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Determination of biogenic amines in wine by multidimensional liquid chromatography with online derivatisation

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An online multidimensional liquid chromatographic system was developed for online clean-up, derivatisation and separation of biogenic amines in wines. The system consists of a cation-exchange precolumn, a derivatisation coil and an analytical column, and a column-switching valve. The entire system can be easily automated. The method proved to be quantitative and sensitive. Limits of detection were below 0.05 mg l^{-1} for all amines and linearity was preserved over the tested range (0.05–15 mg l^{-1}). The method was applied to the analysis of red wines of different origin.

Introduction

Biologically active amines are major determinants of the quality of wines. More than 20 amines have been identified in wines, but the levels are typically low. Levels ranging from a few mg l^{-1} to about 50 mg l^{-1} have been reported.[1,2] Physiologically, the most important amines are histamine, tyramine, phenylethylamine, cadaverine, putrescine and isoamylamine. Biogenic amines are formed primarily from the decarboxylation of amino acids. In wine their formation has been associated with malolactic acid fermentation, in which malic acid is degraded partially to lactic acid under the influence of diverse organisms. The formation of amines depends on factors such as the existence of strains capable of decarboxylising amino acids and on the concentration of amino acids. Favourable bacterial microflora in the winery enhances amino formation, while a vinification operation that prolongs contact of the wine with the lees increases the concentration of amino acids.[1–3]

At present, the amines in wines and other sample matrixes are determined mainly by liquid chromatography, although other chromatographic techniques and capillary electrophoresis are also available.[1,3–11] Practically all the analytical techniques require pretreatment of samples owing to the complexity of the wine matrix and the low concentrations of the amines. Sample clean-up for wines by liquid–liquid extraction or solid-phase extraction has been applied to remove interfering compounds, such as amino acids. Because the aliphatic biogenic amines do not possess chromophore groups strongly absorbing in the UV/VIS region, they have typically been derivatized to obtain UV active or fluorescing derivatives. Pre- or postcolumn or even on-column derivatisation has been applied.[1,3–8,10,11] The most common derivatisation reagents are *o*-phthalaldehyde (OPA), dansyl chloride and dabsyl chloride.[1,3–8,10,11]

Multidimensional liquid chromatography (LC-LC) has been successfully used in the analysis of beverages and biological and environmental samples and other such complex samples.[12–14] The goals in LC-LC are to increase chromatographic resolution, selectivity and sensitivity, to enrich the analytes and to remove disturbing matrix compounds, as well as to protect the analytical column and detector. Often, no previous sample pretreatment is required.

In this study, a method was developed in which the sample clean-up, derivatisation and separation of amines in wine were done in a closed online system. The idea was to combine multidimensional liquid chromatography and online derivatisation. The system consisted of a cation-exchange precolumn, a derivatisation coil and an analytical column, and a column-switching valve. Study was made of parameters affecting each step of analysis such as eluent composition and flow rate, clean-up efficiency, derivatisation conditions, linearity, and limits of detection and quantification. The method was applied to the analysis of amines in red wines of different origin.

Experimental

Chemicals and reagents

Acetonitrile and 1-octane were HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ, USA) and Merck (Darmstadt, Germany), respectively. Methanol was from Riedel-de Haën (Seelze, Germany). Distilled water was de-ionised. NaCl, KCl, and boric acid were from Merck. Histamine, putrescine and mercaptoethanol (ME) were from Sigma (St. Louis, MO, USA); tyramine was from Merck; cadaverine, isoamylamine, phenethylamine, methylamine, ethylamine, hexylamine, spermidine and *o*-phthalaldehyde (OPA) were from Fluka (Buchs, Switzerland).

Samples

Wine samples were diluted 1 : 10 with deionised water, internal standard (hexylamine) was added and the sample was filtered through 0.45 μm filters (Gelman Sciences, Ann Arbour, MI, USA). White wine was used as a blank matrix. Red wines of different origin were analysed.

Liquid chromatography

The LC-LC system with online derivatisation system is presented in Fig. 1. The liquid chromatograph was a Hewlett-Packard 1090 system (Waldbronn, Germany). The pre-column was a 10 mm × 2.1 mm id SCX cation exchange column packed with 10–15 μm particles and the analytical column was a 150 mm × 3.0 mm id XTerra C18 column packed with 3.5 μm particles (Waters, Germany). The column-switching valve was

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a manual 6-port valve (Valco Instruments Co. Inc., Houston, TX, USA). Two Jasco pumps (Jasco, Japan) were used for delivering the washing buffer of the precolumn (Pump 1) and the derivatisation reagent (Pump 2). The reagent was mixed with the effluent from the precolumn in a T-piece and the reaction took place in a 250 mm × 0.25 mm id PEEK coil. A short 5 cm × 2.1 mm id column packed with 5 µm C18 particles (Asahipak, Japan) was used on front of the reagent pump to give back-pressure. A Waters Model 420-AC fluorescence detector with excitation and emission wavelengths of 350 nm and 445 nm, respectively, was used for detection.

Two buffers were used, one for the gradient elution, and the other one for the derivatization mixture. The buffer 1 consisted of 20 mM boric acid at pH 8.2 with 2% NaCl. A 20 mg portion of *o*-phthalaldehyde (OPA) and 1.5 ml of mercaptoethanol (ME) were dissolved in 5 ml of methanol and 1.5 ml of this solution was then dissolved in 23.5 ml of 0.4 M boric acid buffer (buffer 2) at pH 10.5. The final derivatisation reagent consisted thus of 6 mg of OPA and 0.45 ml of ME, corresponding concentrations of 240 mg l^{-1} for OPA and 20 mg l^{-1} for ME. The reagent was prepared daily.

Analysis procedure

Deionised water was used for sample clean-up with a flow rate of 0.7 ml min^{-1} (Pump 1 in Fig. 1). The sample was injected through a 980 µl loop to the pre-column and the precolumn was washed for 3.4 min to remove the majority of matrix compounds directly to waste. After washing, the valve was switched and the amines were eluted in back-flush mode to the derivatisation coil with a mixture of the buffer and acetonitrile (85 : 15, v/v) at a flow rate of 0.25 ml min^{-1}. The flow rate of the reagent was 0.075 ml min^{-1} and it was delivered by pump 2 (Fig. 1). The reagent pump was kept on during the elution of the fraction, after which it was switched off. The gradient elution for the separation of the amines is described in Table 1. During the separation, the precolumn was flushed first with water–methanol mixture (20 : 80, v/v) containing 1% of KCl and then with water, 10 min each.

Results and discussion

Development of the online method required optimisation of the different analytical steps, *i.e.* clean-up, derivatisation and separation. Compared with off-line methods, there are a number of restrictions in choosing the analytical conditions. To keep the system as simple as possible, the same eluent was used for eluting the amines from the precolumn and as the starting eluent in the gradient separation. This meant that the eluent had to be suitable for both the elution and the separation. Since the online derivatisation took place between the precolumn and the analytical column, the composition of the eluent was relevant to the derivatisation reaction.

Cation-exchange clean up

The complexity of the wine matrix makes it difficult to analyse amines directly. Cation-exchange with a strong cation-exchange material (SCX) has been found to provide efficient clean-up for amines in wines.[2,15] The amines are fully protonated in neutral pH, and thus have strong interaction with the SCX material. Clean-up in our system was accordingly done in a short pre-column packed with strong cation-exchange material, and the washing solvent, washing time, elution solvent and elution volume were optimised. Spiked wine samples were used in these tests. In earlier off-line studies,[2,15] the SCX-cartridge has been washed with water or dilute buffer solution, and the amines have been eluted with several millilitres of methanol–salt solution with high ionic strength or methanol–HCl solution. In our online combination, the eluent composition used to elute compounds from the cation-exchange column was the same eluent as that used at the beginning of the gradient elution in the separation. Since derivatisation takes place at the same time, the eluent must also be suitable for the derivatisation reaction. Because of the pH limits of the C18 column, highly acidic or basic solutions could not be used for the elution. Also, a high concentration of methanol would disturb the separation. Therefore, elution with high ionic strength solution was chosen.

After injection of the diluted wine samples, the pre-column was washed before the elution of the amines to remove the majority of disturbing matrix compounds. Especially amino acids, which are present is large quantities in wines, would disturb the analysis because they can react with the derivatising reagent forming fluorescent products. Washing with 10 and 20 mM phosphate buffers at pH 7.2 was tested, but losses of the amines were severe, and washing was subsequently done with water. A washing volume of 3 ml was found optimal. Elution was done with solvent that was also used as the starting composition for the gradient elution. Different ion strengths and acetonitrile concentrations were tested. Boric acid buffer (0.02 M, pH 8.2) with 2% of NaCl and 15% acetonitrile was found to elute the analytes from the precolumn efficiently, in a volume of 0.85 ml. This eluent composition was also compatible with the derivatisation reaction.

The clean-up was efficient: the majority of disturbing matrix compounds were removed, as can be seen from Fig. 2A for a spiked wine sample. The recoveries of the analytes were determined by comparing the results of the online method of a spiked wine sample with those obtained by direct injection of a standard sample, without the pre-column, where the injection volume was the same as the size of the SCX fraction (0.85 ml) (Table 2). For most analytes, the recoveries were at satisfactory

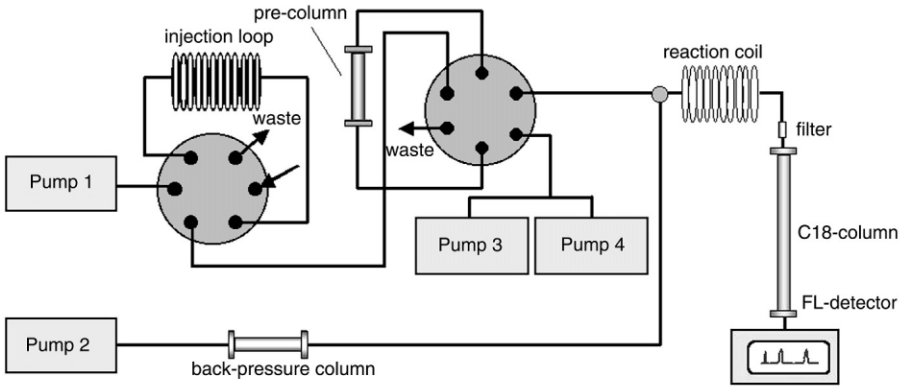

Fig. 1 The LC-LC system with online derivatisation.

levels. Recoveries for methylamine and ethylamine were low. The washing time had a noticeable effect on the recoveries of these two compounds: without washing, the recoveries were at quantitative level, but with even small washing volumes (< 1.5 ml), there was a considerable loss of analytes. Removal of the disturbing matrix compounds required a washing volume of 3 ml, however. The pre-column was conditioned after the elution and before the next analysis.

Table 1 Gradient elution program employed in the analysis of wines

Time/min	%ACN/ 1-octanol	Flow rate[a]/ ml min^{-1}	Steps in the procedure
0	10	0.25	Injection
7.1	15		SCX clean-up
7.2	15		Elution + derivatisation
11.1	15		
11.2	25	0.35	Separation
17.1	30		
27.1	40		
34.1	40		
44	45		
50	45		
60	80		

[a] Pumps 3 and 4 in Fig. 1.

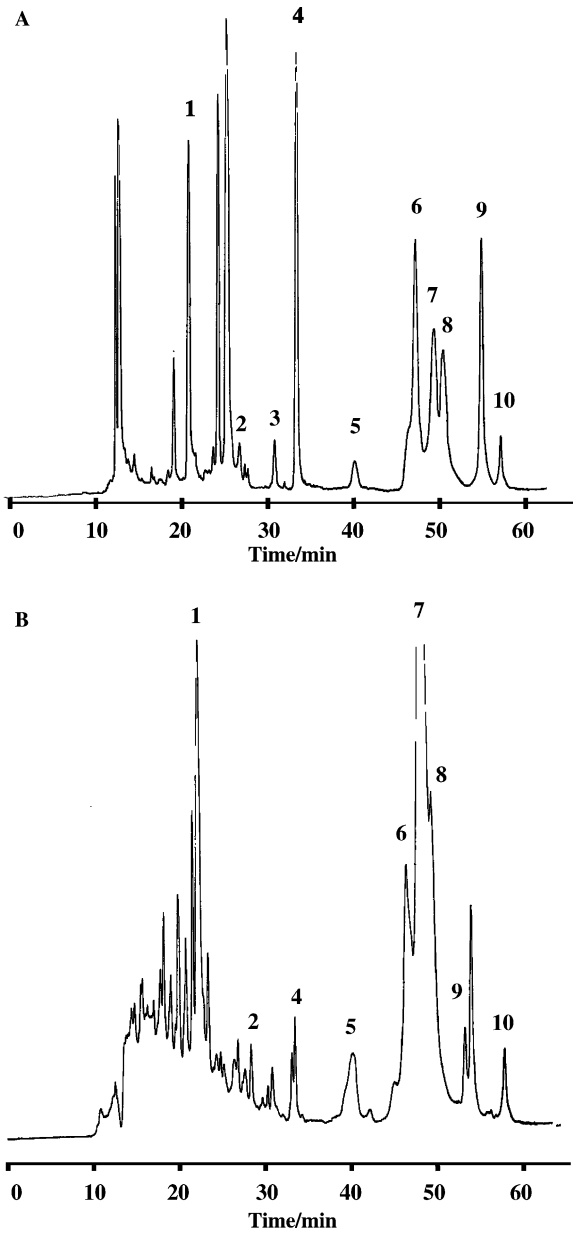

Fig. 2 Chromatograms of (A) a spiked white wine sample (c = 0.5 mg l^{-1}) and (B) a red wine sample. Peaks: 1, histamine; 2, methylamine; 3, ethylamine; 4, tyramine; 5, spermidine; 6, phenethylamine; 7, putrescine; 8, isoamylamine; 9, cadaverine; and 10. hexylamine (ISTD). For analytical conditions, see Experimental and Table 1.

Derivatisation

Online derivatisation was done immediately after the clean-up. The effluent from the pre-column was mixed with the derivatisation reagent (OPA) in a T-piece and the reaction took place in a reaction coil. OPA is well suited for the online derivatisation, because the reaction is fast. A particular benefit of the online derivatisation is that the reaction takes place in a closed system, and since the analytes are immediately transferred to the column, the instability of the OPA derivatives is not a problem.

To find optimal conditions for the reaction, we studied the effect of the concentration of OPA–ME reagent as well as the flow rate of the reagent and the effluent from the SCX column. In addition, the effect of eluent composition (pH, ion strength) was clarified.

The optimal reagent amount was studied in the range 3 to 12 mg for OPA and 0.05 to 0.5 ml for ME, with varying concentrations of methanol in the buffer, and with various flow rates of the reagent. To minimise the band broadening of the analytes due to high organic concentration in the final eluent (effluent from SCX-column mixed with derivatisation reagent), it was necessary to minimise the concentration of methanol in the reagent. The selected methanol concentration in the buffer was 4.8 vol%, at which level the reagent was still well soluble in the solution. The best results were obtained with OPA–ME amount of 6 mg per 0.45 ml, which corresponds to concentrations of 240 mg l^{-1} and 20 mg l^{-1} for OPA and ME, respectively. For the derivatisation reagent, flow rates of 0.05–0.15 ml min^{-1} were tested with three reagent concentrations. For the effluent, flow rates of 0.2–0.35 ml min^{-1} were studied with three reagent concentrations. For the reagent flow rate, 0.05 ml min^{-1} was too slow for efficient mixing of the effluent and reagent. Even with the most concentrated reagent solution the reaction was not quantitative. With flow rates higher than 0.1 ml min^{-1}, band broadening due to increased dilution of the analyte band began to occur. This could be seen in the broadened peaks of the analytes. The optimal flow rate was found to be 0.075 ml min^{-1}. The flow of the reagent was stopped after the sample had reached the column. The flow rate of the effluent has an effect not only on the mixing but also on the time required for the reaction. Best results were obtained when the eluent flow rate during the reaction was decreased to

Table 2 SCX recovery, linearity (in the range 0.05–15 mg l^{-1}), limits of quantification (LOQ, mg l^{-1}) and the relative standard deviation of peak areas (c = 0.5 mg l^{-1}, n = 4)

Analyte	SCX recovery (%)	r^2	LOQ	RSD (%)
Histamine	70.0	0.999[a]/0.989[b]	0.005	6.0
Methylamine	19.3	0.966	0.005	7.0
Ethylamine	11.2	0.954	0.025	4.1
Tyramine	73.1	0.992	0.04	5.0
Spermidine	84.7	0.968	0.005	2.2
Phenylethylamine	88.3	0.999	0.05	5.7
Putrescine	101.1	0.948	0.005	14.5
Isoamylamine	60.9	0.984	0.005	15.9
Cadaverine	93.0	0.980	0.005	8.1

[a] At range 0.05 to 5 mg l^{-1}. [b] At 5 to 20 mg l^{-1}.

Table 3 Amount of amines in red wines (mg l^{-1})

Analyte/wine	Hungary	Romania	Spain	Hungary	Bulgaria	Italy	Macedonia	USA	Crete	Chile	Spain	Italy
Histamine	2.8 ± 1.4	2.2 ± 1.3	6.9 ± 4.3	1.33 ± 0.5	0.2 ± 0.1	7.4 ± 1.8	0.1 ± 0.01	0.3 ± 0.01	0.3 ± 0.06	7.0 ± 2.0	1.6 ± 0.5	4.6 ± 1.3
Methylamine	1.3 ± 0.4	< 0.05	4.5 ± 0.8	0.6 ± 0.13				0.3 ± 0.05		0.8 ± 0.04		
Ethylamine					1.1 ± 0.3	2.8 ± 0.8	6.5 ± 2.0	0.8 ± 0.2	1.8 ± 0.08	6.0 ± 1.8		2.4 ± 0.5
Tyramine	< 0.05	2.9 ± 2.4	1.4 ± 0.3	< 0.05	0.3 ± 0.03	4.8 ± 1.2	< 0.05	0.8 ± 0.06	< 0.05	11.5 ± 1.1	2.3 ± 0.7	9.1 ± 2.5
Spermidine	< 0.05		< 0.05					2.0 ± 0.2		0.3 ± 0.08		
Phenylethylamine	2.0 ± 0.06	0.3 ± 0.2	< 0.05	0.2 ± 0.003		1.6 ± 0.4		1.8 ± 0.4	1.0 ± 0.05	< 0.05		
Putrescine	11.8 ± 4.1[a]	2.1 ± 1.0[a]	< 0.05	7.6 ± 1.4	< 0.05	9.2 ± 0.4			6.9 ± 0.6	4.7 ± 1.4	0.2 ± 0.04	0.1 ± 0.04
Isoamylamine								0.8 ± 0.2				< 0.05
Cadaverine	< 0.05	< 0.05	2.2 ± 0.5	3.5 ± 0.19		< 0.05	< 0.05	0.8 ± 0.3	< 0.05	< 0.05	< 0.05	< 0.05

[a] Closely eluting matrix peak, might have disturbed quantification.

0.25 ml min^{-1}. To speed up the analysis, the flow rate was increased to 0.35 ml min^{-1} after the reaction.

The eluent composition had a marked effect on the derivatisation reaction. With too high buffer concentration (> 0.15 M) in the eluent, the pH of the eluent–derivatisation reagent dropped too low to ensure efficient derivatisation. On the other hand, the lifetime of the column will be prolonged if the pH of the eluent is not too high. However, high ionic strength is required for the elution of the amines from the precolumn. Several buffer solutions of different pH and ion strength were tested, and the pH of the eluent–derivatisation reagent was measured.

The online derivatisation gave comparable results to those obtained with off-line derivatisation in a test tube. No significant band broadening was observed due to the derivatisation reagent or to the extra volume in the reaction coil.

Quantitative analysis

To determine the suitability of the method for quantitative analysis, we studied sensitivity, linear range, repeatability and limits of detection and quantification. The sensitivity and repeatability of the system were good (Table 2). The linearity was studied in the range from 0.05–15 mg l^{-1}. The method was linear for all the analytes except histamine, for which two different calibration curves were used (0.05–5 mg l^{-1} and 5–20 mg l^{-1} (Table 2). Very low detection and quantitation limits were obtained (LOD < 0.01 mg l^{-1} and LOQ < 0.05 mg l^{-1}) for all the analytes. Detection limits could be decreased even further by increasing the amount of sample injected. The capacity of the precolumn was large enough for sample sizes up to 10 ml. For example, when the sample loop was increased from 0.6 ml to 2 ml, the detection limits fell below 1 μg l^{-1}. On the other hand higher concentrations (>5 mg l^{-1}) then overloaded the analytical column, and the separation suffered. In the end, a loop size of 980 μl was chosen.

Several red wines were analysed for amines. Calibration curves used in the quantitation were obtained by running spiked samples through the whole online system. The results are presented in Table 3. A chromatogram of a red wine sample is shown in Fig. 2B. All the wines contained histamine and tyramine. The total amine concentrations in wine ranged from 1.6–30 mg l^{-1}.

Conclusions

The LC-LC system with online derivatisation allowed the whole analysis to be performed in a closed online system. The only manual pretreatment was dilution of the sample and filtration. The system proved to be highly sensitive and, it would be easy to increase the sensitivity even further simply by increasing the sample size. The system also can be easily automated.

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