEC	HEC
Concentration (%, w/v)	0.10	0.05	0.10
pH	6.0	4.3	4.1
Solvent	Water	Water	Water

2.2. Chemicals

Potassium hydroxide, 2-(*N*-morfoline)ethanesulfonic acid (MES), γ-aminobutyric acid (GABA), ε-aminocapronic acid (EACA), tetrabutylammonium chloride (TBA^+Cl^-), tris(hydroxymethyl)aminomethane (TRIS), bis-amino-tris(hydroxymethyl)methane (BIS-TRIS), β-alanine, acetic acid, hydroxyethylcellulose (HEC), magnesium sulfate, hydrochloric acid, histamine and tyramine were obtained from Sigma (Sigma, St. Louis, MO, USA). 2-phenylethylamine was purchased from Fluka (Buchs, Switzerland). All chemicals used were of analytical grade or additionally purified by the usual methods. Deionised water was obtained from the 2-steps purification system PRO-PS (Labconco, Kansas City, UK, USA) and Simplicity (Millipore, Molsheim, France).

2.3. Preparation of working solutions and wine samples

The electrolytes were prepared by dissolving appropriate amounts of chemicals in deionised water. All working solutions were filtered before the use through a 0.45 μm membrane micro-filter (Millipore). All electrolytes contained hydroxyethylcellulose to suppress the EOF. The composition of final electrolyte system used for the cITP and CZE separation is given in Table 1.

The stock solutions of BAs standards were prepared by dissolving of histamine, 2-phenylethylamine or tyramine in methanol with an addition of hydrochloric acid to reach the concentration of each analyte to be 1 g L^{-1}. The working standard solutions were prepared by diluting of the stock solutions of BAs standards in deionised water. A magnesium sulfate at the concentration level at 0.5 mmol L^{-1} was added to the samples to suppress the adsorption of analytes on a labware and/or the inner walls of the separation system. The peaks were identified by spiking a standard mixture with the standards of BAs.

Red wine samples were purchased from a local supermarket. Wine samples were prepared by the dilution in the deionised water from 5 to 10 times. The diluted samples were filtered through a 0.22 μm membrane micro-filter (Millipore) before the filling to the vials of the autosampler.

3. Results and discussion

3.1. cITP–CZE–UV method optimization

Tyramine, 2-phenylethylamine and histamine have all the basic characters and their pK_a values are 10.67 [30], 9.90 [30] and 9.75 [31], respectively. Hence, their preconcentration and the final separation were carried out in a cationic mode in the weakly

acidic electrolytes. Firstly, the suitable leading and terminating electrolytes for the ITP step – in which the on-line sample preconcentration of analytes was realized – were chosen.

For the reason that sodium ions are naturally present in the wine samples, this ion was firstly studied as a potential leading ion. Unfortunately the effective mobility of histamine was higher than the effective mobility of sodium ion. To decrease the effective mobility of histamine the influence of pH in the range from 4.0 to 5.5 (the changes in the degree of protonation of histamine) was checked. However, the histamine ion was faster than sodium ion under the all studied experimental conditions. As a next step the potassium ion was studied as the leading ion. The effective mobilities of tyramine, 2-phenylethylamine and histamine under studied conditions were lower than the effective mobility of this ion. Therefore potassium ion was chosen as leading ion for the ITP step.

In the next step a suitable composition of the termination electrolyte was chosen. Several ions, i.e., $TRIS^+$, TBA^+ and $EACA^+$ were studied as the potential terminating ions. Unfortunately the effective mobility of $TRIS^+$ was higher than the effective mobility of tyramine under the studied conditions (the different pH – in the range from 5.7 to 7.1 – and composition of electrolyte). The solubility of TBA^+Cl^- in water is very low, thus it was not possible to prepare the terminating electrolyte with adequate concentration without the using of organic additives. However the higher concentration of organic additives in the electrolyte can generate the high separation voltage and the Joule heating. The Joule heating can cause, for example, a formation of bubbles and a collapse of the analysis. The effective mobility of $EACA^+$ was (by using the electrolyte with pH 4.3) lower than effective mobilities of studied BAs and for this reason $EACA^+$ was chosen as the terminating ion for the ITP step. The final compositions of the electrolytes used for the ITP step are presented in Table 1. The ITP separation of model mixture of tyramine, 2-phenylethylamine and histamine is presented in Fig. 2.

Afterwards the choice of suitable background electrolyte for the CZE separation step was provided. During the method optimization several background electrolytes with different composition, concentration and pH values were studied, e.g., β-alanine$^+$, $GABA^+$, $TRIS^+$ and $BIS\text{-}TRIS^+$ as co-ions and acetate$^-$ and MES^- as counterions. The best separation was obtained with the background electrolyte presented in Table 1.

Subsequently a suitable detection wavelength (in the UV range) was chosen for the detection of BAs analytes. The detection was carried out at 200, 220, 254 and 280 nm. The best S/N ratio was obtained at 280 nm wavelength. It can be assumed that a less number of compounds (in a real sample) absorb a light at this wavelength in the comparison with the other potentially used wavelengths. The CZE separation of model mixture of tyramine, 2-phenylethylamine and histamine is presented in Fig. 3.

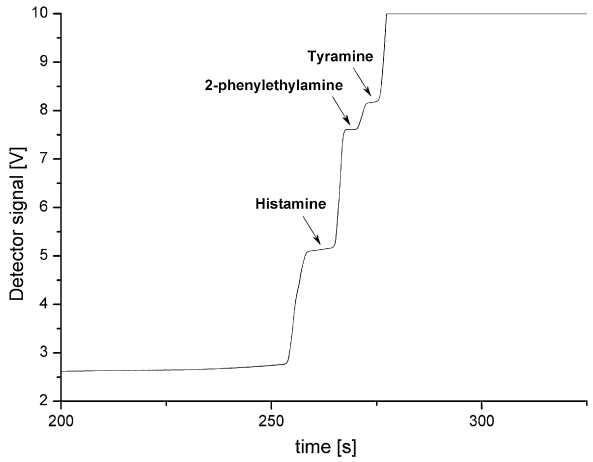

Fig. 2. The ITP isotachopherogram (contactless conductivity detector) of the standard mixture of histamine, 2-phenylethylamine and tyramine (the concentration of each analyte was 0.2 mg L^{-1}). For the composition of electrolytes, see Table 1.

Finally, the preconcentration factor of cITP step was calculated (from the standard solutions of biogenic amines). For the

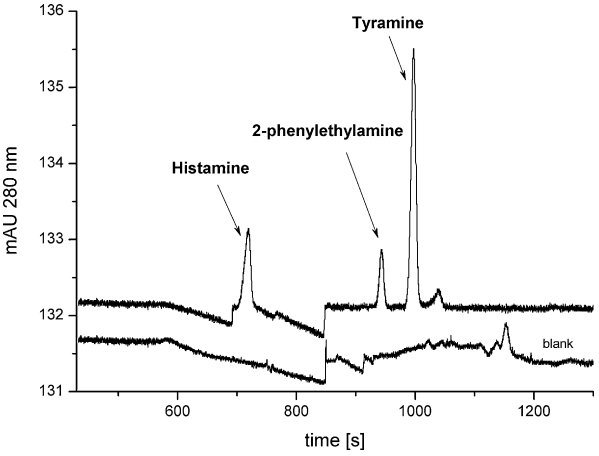

Fig. 3. The CZE electropherogram (from the cITP–CZE–UV separation) of the standard mixture of histamine, 2-phenylethylamine and tyramine (the concentration of each analyte was 0.5 mg L^{-1}). For the composition of electrolytes, see Table 1.

Table 2
The validation parameters of the developed cITP–CZE–UV method.

	Histamine	Tyramine	2-phenylethylamine
Limit of detection (mg L^{-1})[a]	0.35	0.37	0.33
Limit of quantification (mg L^{-1})[a]	0.52	0.55	0.49
Regression equation	$y = 1680.9x - 71.49$	$y = 3757x - 332.18$	$y = 758.3x - 67.93$
r	0.9997	0.9997	0.9998
r^2	0.9995	0.9995	0.9996
N[b]	13,500	22,620	17,800
H (μm)[c]	11.70	7.10	9.00
Repeatibility	2.60	2.10	2.00
Recovery (%)[d]	92.10	95.50	96.40
Accuracy (RE) (%)	−7.90	−4.50	−3.60
RSD_{tm} (%)[e]	1.10	0.80	0.60

[a] LODs and LOQs were calculated by QC Expert 2.5 (IUPAC method).
[b] The values were calculated by term: $N = 5.545 (t_R/w_{1/2})$.
[c] The values were calculated by term: $H = L/N$.
[d] The values are related to the concentration 1 mg L^{-1}.
[e] The values are related to the concentration 0.2 mg L^{-1} and were calculated from five replicate measurements.

Table 3
The comparison of developed cITP–CZE–UV method with previous reported methods used for the determination of biogenic amines.

	cITP–CZE–UV	CZE–UV	CZE–CCD	HPLC-UV/fluorescence det. (FD)	HPLC–FD	GC–MS
Separation time (min)	<30	<9	<10	<20	<40	<12
Total analytical time (min)	35	15	15	35	45[a]	18[a]
Limit of detection	0.35 mg L^{-1} (HA) 0.37 mg L^{-1} (TA) 0.33 mg L^{-1} (PEA)	2.0 mg kg^{-1} (HA) 6.0 mg kg^{-1} (TA)	0.34 mg L^{-1} (HA) 0.69 mg L^{-1} (TA)	UV: 0.5 mg kg^{-1} (HA) 1.5 mg kg^{-1} (TA) FD: 1.0 mg kg^{-1} (HA)	0.07 mg L^{-1} (HA) 0.03 mg L^{-1} (TA) 0,04 mg L^{-1} (PEA)	About 0.01–0.03 mg L^{-1}
Reagent consumption (ml)[b]	100[c]	10[c]	50	350[d]	500[d]	–
References	Developed method	[17]	[8]	[17]	[7]	[11]

[a] Does not include the derivatization step of analytes.
[b] Approximate amount related to 10 analysis.
[c] Water-based electrolytes.
[d] Organic-based mobile phases.

calculation of the preconcentration factor, the comparison (the ratio) of LODs obtained by the CZE–UV method and LODs obtained by the cITP–CZE–UV method was used. The achieved preconcentration factors of the ITP step were about 4500 for all analytes and allowed to improve LODs and LOQs for the separation of selected biogenic amines.

3.2. Method validation

The calibration curves of histamine, 2-phenylethylamine and tyramine were constructed from peak areas using six calibration solutions in the range from 0.2 to 10.0 mg L^{-1} (calibration points: 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 mg L^{-1}). Each calibration point was measured seven times.

Limits of detection (LODs) and limits of quantification (LOQs) were evaluated from calibration curves by using the statistical software QC Expert 2.5 (Trilobyte Statistical Software, Pardubice, Czech Republic). To the calculation of LODs and LOQs the IUPAC method was used. The calculating of LOD (lowest concentration of analyte, which can be detected) by IUPAC method is based on the theory of hypothesis testing and the probabilities of the false positives (α) negatives (β). LOQ (lowest concentration of analytes, which can be quantified) is by IUPAC method defined as specified value for the relative standard deviation [33].

The method recovery values were calculated from the individual regression equations of analytes. The calculated values of LODs, LOQs and method recoveries are presented in Table 2.

Precision was evaluated according to the ICH guideline [32] as the repeatability which is expressed via relative standard deviation of peak areas measured within the concentration range of calibration line. Evaluated repeatability is acceptable for all BAs studied (for details see Table 2).

Good linearity of the calibration lines is indicated by the values of correlation coefficient (r) and coefficient of determination (r^2). Accuracy (expressed via relative error, RE) of cITP–CZE method was evaluated according to the ICH guideline [32] using the recovery of BAs at three concentration levels. Recoveries of all BAs were slightly below 100% and RE values calculated from these values indicated very good accuracy of the proposed cITP–CZE method (for details see Table 2).

The separation efficiency was obtained due to the number of theoretical plates (N) and the height of equivalent to a theoretical plate (H). These parameters were calculated for each analyte. The values of N were in the range from 13,500 to 22,600 and the values of H were in the range from 7.10 to 11.70 μm. The calculated values of N and H indicate sufficient separation efficiency for all analytes.

3.3. Analysis of red wine samples

The developed on-line cITP–CZE–UV method was used for the determination of histamine, 2-phenylethylamine and tyramine in seven red wine samples from Czech Republic. The selected analytes were determined in the three varieties of red wines, namely Zweigeltrebe, Cabernet-Sauvignon and Frankovka.

Firstly the analysis of diluted and filtered wine samples was provided. The CZE separations of two selected real samples are shown in Fig. 4. The quantification of histamine, 2-phenylethylamine and tyramine in wine samples was performed by using the method

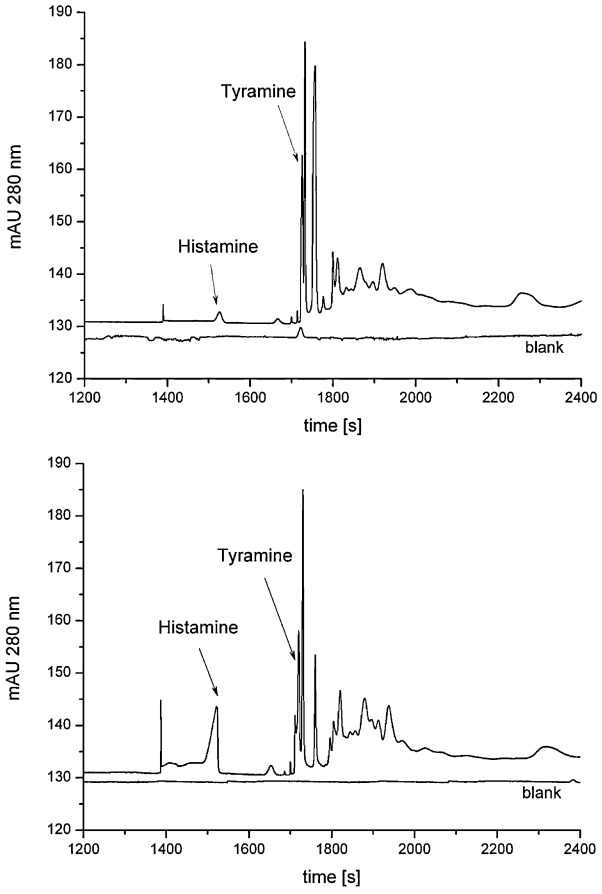

Fig. 4. The CZE electropherograms (from the cITP–CZE–UV separation) of red wine samples: (A) 7 x diluted sample and (B) 5 × diluted sample. For the composition of electrolytes, see Table 1.

of standard additions (additions of BAs at 0.2, 0.4 and 0.6 mg L^{-1} concentration, respectively).

The determined concentrations of histamine and tyramine in the analyzed red wine samples were in the range from 2.0 to 7.0 mg L^{-1} and from 1.5 to 2.5 mg L^{-1}, respectively. The average concentration was 5.3 mg L^{-1} for histamine and 1.8 mg L^{-1} for tyramine. 2-phenylethylamine was not found in the selected red wine samples.

The matrix effect was determined by the comparison of calibration curve slopes of the matrix standards (red wine spiked by standard mixture of BAs) and the aqueous standards (water spiked by mixture of BAs). The matrix effect of the developed cITP–CZE method is not significant for the reason that calibration curve slopes are not statistically different ($\alpha = 0.05$).

4. Conclusion

The automated on-line cITP–CZE–UV method for the separation and determination of tyramine, 2-phenylethylamine and histamine was developed. Potassium ion served as leading ion and ε-aminocaproic acid served as terminating ion in ITP step, while γ-aminobutyric acid served as background ion in CZE step. The analytical characteristics of the proposed method, namely limits of detection and quantification, method recoveries, the values of repeatability of the migration time and peak area, were evaluated. The evaluated limits of detection were below 0.4 mg L^{-1} level what were sufficient for monitoring of selected biogenic amines in the red wine samples.

In the comparison with previous methods (chromatographic and electrophoretic) [6,14,17] used for the determination of selected biogenic amines, presented method provides comparable LODs and LOQs. Although in some cases presented methods [7,11] give lower LODs and LOQs, they use the additional sample preparation step such as derivatization of analytes. That could be non-quantitative and time consuming procedure. Difficulties of derivatization pretreatment include, e.g., forming unstable derivatives and presence of interfering peaks (corresponding to the reagents used for the derivatization).

The analysis times of developed method and previous methods are comparable, however do not included sample pretreatment (e.g. preconcentration or/and derivatization of analytes) which is not necessary for the on-line cITP–CZE–UV method. Moreover the proposed method provides lower reagents consumption (mainly the consumption of organic solvents) what makes it environmentally friendly. For more details see Table 3.

The developed automated cITP–CZE–UV method was successfully applied for the determination of tyramine, 2-phenylethylamine and histamine in seven red wine samples. The range of concentration for histamine and tyramine found in wine was from 2.0 to 7.0 mg L^{-1} and from 1.5 to 2.5 mg L^{-1}, respectively. The measurements carried out on the red wine samples do not detect the presence of 2-phenylethylamine. The proposed automated cITP–CZE–UV method can be used for routine control of biogenic amines in the red wine samples.

Acknowledgements

This work was supported by a grant from the Slovak Research and Development Agency (No. VVCE-0070-07), the grants of Slovak Grant Agency, Nos. 1/0882/09 and 1/0546/10, the Operational Program Research and Development for Innovations–European Regional Development Fund (project CZ.1.05/2.1.00/03.0058 of the Ministry of Education, Youth and Sports of the Czech Republic) and the Operational Program Education for Competitiveness–European Social Fund (project CZ.1.07/2.3.00/20.0018).

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