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Short communication

Ion-pair high-performance liquid chromatographic determination of biogenic amines and polyamines in wine and other alcoholic beverages

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Abstract

An optimised ion-pair reversed-phase high-performance liquid chromatographic method with automatic *o*-phthalaldehyde post-column derivatization and spectrofluorometric detection for the same-run separation and quantification of 12 biogenic amines and polyamines in alcoholic beverages has been validated. The reliability of the method was satisfactory in terms of linearity (from 0.5 to 15 mg/l), precision (relative standard deviation below 5%), recovery (from 98.7 to 101.1%), and sensitivity (detection limit between 0.03 and 0.06 mg/l). The automatic accomplishment of the derivatization step reduces time and effort of analysis, especially thanks to the easy preparation of the sample.
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1. Introduction

Biogenic amines and polyamines are organic bases of low molecular mass mainly originated by decarboxylation of amino acids, although other reactions are also involved in polyamine synthesis. These compounds can be degraded through different enzymatic pathways (oxidation and acetylation) during metabolic processes in animals, plants, and microorganisms [1]. In food, aromatic biogenic amines such as histamine, tyramine, and phenylethylamine are generally undesired due to their vasoactive and/or psychoactive properties. Individuals suffering from histamine intolerance, due to a reduced activity of amino-oxidase enzymes, and individuals under antidepressive treatment with monoamino-oxidase inhibitor (MAOI) drugs are particularly sensitive to these biologically active amines. In addition, amino-oxidases are competitively inhibited by other biogenic diamines (putrescine and cadaverine) and alcohol, which can enhance the toxicity of aromatic biogenic amines in wine [2]. Typical symptoms caused by food histamine include nausea, sweating, headache, and hyper- or hypotension. Tyramine and phenylethylamine, have also been incriminated as responsible for food-induced migraine and hypertensive crisis due to MAOI interaction [3].

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Considerable amounts of some biogenic amines can appear during food fermentation processes or food storage under certain conditions if amino acid-decarboxylase positive microorganisms are present. However, it has not been fully established in which proportion such biogenic amine accumulation is due to contaminant microorganisms or to microorganisms responsible for the fermentative processes [4,5]. Histamine, tyramine, phenylethylamine and putrescine are the most important amines in wine, although in variable amounts depending on the wine. The variability of the amine contents in wine could be explained on the basis of differences in the winemaking process, time and storage conditions, raw material quality, and possible microbial contamination during winery operations [6].

In spite of the toxicological implications, no legal limit has been defined for biogenic amines in wines. Some countries have established rough guidelines concerning maximum recommended levels for histamine, which are quantitatively much lower than in other food (such as fish) due to the presence of alcohol. A maximum of 2 mg/l has been proposed in Germany, 5–6 mg/l in Belgium, 8 mg/ml in France, and 10 mg/l in Switzerland [7]. To date, no recommendation has been described for tyramine or any other biogenic amine in wines and alcoholic beverages.

Analytical determination of biogenic amines and polyamines is not simple because of their structure and because they are usually present at low levels in a complex matrix. High-performance liquid chromatography (HPLC) is the technique most extensively used to determine biogenic amines and polyamines in wine due to its high resolution, sensitivity, great versatility, and simple sample treatment. Biogenic amines and polyamines do not exhibit satisfactory absorption at the visible or ultraviolet wavelengths, nor do they exhibit fluorescence. Therefore, chemical derivatization is usually applied for their analysis by HPLC, increasing the selectivity and sensitivity of detection [8].

The aim of this work was to optimise and validate a rapid, precise, and versatile method by HPLC, using post-column derivatization with *o*-phthalaldehyde associated with mercaptoethanol (OPA/ME), for the determination of 12 amines in wine and other alcoholic beverages. For method validation the following parameters were tested: range of linearity, precision in terms of reproducibility, accuracy as recovery percentage, and sensitivity as detection and quantification limits. Furthermore, instrumental precision, of both peak areas and retention times, were estimated.

2. Experimental

2.1. Samples

All samples were purchased from Spanish retail stores. For the method validation test red wine was used, but other alcoholic beverages, such as white wine, cider, Catalan sparkling wine (“cava”) and red vermouth were also used to check the recovery of the method.

The samples were filtered through a 0.45 μm filter, and cava and cider samples were previously degassed in an ultrasonic bath.

2.2. Equipment

The liquid chromatograph consisted of a Waters 600E system controller pump (Waters Chromatography, Milford, MA, USA), a Waters 715 autosampler and a Waters RDM post-column reaction equipment were used with a spectrofluorometric detector (Kontron Instruments, Everett, MA, USA). The Waters RDM was connected to a zero dead volume mixing T installed between the column outlet and the detector. Data acquisition was accomplished with the Millennium 2010 version 2.10 system (Waters Chromatography). The chromatographic separations were carried out using a Nova-Pak C_{18} column (15×0.39 cm), 4 μm particle size (Waters Chromatography).

2.3. Chemicals

Ultrapure water was obtained from a Milli Q-System (Millipore, Milford, MA, USA). Methanol and acetonitrile were LC grade (Scharlau, Barcelona, Spain). Biogenic amine and polyamine standards, histamine (HI) dihydrochloride, tyramine (TY) free base, β-phenylethylamine (PHE) hydrochloride, serotonin (SE) creatinine sulfate, tryptamine (TR)

hydrochloride, octopamine (OC) free base, dopamine (DO) free base, cadaverine (CA) dihydrochloride, putrescine (PU) hydrochloride, agmatine (AG) sulfate, spermine (SM) tetrahydrochloride, spermidine (SD) trihydrochloride, were from Sigma (St. Louis, MO, USA). A concentrated 1000 mg/l stock solution of each amine as a free base was prepared in 0.1 *M* HCl. A 50 mg/l intermediate solution including all biogenic amines and polyamines was prepared in 0.1 *M* HCl from the stock solution. Calibration standards (ranging from 0.25 to 15.00 mg/l) were prepared in 0.1 *M* HCl from the intermediate standard solution. Finally, they were filtered through a 0.45 μm filter, stored in a refrigerator and protected from light.

2.4. Chromatographic conditions

Mobile phase consisted of the eluent A as a solution of 0.1 *M* sodium acetate (Merck, Darmstadt, Germany) and 10 m*M* sodium octanesulfonate (Romil, Cambridge, UK) adjusted to, pH 5.30 with acetic acid (Merck); and eluent B was a mixture of solvent B–acetonitrile (6.6:3.4), where solvent B was a solution of 0.2 *M* sodium acetate and 10 m*M* sodium octanesulfonate solution adjusted to pH 4.5 with acetic acid. Mobile phases were filtered and degassed before use. The post-column derivatizating reagent was prepared as follows: 15.5 g of boric acid and 13.0 g of potassium hydroxide (Panreac, Barcelona, Spain) were dissolved in 500.0 ml of water, 1.5 ml of 30% Brij (Merck) and 1.5 ml of 2-mercaptoethanol (Merck) as a reducing agent were added; finally, 0.1 g of OPA (Merck) dissolved in 2.5 ml of methanol was added and the above buffer solution. The derivatizating reagent, filtered and degassed before use, was prepared fresh daily and protected from light.

The gradient program was implemented as follows: time=0 min, A–B (80:20); time=30 min, A–B (50:50); time=44 min, A–B (40:60); time=46 min, A–B (20:80); time=50 min, A–B (80:20). The last step was to re-equilibrate the column to the initial conditions during 10 min more. The increase of eluent B was programmed according to a second-order exponential curve.

The flow-rate of the mobile phase was 1.0 ml/min, and the flow-rate of the derivatizating reagent was 0.4 ml/min. Mobile phase and the derivatizating agent were filtered and degassed before use. The column temperature was set at 40 °C and the post-column reaction equipment was kept at room temperature. Automatic injection (20 μl) of standard solutions or samples was carried out when the eluate was alkaline (pH 10.5–11.0) indicating the presence of derivatization reagent and a steady base line was recorded. Fluorimetric detection at 340 nm for excitation and 445 nm for emission was used.

2.5. Calculations

The concentration of each biogenic amine or polyamine was obtained directly by interpolation of the peak area in the correspondent linear calibration curve (peak area against amine concentration) between 0.50 and 15.00 mg/l. Samples were diluted with water when they were too concentrated.

2.6. Statistical analysis

All statistical tests were performed by means of the Statistical Software Package for Windows SPSS, version 9.0 (SPSS, Chicago, IL, USA). For checking the reliability of the method analysis of variance of linear regression, the *t*-test for mean comparison, and the Cochran test for variance homogeneity were applied.

3. Results and discussion

The method, based on the HPLC procedure used to determine biogenic amines in beers [9], requires the use of a reversed-phase stationary column, a post-column reaction system for OPA/ME derivatization, and provides a good separation of amines from possible interferences. Scan analysis of biogenic amine and polyamine standards indicated that the detection of those compounds was optimum at 340 (excitation wavelength) and 445 nm (emission wavelength). The preliminary trials to optimise the HPLC procedure demonstrated that the retention times of biogenic amine standards were pH dependent. Particularly, the pH of eluent A was critical for the steadiness of the elution of the compounds throughout the analyses. Moreover, a constant oven temperature was set up to improve the precision as

well as reduce the time of the analysis in comparison to other similar procedures [9,10]. The elution program developed provided chromatograms of high-resolution peaks, allowing a complete pattern of 12 amines (aromatic, diamines and polyamines) in a single run of less than 1 h. The chromatograms of a standard solution (10 mg/l) and a red wine sample are shown in Fig. 1. As can be observed, chromatograms were simple without interferences, and the identification was certain. Amine identification was made on the basis of retention time by comparison with standard solution. Relative standard deviations (RSDs) of retention times ranged from 0.26 to 2.20%. Moreover, due to the low variability of the retention time and peak area (data not shown), no internal standard was needed for amine quantification.

The samples chosen for the validation of the method were of red wines because they have the most complex matrices in comparison with other alcoholic beverages. However, the recovery trial was also performed with other alcoholic beverage samples.

3.1. Linearity

Detector response in the corresponding calibration curves (eight points from 0.50 to 15.00 mg/l) was linear. Linearity was verified by analysis of the variance of the regression and by the calculation of the RSD of the response factors (peak area/concentration). Least-squares analysis produced a correlation coefficient of $r \geq 0.9990$ for HI, TY, OC, DO, PU, CA, AG, SD and SM ($P<0.001$), and $r \geq 0.9950$ for SE, PHE and TR ($P<0.001$). Coefficient of determination (r^2) was higher than 99.50% for all standard curves, except for SE, which was 99.01%. Furthermore, the RSD of the response factors was less than 2.0% in all cases, ranging from 0.38 to 1.69%.

3.2. Precision

Eight determinations of a sample of red wine were performed using the same reagents and apparatus on the same day to evaluate method repetitiveness. Since no red wine contained all 12 amines, two different samples were prepared by adding known amounts of all biogenic amines (4.0 and 7.5 mg/l, respectively) to test the precision at two levels of concentration. Table 1 shows that the RSDs obtained for all amines were always less than 5.0%, which were satisfactory according to the Horwitz formula for intralaboratory studies [11].

3.3. Recovery

Recovery was tested by the standard addition procedure using two addition levels (4.0 and 7.5 mg/l) for each amine. Eight determinations were carried out for each addition level. Results obtained are shown in Table 1. Cochran's *C*-test was used to verify that the recovery values showed the same variance irrespective of the amine content (addition level) of the sample ($P>0.05$). In addition, Student's *t*-test was applied to compare the experimental recovery with the optimal theoretical value of 100% in each level of concentration and also for global recovery for each amine. The *t*-test showed that the mean recovery found for each amine did not differ ($P>0.05$) from 100%, and ranged from 98.3 to 102.5% depending on the amine.

In addition, the recovery of the method was tested using different types of alcoholic beverages such as white wine, cider, Catalan sparkling wine (''cava'') and red vermouth. Samples of each kind of beverage were analysed before and after the addition of one level of concentration (5 mg/l). Results of recovery ranging from 98 to 101% are shown in Table 2.

3.4. Sensitivity

The detection limit (DL) and the determination limit (DtL) were both estimated from the regression curve obtained with low concentration standards of biogenic amines and polyamines (from 0.25 to 2.00 mg/l, except for SM where it was from 0.80 to 2.00 mg/l) [12]. DLs were below 0.03 mg/ml for HI, PHE, DO, AG and SD, 0.04 mg/ml for TY and SE, 0.06 mg/ml for OC, TR, PU and CA, and 0.20 mg/ml for SM. DtLs were also satisfactory, being below 0.15 mg/ml for all amines except for OC and PU (below 0.20 mg/ml), and SM (0.50 mg/ml). All DLs and DtLs obtained by extrapolation for each amine were confirmed by the analysis of a standard solution at those level concentrations.

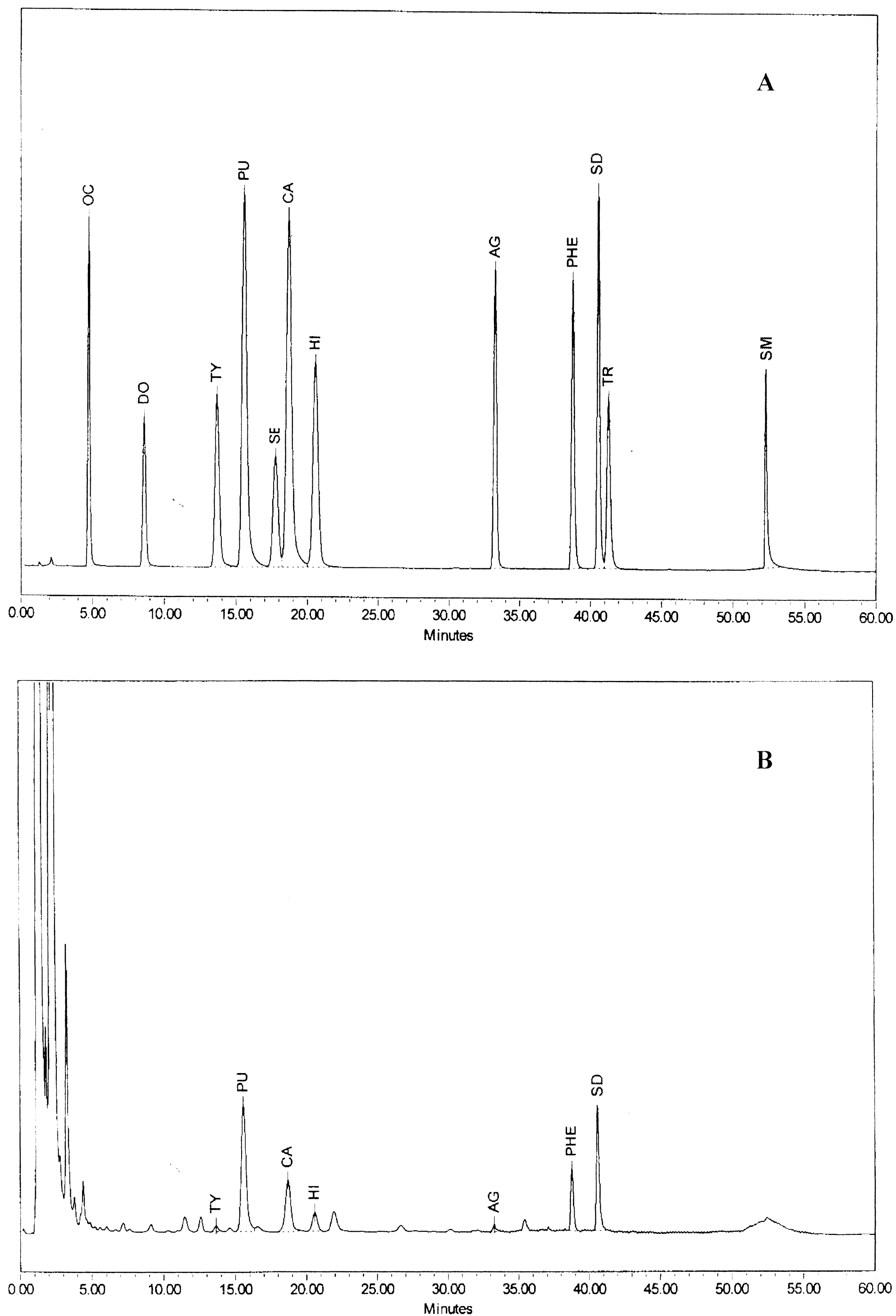

Fig. 1. Chromatograms of a biogenic amine and polyamine standard solution of 10 mg/l (A) and a red wine (B). Abbreviations: OC, octopamine; DO, dopamine; TY, tyramine; PU, putrescine; SE, serotonin; CA, cadaverine; HI, histamine; AG, agmatine; PHE, phenylethylamine; SD, spermidine; TR, tryptamine; SM, spermine.

Table 1
Precision and recovery of the method for determination of biogenic amines and polyamines in wine

	Addition level I[a]				Addition level II[b]				CI95[c] global recovery (%)
	Initial content (mg/l)	Content after addition (mg/l)	RSD[d] (%)	Recovery (%)	Initial content (mg/l)	Content after addition (mg/l)	RSD (%)	Recovery (%)	
TY	4.58	8.77	0.65	102.2	3.83	11.36	1.27	100.3	95.2–99.4
HI	6.31	10.33	1.21	100.2	5.82	13.48	1.50	101.2	98.6–100.0
PHE	0.36	4.42	1.73	101.4	0.85	8.20	1.79	98.3	99.1–101.4
SE	nd[e]	4.08	2.89	102.1	nd	7.58	3.99	101.1	97.0–100.1
OC	nd	4.00	0.84	100.0	nd	7.49	2.73	99.9	99.2–101.1
DO	nd	4.01	1.03	100.2	nd	7.44	1.01	99.2	99.8–100.8
TR	nd	3.97	0.90	99.2	nd	7.51	1.55	100.2	99.7–101.0
PU	21.38	25.18	3.60	99.2	16.11	23.97	0.25	101.6	98.3–101.2
CA	0.30	4.27	3.25	99.4	0.51	7.87	1.80	98.4	99.9–102.5
AG	0.12	4.11	0.70	99.6	nd	7.52	0.74	100.3	99.7–100.4
SD	0.24	4.32	0.89	101.8	0.55	7.98	1.62	99.2	98.6–100.4
SM	nd	4.00	0.85	100.0	nd	7.44	0.77	99.2	100.0–100.8

[a] 4.0 mg/l of each amine.
[b] 7.5 mg/l of each amine.
[c] CI95: confidence interval (95%) of the mean recovery from both addition levels.
[d] RSD: Relative standard deviation for the eight determinations.
[e] nd: Not detected.

3.5. Lack of interferences

A standard solution of 20 amino acids and of other primary amines usually found in wine and alcoholic beverages (i.e., methylamine, ethanolamine, etc.) was injected using the same chromatographic conditions described above. Neither amino acids nor these amines coeluted with any biogenic amine, showing smaller retention times than the biogenic amines due to its more polar nature. Moreover, proline (one of the major amino acids in wine) does not interfere because it does not react with OPA. The lack of

Table 2
Recovery after 5 mg/l addition of each biogenic amine and polyamine in alcoholic beverages

	White wine		Cider		"Cava"		Black vermouth	
	Initial content (mg/l)	Recovery (%)	Initial content (mg/l)	Recovery (%)	Initial content (mg/l)	Recovery (%)	Initial content (mg/l)	Recovery (%)
TY	2.53	98.5	0.73	99.6	nd	100.0	3.55	99.8
HI	1.10	98.9	nd	100.2	nd	99.9	0.74	100.6
PHE	nd[a]	100.0	nd	100.9	nd	100.2	nd	100.2
SE	nd	100.9	nd	100.7	nd	98.8	nd	99.7
OC	nd	100.8	nd	100.0	nd	99.3	nd	100.4
DO	nd	100.6	nd	100.5	nd	99.9	nd	99.8
TR	nd	100.9	nd	101.1	nd	100.9	nd	101.6
PU	6.06	99.4	0.73	99.7	2.46	99.7	9.16	101.8
CA	0.02	101.2	nd	100.5	nd	100.0	nd	100.2
AG	nd	99.4	nd	101.5	nd	100.3	nd	99.8
SD	nd	100.9	nd	100.3	0.63	100.0	nd	100.8
SM	nd	100.6	nd	99.5	nd	99.6	nd	99.6

[a] nd: Not detected.

interferences by amino acids and other amines was also verified in the method described by Izquierdo-Pulido et al. [9] for biogenic amine analysis in beers.

4. Conclusion

The use of an elution gradient for amine analysis makes it possible to avoid pre-treatments of the samples to remove polyphenolic compounds and other substances that could interfere in the analysis. Filtration of the samples is the only step required before the analytical determination. The simple preparation of the sample and the on-line post-column derivatization of the amines considerably reduce analysis time and effort. The method described in this paper has been demonstrated as a reliable procedure to analyse biogenic amines and polyamines in wine and alcoholic beverages, showing satisfactory linearity, precision, accuracy and sensitivity.

5. Nomenclature

AG	Agmatine
CA	Cadaverine
DO	Dopamine
HI	Histamine
OC	Octopamine
PHE	Phenylethylamine
PU	Putrescine
SE	Serotonin
SD	Spermidine
SM	Spermine
TR	Tryptamine
TY	Tyramine

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