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Short communication

# Determination of biogenic amines in wines by high-performance liquid chromatography with on-column fluorescence derivatization

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**Abstract**

An on-column fluorometric derivatization method was developed for determining eight biogenic amines by HPLC and fluorometric detection. The derivatization was carried out by pumping the *o*-phthalaldehyde reagent through the column together with other solvents used as mobile phases. The on-column reaction conditions (pH, borate and *o*-phthalaldehyde concentrations) were optimized. The matrix effect, the thiol used in the derivative reagent and the effect of the temperature on the separation process were also studied. Solid-phase extraction was applied prior to the derivatization procedure as a clean-up of wines by using 2 different commercially available SAX and $C_{18}$ cartridges to improve the accuracy and the precision of the method. The linearity range (0.5–15 mg $l^{-1}$) and detection limits (between 100 and 300 μg $l^{-1}$) of this method were similar to the standard pre-column automated *o*-phthalaldehyde derivatization method. The method was checked with several red wines from Tarragona by comparing the results with the standard pre-column method.

*Keywords:* Derivatization, LC; Wine; Amines; Methylamine; Histamine; Ethylamine; Tyramine; Putrescine; Cadaverine; Phenethylamine; 3-Methylbutamine

## 1. Introduction

Biogenic amines are found in various foods and beverages, such as wine, beer, fish and meat, normally as a result of enzymatic degradation or fermentation processes [1–3].

They can cause direct or indirect toxicity when their concentration levels are high. For this reason, several countries have established regulations for their intake content in various kinds of foods [4]. Furthermore, in alcoholic beverages it is important to take into account the synergic effect that there seems to be between the amines and ethanol [5].

Many analytical methods based on different techniques have been described for determining the biogenic amine content in foods, but high-performance liquid chromatography (HPLC) is the one preferred, using either pre-column [6–8] or post-column [9] derivatization procedures, using dansyl chloride (DnsCl) [10] or *o*-phthalaldehyde (OPA) [11–13], among others [14,15] as fluorescence labelling reagents.

The most important advantage that OPA has over other derivatization reagents is that it reacts with

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amines quickly, and enables the biogenic amines to be detected at fmol levels. However, it has the drawback that it only reacts with primary amines to give unstable derivatives. For this reason, the pre-column methods have certain drawbacks, such as the need for carefully controlled reaction conditions, thus affecting the reproducibility, and expensive equipment such as an automatic injector in the HPLC system for derivative formation. In the post-column methods, an additional pumping system is needed to carry the analyte from the column to the detector. The on-column methods avoid the problems that other methods have, and the results obtained seem to be satisfactory.

Since OPA has the most commonly accepted advantages, an on-column derivatization method [16,17] has been developed to simplify the instrumentation usually needed in other derivatization procedures.

The column used is the determining factor in the on-column methods, because the derivatization must be performed in it. When using OPA as the derivatization reagent, the analytical column has to be resistant to alkaline mediums. Therefore, a polymeric reversed-phase column was selected as it is tolerant towards alkaline eluent.

Furthermore, it is difficult to determine amines in wines because the matrix is very complex. Therefore, clean-up or preconcentration steps must be undertaken prior to chromatography. Many analytical methods include these steps, by using mainly liquid–liquid extraction (LLE) [18,19] or solid-phase extraction (SPE) [6,10,13] when isolating biogenic amines in wines.

LLE procedures use different mixtures of organic solvents to improve selectivity with good results, although this usually involves tedious preparation steps. On the other hand, SPE has been performed with several stationary phases based on anionic [20] or cationic [6,14] exchangers and octadecylsilane groups [9], as well as combinations of both [13].

The aim of this work is to find an on-column derivatizing method for simultaneously determining eight enologically important biogenic amines. A polymer-supported $C_{18}$ column with a mobile phase containing the OPA reagent was used, and a clean-up step using anionic and octadecylsilane cartridges was introduced before separating the studied compounds.

## 2. Experimental

### 2.1. Chemicals and reagents

The eight amines studied were: methylamine, histamine, ethylamine, tyramine, putrescine, cadaverine, phenethylamine and 3-methylbutylamine, all of which were supplied by Aldrich-Chemie (Beerse, Belgium). An individual standard solution of 2000 mg $l^{-1}$ of each amine was prepared in HPLC-grade acetonitrile (Scharlau, Barcelona, Spain) and stored in darkness at 4°C. Solutions for further studies were prepared by diluting these standard solutions with water which was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

The acetonitrile (ACN), tetrahydrofuran (THF) and sodium tetraborate used in the preparation of the chromatographic mobile phase were of HPLC grade (Scharlau). N-Acetylcysteine (NAC) and OPA were supplied by Fluka Química (Madrid, Spain), whereas $\beta$-mercaptoethanol (ME) was supplied by Aldrich-Chemie (Beerse, Belgium).

### 2.2. Equipment

Chromatographic experiments were performed using a Hewlett-Packard (Waldbronn, Germany) 1050 liquid chromatograph with a HP Model 1046A fluorescence detector. The samples were injected with a HP Series 1050 automatic injector. Separation was performed using an Asahipack OP-50 cartridge (250×4.6 mm I.D., particle size 5 μm) supplied by Hewlett-Packard. Chromatographic data were collected and recorded on a HP Vectra 5/100 implemented with the Chemstation software version B.01.02.

### 2.3. High-performance liquid chromatographic method

Three solvent reservoirs containing the following eluents: (A) 5 m*M* borate solution (pH 9) with 1% THF, (B) 5 m*M* borate solution (pH 9) with 12 m*M* OPA–NAC, and (C) ACN were used to separate all the amines with the HPLC gradient elution shown in Table 1. The flow-rate was fixed at 0.8 ml $min^{-1}$, so as not to exceed the upper limit of the column pressure. Eluents (A) and (B) were filtered through a

Table 1
HPLC elution programme used in the amines chromatography

| Time (min) | %A | %B | %C |
|---|---|---|---|
| 0 | 70 | 15 | 15 |
| 10 | 70 | 15 | 15 |
| 11 | 80 | – | 20 |
| 27 | 80 | – | 20 |
| 28 | 75 | – | 25 |
| 40 | 75 | – | 25 |

A: 5 m*M* borate (pH 9)/1% THF. B: 12 m*M* OPA–NAC/5 m*M* borate (pH 9). C: ACN.

0.45-μm nylon membrane (Millipore). The eluted OPA derivatives were detected by monitoring their fluorescence using 340 nm and 450 nm as the excitation and emission wavelengths, respectively. The analytical chromatographic column was thermostated at 40°C throughout the experiment. Under these conditions all eight amines were eluted in less than 40 min.

### 2.4. Solid-phase clean-up

Solid-phase extraction (SPE) experiments were performed using a Visiprep DL disposable-liner solid-phase extraction vacuum manifold with individual flow control valves from Supelco (Bellefonte, PA, USA) which enabled twelve SPE tubes to be dried at a time.

$C_{18}$ and SAX cartridges (500 mg, Varian, Harbor City, CA, USA) were used to remove interfering compounds such as amino acids and polyphenols from wine. They were activated with two fractions of 3 ml of methanol, and further conditioned with two fractions of 5 ml of Milli-Q-purified water and dried before sample elution. After the sample had been eluted, water was passed through the cartridge so that the same volume was obtained and reproducibility was not lost.

## 3. Results and discussion

The determination of biogenic amines by HPLC has usually been carried out with fluorescence derivatization reagents, such as OPA, mainly because they react quickly with amines, as has been already mentioned.

In previous works, OPA has been used with pre-column HPLC techniques, which require additional expensive equipment such as an automatic injector, among other things. When on-column derivatization procedures are used, the reaction is completed when the OPA contained in the mobile phase reaches the analyte in the injection loop. OPA and borate concentrations, the thiol used in the indole formation and the percentage of acetronitrile, pH and column temperature in the mobile phase, were the parameters studied to optimize the OPA-labelling reaction. All the experiments were carried out by analysing a red wine fortified with 10 mg $l^{-1}$ of the amines.

In the first part of this report the separation of the compounds was optimized. Given that the eight OPA derivatives obtained from amines have different polarities, it was necessary to vary the percentage of organic solvent in the mobile phase. A single linear gradient elution could not be used because of the baseline drift, but it was alternated with several isocratic elutions at different chromatographic times. Besides the OPA eluent which was halted at minute 11 to decrease the baseline drift as the ACN percentage increased, the mobile phases used were always made up of different ACN and borate fractions so that the alkalinity needed for the derivatization and the polarity were suitable for eluting the analytes.

Before gradient optimization, parameters such as the active thiol which are indispensable for the OPA-labelling reaction were studied. β-Mercaptoethanol has been widely used [7,12], although NAC [16,17] as well as other thiols [21] have also been used. In this work, NAC was selected because it results in a more stable baseline with only a faint odour, whereas the β-mercaptoethanol is a smelly liquid and difficult to handle.

Since the most polar amine derivatives can coelute with other polar compounds present in wine, such as polyphenols and aminoacids, the proportion of organic solvent during the initial chromatographic separation must be enough for them to be separated. Several percentages of ACN were tested and the best results were obtained with an initial proportion of ACN of 15%.

As the reaction between amines and OPA is almost

instantaneous, the OPA derivatives are formed when the analyte comes into contact with the mobile phase. For this reason, the OPA eluent does not need to be continuously pumped throughout the chromatogram, and at minute 11 the percentage of OPA is eliminated. The change in the baseline fluorescence is observable because a peak appears in the middle of the chromatograms.

Afterwards, other parameters which are important in the OPA-labelling reaction and the separation of the compounds analyzed, were examined. First of all, it must be taken into account that fortified wines will be optimized in order to ensure the efficacy of the analytical method proposed for the samples analyzed. For all the experiments, a red wine spiked with 10 mg $l^{-1}$ of the biogenic amines was used, and subsequently checked with amine standard solutions.

The OPA–NAC concentration was the first variable tested. The molar ratio of OPA and NAC was always set at equivalence, so they were optimized together. Firstly, the concentration had to be enough to derivatize the amines when large amounts of other compounds that could be derivatized appeared in real samples. The concentrations tested ranged between 0.5 and 5 m*M* according to other studies [16,17]. The capacity factors ($k'$) and the areas of the amines in the fortified wine were represented versus OPA–NAC reagent concentration and no significant differences were observed for $k'$ at different concentrations. For histamine there was a little change in the response, but there was no change in the responses of the rest of the amines between 2 and 5 m*M*. For this reason, 2 m*M* OPA–NAC was chosen as it is enough to derivatize all the amino compounds in real samples

The pH of the mobile phase was the second parameter tested. The values tested were between 8 and 11 in order to ensure the formation of derivatives, since the OPA derivatization of amines is generally best carried out at alkaline pH. The area response variations show that at pH values lower than 9 and higher than 10, most of the amines had lower responses. Since best results were obtained between 9 and 10, pH 9 was chosen for subsequent work.

The concentration of borate was also checked at the optimized pH. The concentrations tested ranged between 5 and 50 m*M*, since these were the concentrations used in previous studies. As in the previous variables, the $k'$ and the variation of the areas were represented versus the variable studied. The $k'$ increased as the borate concentration rose. Furthermore, similar areas were obtained at different concentrations. Since 15 m*M* borate did not significantly decrease the derivative responses, and the retention times slightly increased as the borate concentration rose, it was fixed as the optimum.

The last parameter examined was the column temperature. The values tested were 35°C, 40°C and 45°C. At higher values the resolution of the chromatographic peaks did not improve and the column durability would decrease. The $k'$ and the area versus temperature show that there were no differences among the temperatures tested, although above 45°C there was a slight tendency to decrease. Symmetrical peaks were obtained at all temperatures. Neither did the temperature affect the derivatization yield, the areas being similar in all cases. Thus, 40°C was fixed for subsequent analysis.

When experimental conditions were determined, a sample aliquot of 5 μl was subjected to HPLC determination. Fig. 1a shows the chromatogram of the analysis of 1 mg $l^{-1}$ of amine standard solution under the conditions outlined. As can be seen, two baseline distortions appear as a consequence of the percentage of organic solvent changes, although they do not interfere with the peaks of the analytes. When dealing with wines, the attenuation was changed in these zones in order to obtain a good baseline. This did not affect the chromatographic areas of the analytes, as it was done exactly at the chromatographic times corresponding to the aforementioned distortions, and the initial attenuation was returned to immediately afterwards.

Fig. 1b and Fig. 1c show the chromatograms obtained when a red wine and the same sample spiked with 10 mg $l^{-1}$ of the amines were analyzed. The first one shows that histamine, methylamine, ethylamine and putrescine are present in this wine, and this is confirmed with a subsequent standard addition. As can be seen, at low concentrations, the peak that elutes between histamine and methylamine prevents them from being quantified. Furthermore, ethylamine is overlapped by another unknown peak. Therefore, to determine these analytes a clean-up step had to be done to prevent these interferences.

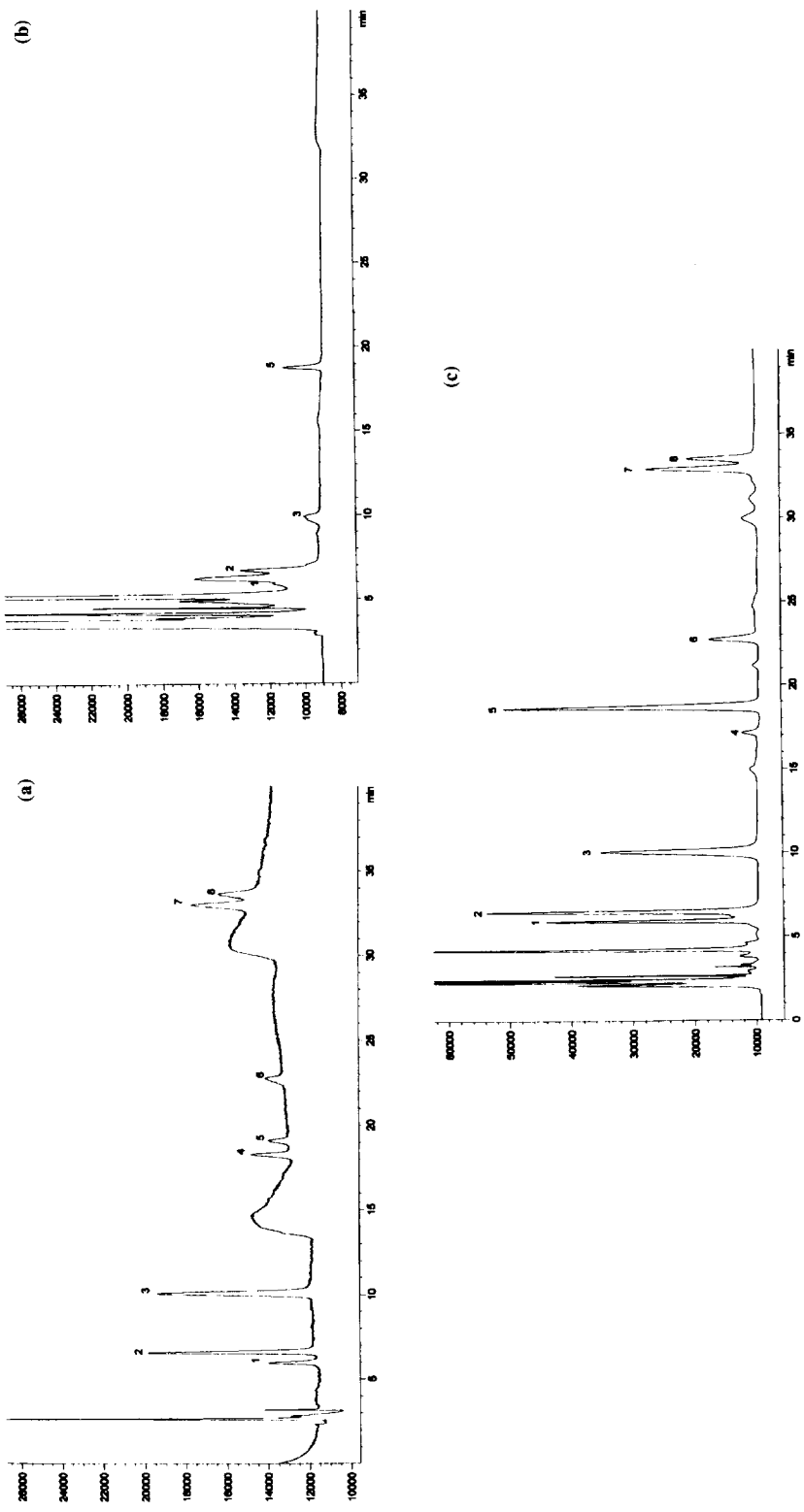


Fig. 1. (a) Chromatogram of a standard sample of 1 mg $l^{-1}$ of the biogenic amines after on-column derivatization with OPA: (1) histamine, (2) methylamine, (3) ethylamine, (4) tyramine, (5) putrescine, (6) cadaverine, (7) isoamylamine, and (8) phenethylamine. (b) Chromatogram obtained from the on-column derivatization of a red wine without SPE treatment. (c) Chromatogram obtained from the on-column derivatization of a red wine spiked with 10 mg $l^{-1}$ of the biogenic amines and without SPE treatment.

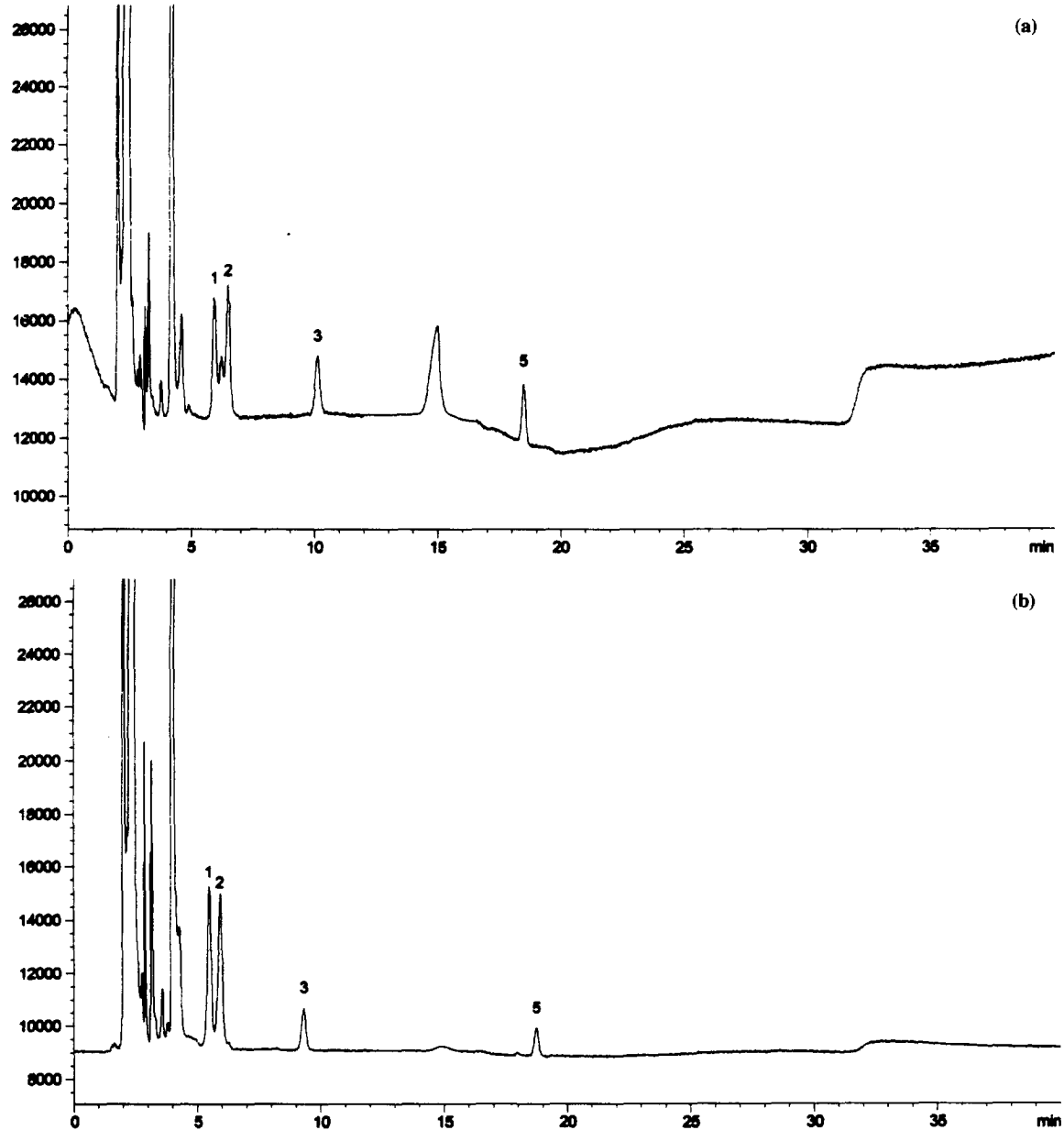


Fig. 2. Chromatogram obtained from the on-column derivatization of the same red wine as shown in Fig. 1b after: (a) $C_{18}$, and (b) SAX clean-up. For peak assignation, see Fig. 1.

Two solid-phase extraction cartridges, a non-polar ($C_{18}$) and a strong anion-exchanger (SAX) were used. In previous studies [13], octadecylsilane ($C_{18}$) and quaternary ammonium groups had been used to remove large amounts of polyphenols from samples by slight modification of the sample pH.

*$C_{18}$ cartridges*: The stationary phase was used to eliminate non-polar compounds. A 5-ml volume of a red wine was processed without treatment. Polyphenolic and other non-polar compounds were retained in the cartridge, while the elute conserved the amines. A 5-μl aliquot was then injected.

Table 2
Percentage of recoveries with octadecylsilane and ammonium anion exchangers ($R$ and R.S.D. in %)

| Amine | $C_{18}$ | | SAX | |
|---|---|---|---|---|
| | $R$ | R.S.D. | $R$ | R.S.D. |
| Histamine | 70 | 7.2 | 100 | 3.1 |
| Methylamine | 65 | 8.5 | 98 | 5.3 |
| Ethylamine | 105 | 4.5 | 95 | 4.5 |
| Tyramine | 102 | 4.3 | 100 | 3.5 |
| Putrescine | 100 | 3.9 | 75 | 6.6 |
| Cadaverine | 80 | 5.7 | 100 | 3.0 |
| Isoamylamine | 100 | 3.5 | 102 | 4.1 |
| Phenethylamine | 105 | 4.1 | 103 | 4.5 |

Results from triplicate analyses of a red wine spiked with 5 mg $l^{-1}$ of the amines. Conditions given in the text.

Fig. 2a shows the chromatogram obtained when the $C_{18}$ elute of the red wine shown in Fig. 1b was injected. As can be seen, the peak that appeared between histamine and methylamine decreased and both peaks were accurately quantified. Furthermore, the peak corresponding to ethylamine was well defined.

*SAX cartridges*: The same study was carried out but this time the ionic compounds were eliminated. A 5-ml portion of a red wine was adjusted to pH 8 in order to retain polyphenolates and amino acids which had been converted into their anionic forms. As in the previous experiment, 5 μl of the elute was then injected.

Fig. 2b shows the chromatogram of the same red wine shown in Fig. 1b, but after SAX treatment. As can be seen, all of the peaks of interest are well defined, as most of the interferences were prevented.

Table 2 gives the recoveries obtained when the clean-up step was done on a red wine spiked with 5 mg $l^{-1}$ of the amines. As expected, both SPEs showed good results. However, histamine, methylamine and cadaverine were better recovered when SAX treatment was applied, perhaps due to the anion exchangers selectively retaining polyphenolates and some fairly ionized amino acids at alkaline pH. Putrescine was also better recovered when $C_{18}$ extraction was applied. It can be observed that the histamine/methylamine interference shown in the chromatogram in Fig. 1b has almost disappeared in Fig. 2a and has been completely removed in Fig. 2b, with no important analyte losses.

Thus, in order to verify the linearity of the response of the different derivatives, including SAX treatment, at the previously specified wavelengths for the working concentrations, standard solutions of amines that ranged between 0.1 and 15 mg $l^{-1}$ were prepared and injected. The linearity was calculated by means of an external standard method. Linear least-squares regression was used to calculate the slope, intercept and correlation coefficient ($r^2$) by using the ULC computer program [22]. All the calibration lines showed ranges of linearity ($r^2 > 0.995$) between 0.5 and 15 mg $l^{-1}$.

The detection limit was calculated from the amount of amines required to give a signal-to-noise ratio of 3 and was found to be between 0.1 and 0.3 mg $l^{-1}$ for all the biogenic amines. The quantification limit was established at 0.5 mg $l^{-1}$ and was fixed as the first point in the calibration graphs.

The reproducibility and the repeatability of the on-column derivatization technique were considered either with 1 mg $l^{-1}$ of an amine standard solution in acetonitrile or an amine-free red wine spiked with 1 mg $l^{-1}$ of each amine. The repeatability analyses ($n=5$) of amines gave relative standard deviations (R.S.D.s) below 5% for the peak area and below 1% for the retention times, in both cases. The repro-

Table 3
Mean (mg $l^{-1}$) of the amine concentrations obtained from the triplicate analyses of 5 red wines from Tarragona

| Amine | Sample 1 | | Sample 2 | | Sample 3 | | Sample 4 | | Sample 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b | a | b |
| Histamine | 1.6 | 1.9 | 1.0 | 1.8 | 1.3 | 1.6 | 2.4 | 3.0 | 0.6 | 0.9 |
| Methylamine | 1.1 | 1.2 | 0.9 | 1.0 | 1.0 | 1.4 | 3.8 | 4.3 | 1.1 | 1.7 |
| Ethylamine | 1.4 | 1.9 | 1.3 | 1.5 | 0.8 | 1.0 | 1.3 | 1.7 | 1.2 | 1.7 |
| Putrescine | 5.3 | 6.1 | 4.3 | 5.0 | 5.0 | 5.9 | 4.1 | 5.0 | 4.8 | 5.2 |

Quantification after applying (a) the combined on-column/SAX method, and (b) the pre-column automated method.

ducibility was examined in the same way but the analysis took place over different days. The results were slightly higher than for the repeatability and did not exceed 10% in any case.

The method was applied to determine the biogenic amine content of five red wines from Tarragona (see Table 3). The results were compared with the ones obtained by automatic injection of the amines after OPA derivatization, as this is the method which is usually used in this kind of determination [6,7,13]. The two sets of data showed no differences between them, all having R.S.D.s lower than 5%. Histamine, methylamine, ethylamine and putrescine were found in all the samples analyzed whereas none showed contents of tyramine, isoamylamine or phenethylamine over their detection limits.

## 4. Conclusions

The method described seems to be suitable for the determination of eight biogenic amines in wines. It involves an easy, fast clean-up step with strong anion exchanger cartridges to avoid chromatographic interferences.

The on-column derivatization technique appears to be a good alternative to the classical pre-column derivatization procedure, since it obtains good results with simpler equipment, which is especially important in enological laboratory work.

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## References

[1] M.C. Vidal and A. Mariné, Rev. Agroquím. Tecnol. Aliment., 25 (1983) 59.
[2] R.E. Subden, R.G. Brown and A.C. Noble, J. Chromatogr., 166 (1978) 310.
[3] S. Moret and R. Bortolomeazzi, J. Chromatogr., 591 (1992) 175.
[4] O. Busto, J. Guasch and F. Borrull, J. Int. Sci. Vigne Vin, 30 (1996) 85.
[5] H. Suomalainen, K. Eriksson and L. Nykkanen, Am. J. Enol. Vitic., 25 (1974) 179.
[6] O. Busto, M. Mestres, J. Guasch and F. Borrull, Chromatographia, 40 (1995) 404.
[7] M.J. Pereira and A. Bertrand, Bull. OIV, 765–766 (1994) 918.
[8] T. Bauza, A. Blaise, J.P. Mestres, P.L Teissedre, F. Daumas and J.C. Cabanis, Sci. Aliment., 15 (1995) 367.
[9] M. Izquierdo, M.C. Vidal and A. Mariné, J. Assoc. Off. Anal. Chem., 76 (1993) 1027.
[10] O. Busto, Y. Valero, J. Guasch and F. Borrull, Chromatographia, 38 (1994) 571.
[11] Ch. Tricard and J.M. Cabazeil, FV OIV, 850 (1990).
[12] P. Lehtonen, M. Saarinen, M. Vesanto and M. Riekkola, Z. Lebensm. Unters Forsch., 194 (1992) 434.
[13] O. Busto, J. Guasch and F. Borrull, J. Chromatogr. A, 718 (1995) 309.
[14] O. Busto, M. Miracle, J. Guasch and F. Borrull, J. Liq. Chromatogr., (1996) in press.
[15] O. Busto, J. Guasch and F. Borrull, J. Chromatogr. A, 737 (1996) 205.
[16] K. Saito, M. Horie, K. Nakagomi and H. Nakazawa, Anal. Sci., 8 (1992) 675.
[17] K. Saito, M. Horie and N. Nose, J. Chromatogr., 595 (1992) 163.
[18] P. Lehtonen, Z. Lebensm. Unters Forsch., 183 (1986) 177.
[19] K. Mayer and G. Pause, Bull. OIV FV, 850 (1990).
[20] M. Calull, R.M. Marcé, J. Fábregas and F. Borrull, Chromatographia, 31 (1991) 133.
[21] J. Kehr, CMA/Microdialysis AB, Application Note 16, 1993.
[22] R. Boqué and F.X. Rius, J. Chem. Educ., 70 (1993) 230.