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Determination of biogenic amines in wine after precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

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Abstract

AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) was used as an alternative to the most common derivatization reagent, OPA (*o*-phthaldialdehyde), to determine sixteen biogenic amines in wines. OPA and AQC were compared in terms of the range of linearity, the limit of detection and the sensitivity (calibration line slopes) resulting from the application of each of the derivatization reagents. Sample handling is minimal because derivatization is fully automated by means of an injection programme. After derivatization, the derivatives were analysed by reversed-phase liquid chromatography with gradient elution and fluorimetric detection. All the amines studied eluted in less than 25 min under the optimum conditions established. Different variables that affect derivatization and separation were optimized. Good linearity of the responses was obtained between 0.05 and 10.00 mg l^{-1} depending on the amine derivative. The detection limits ranged between 5 and 50 $\mu g\ l^{-1}$ for standard solutions and between 100 and 500 $\mu g\ l^{-1}$ for wines. The method was successfully applied to the determination of the above amines in several types of wines from the Tarragona region.

Keywords: Derivatization, LC; Wine; Food analysis; Amines; Biogenic amines; Aminoquinolylhydroxysuccinimidyl carbamate

1. Introduction

Biogenic amines can be a food poisoning hazard. They are commonly found in fermented foods, e.g., cheese, dry sausages and wine (mainly red wine) and some of them are usually assumed to be involved in the development of alcohol dependence [1] and may also cause unnatural or toxic effects when consumed in large amounts [2].

There is a great diversity of biogenic amines in food and particularly in wine, where histamine, putrescine, cadaverine and tyramine have been studied much more than other amines, because

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they can elicit physiological responses in humans [3].

Current research is aimed at achieving good methods for determining low contents of biogenic amines in wine which are both repeatable and reproducible [4]. To this end, amines have been determined in a variety of ways, including fluorimetric [5,6] and gas–liquid chromatographic (GLC) [3,7–10] methods. However, the most commonly used method is HPLC, although ion partition column chromatography [11] and ion-pair HPLC have also been proposed [12]. The separation of underivatized amines by ion-pair chromatography is a well known technique [13] but it has the drawback that amines such as putrescine and cadaverine cannot be detected as they lack a suitable chromophore, whereas others such as histamine must be detected at wavelengths that are not selective, so the risk of interfering matrix components cannot be neglected, especially when dealing with wines. In addition, ion-exchange HPLC and post-column derivatization are time consuming and more expensive [14].

In order to prevent matrix interference and so enhance detection sensitivity when using HPLC, derivatization reactions with dansyl chloride (Dns-Cl) [15–19] and *o*-phthaldialdehyde (OPA) [4,5,18,20–24] in the presence of 2-mercaptoethanol are commonly used. Phenyl isothiocyanate (PITC) [25], fluorenyl methyl chloroformate (FMOC-Cl) [14,26], benzoyl chloride [27] and fluorescamine [18,28] are other derivatizing agents used in these determinations. However, the use of these reagents involves a number of drawbacks. PITC derivatives cannot be detected by fluorescence, and the methods used for them are less sensitive than those for other derivatization reagents. FMOC gives good sensitivity and stability of its derivatives, but it produces multiple products from a single amine, so it is not suitable when dealing with complex samples or multiple standards. Dns-Cl and benzoyl chloride are non-specific reagents, since they react not only with primary and secondary amino groups, but also with phenols, aliphatic alcohols and some sugars. Further, the relatively long reaction time makes automatic pre-column derivatization impossible. As the benzoylated amines do not fluoresce, these compounds are generally detected with the less sensitive UV detector.

Using OPA instead of fluorescamine has more advantages, as it reacts with primary amines in a few minutes and forms strong fluorescent derivatives, which increase the method's selectivity with fluorimetric detection. This reaction can easily be automated because of the short reaction time [4,21,29,30]. However, OPA derivatives are not very stable, they do not allow for clean-up after the derivatization processes and strict derivatization conditions are also necessary. Further, the structures of these products have only been fully ascertained for reactions in the presence of a thiol such as mercaptoethanol. The reaction also seems to be strongly dependent upon structural and other factors since many amines, notably histamine, do not require mercaptoethanol to produce highly fluorescent products; in fact, mercaptoethanol will decrease the intensity of fluorescence [31]. Some investigators have worked on improving the determination of histamine using derivatizing agents such as luminarin 1 or 2, but the derivatization conditions are very strict and time consuming [32].

In this paper, a precolumn derivatization reagent is proposed in order to avoid the drawbacks of reagents such as Dns-Cl and OPA. 6-Aminoquinolyl-N-hydroxysuccimidyl carbamate has already been reported as being of use in the precolumn derivatization of primary and secondary amino acids [33,34]. Here we report a rapid method for determining sixteen primary and secondary biogenic amines in wines.

2. Experimental

2.1. Chemicals and reagents

The sixteen amines studied were ammonia, ethanolamine, methylamine, histamine, ethylamine, pyrrolidine, isopropylamine, propylamine, tyramine, isobutylamine, putrescine, cadaverine, phenethylamine, 3-methylbutylamine, amylamine and hexylamine, all of which were supplied by

Aldrich-Chemie (Beerse, Belgium). An individual stock standard solution of 2000 mg l^{-1} of each amine was prepared in HPLC-grade methanol (Scharlau, Barcelona, Spain) and stored in darkness at 4°C. Solutions for further studies were prepared by diluting these stock standard solutions with water purified using a Milli-Q system (Millipore, Bedford, MA, USA). A solution of 2000 mg l^{-1} of heptylamine as internal standard was prepared and diluted 1000-fold.

The methanol and tetrahydrofuran used in the chromatographic analysis and the sodium acetate used to prepare the mobile phase were of HPLC grade (Scharlau). For the automatic derivatization method an AccQ·Fluor Reagent Kit (Waters, Milford, MA, USA) was used.

2.2. Equipment

Chromatographic experiments were performed using a Hewlett-Packard (Waldbronn, Germany) Model 1050 liquid chromatograph with an HP Model 1046A fluorescence detector. The samples were derivatized and injected with an HP Series 1050 automatic injector. Separation was performed using a Spherisorb ODS-2 cartridge (250 × 4.6 mm I.D., particle size 5 μm) preceded by a Spherisorb ODS-2 precolumn, both thermostated at 65°C, and supplied by Hewlett-Packard. Chromatographic data were collected and recorded on an HP ChemStation version A.01.01.

2.3. High-performance liquid chromatographic method

Two solvent reservoirs containing (A) 0.05 *M* sodium acetate solution in 1% tetrahydrofuran and (B) methanol were used to separate all the amines with gradient elution. The programme used was as follows: initial isocratic elution at 25% methanol for 5 min, followed by linear gradient elution from 25 to 80% methanol until 25 min. The column was then cleaned up by eluting 100% of methanol for 3 min. Finally, the programme ended by returning to initial conditions and stabilizing the corresponding mobile phase in 2 min. The eluted AQC derivatives were detected by monitoring their fluorescence using 250 and 395 nm as the excitation and emission wavelengths, respectively. The flow-rate was fixed at 1 ml min^{-1}. Under these conditions all sixteen amines were eluted in less than 25 min.

2.4. Derivatization

The derivative reagent was formed by reconstituting the 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate with acetonitrile (final concentration approximately 10 m*M* according to the specifications of the Waters Fluor Reagent Kit). The alkalinity needed to perform the derivatization is obtained by using the borate buffer (pH 8.8) supplied by Waters in the same Fluor Reagent Kit (AccQ·Tag).

The derivatization was fully automated by means of an injection programme. The injection system mixes the reagents automatically after cleaning the injection needle three times with acetonitrile in order to ensure the repeatability of the results. The AQC, the borate buffer and the sample are drawn sequentially into the injection needle. In order to ensure that the sample is fully mixed with the borate buffer, there is a previous mixing step before adding the AQC reagent. All the reactants are then mixed by drawing them back and forth in the injection seat. Finally, the mixture is injected into the column and separated using gradient elution. The steps in the derivatization sequence are summarized in Table 1.

3. Results and discussion

Biogenic amines have traditionally been analysed chromatographically by making them react with different derivatizing agents that produce fluorescence and thus improve the selectivity of established methods. AQC has been successfully used to derivatize alkylamines [33] and amino acids [33–35]. The ease of sample derivatization, the stability of the derivatives and its sensitivity to all the amines studied encouraged us to use it for determining biogenic amines in wines.

The same solvents as used in previous work

Table 1
Injection programme for the derivatization of biogenic amines with AQC

Step	Action	Amount (μl)	Details	Substance
10	Draw	2	Air	
20	Draw	20	From Vial 1	ACN[a] for needle wash
30	Eject	20	Into Vial 4	Empty
40	Draw	20	From Vial 1	ACN for needle wash
50	Eject	20	Into Vial 4	Empty
60	Draw	20	From Vial 1	ACN for needle wash
70	Eject	20	Into Vial 4	Empty
80	Draw	10	From Sample	
90	Eject	10	Into Seat	
100	Draw	0	Vial 1	ACN for needle wash
110	Draw	6	Vial 2	Borate buffer
120	Eject	6	Into Seat	
130	Mix	16	Needle in Seat	Ten cycles
140	Draw	0	From Vial 1	
150	Draw	0.5	From Vial 3	AccQ·Tag (derivatizing agent)
160	Eject	0.5	Into Seat	
170	Mix	16.5	Needle in seat	Ten cycles
180	Wait	5 min		
190	Inject			Start analysis

[a] Acetonitrile.

[30] with OPA as derivatization reagent for wines were used as the mobile phase. They were chosen because unstable organic modifiers such as triethanolamine, which is used in similar analyses to reduce band broadening, reduces the life of the column, as we have observed before.

Since AQC derivatives are more polar than OPA derivatives, the percentage of methanol in the mobile phase at the start had to be decreased to obtain good resolution among the first chromatographic peaks. Different compositions of the mobile phase were tested and 25% MeOH was adopted as the best initial conditions. This percentage should not be decreased as it is important not to lengthen the time of the chromatography if possible. Further, an increase in the percentage of methanol in the initial mobile phase would produce an overlap between the peaks corresponding to amino acids and those recorded for the first biogenic amine derivatives.

During the experiments it became clear that the life of the column was strongly dependent on the mixture of the AccQ·Tag reagent with the mobile phase, which is not observed with OPA. This phenomenon is probably caused by the gradual hydrolysis of the bonded phase. However, more than 150 analyses can be carried out with an octadecylsilane cartridge with no important reduction in the performance of the column.

The temperature of the analysis was also studied in order to achieve the best resolution among the chromatographic peaks. Different temperatures between 40 and 70°C were tested and compared in terms of resolution. As a result of these experiments, the temperature was fixed at 65°C as all of the amine derivatives were well resolved.

The derivatization was also optimized by varying two variables: reaction time and volume of concentrated AccQ·Tag reagent. A modification of the derivatization system developed for amino acid compounds by Díaz et al. [34] was applied to achieve the best derivatization conditions. A 10-μl volume of 1 mg l^{-1} standard were automatically mixed with 6 μl of borate buffer solution and with 0.5 μl of reconstituted AQC reagent. Because the reaction takes place almost instantaneously, several experiments of between 1 and 5 min were carried out. The results of

10-min experiments were also considered and compared with the best responses of the 1–5-min experiments. The response of the amine derivatives at 1 min is very poor, so it is concluded that the reaction cannot be completed in such a short time. The responses increased with reaction time up to a maximum of 5 min. The sensitivity was no better at 10 min, confirming that the reaction was complete after 5 min.

Several micro-volumes ranging from 0.1 to 2 μl of the concentrated derivative reagent were also tested and compared by derivatizing samples that contained concentrations that were slightly higher than those which were found to be present in wines according to our previous experiments. The derivative responses decrease at volumes below 0.5 μl, maybe owing to the lack of accuracy of the injection needle at volumes close to its lower limit, do not increase at volumes over 0.5 μl and the excess of the reagent peak overlaps the peaks that elute near its retention time, so 0.5 μl of the AQC reagent was used to derivatize the samples injected.

Finally, the sample volume was also studied. Experiments were performed at 1, 5, 10, 15 and 20 μl and the best results were obtained when 10 μl of sample were injected.

As Table 1 shows, the derivatization programme was optimized by repeating the mixing step twice. Here, the excess of the AccQ·Tag reagent is the limiting factor. As the peak of the reagent may overlap several peaks if it is increased, we must control its amount without decreasing the derivatization yield. The sample is homogenized with borate buffer and the AccQ·Tag reagent is added at the end. In this way, the derivatization reagent need not be added twice, as in previous studies [30].

The chromatogram that was obtained by automatically injecting a standard solution of 1 mg l^{-1} of the sixteen amines under the conditions specified above is shown in Fig. 1. As can be seen, good resolution was obtained for the sixteen peaks, even when the excess of reagent in the middle of the chromatogram was taken into account. The reaction between AQC and the biogenic amines gave higher derivative responses than when derivatization was carried out with

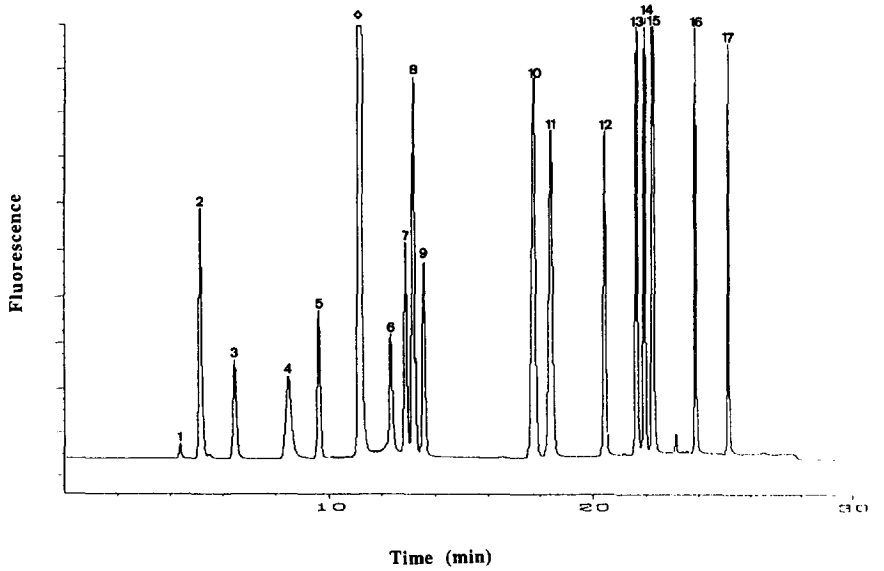

Fig. 1. Optimum chromatographic separation of the AQC–amine derivatives. The standard solution of the biogenic amines was about 1 mg l^{-1}. Peaks: 1 = ammonia; 2 = ethanolamine; 3 = methylamine; 4 = histamine; 5 = ethylamine; 6 = pyrrolidine; 7 = isopropylamine; 8 = propylamine; 9 = tyramine; 10 = butylamine; 11 = putrescine; 12 = cadaverine; 13 = phenethylamine; 14 = 3-methylbutylamine; 15 = amylamine; 16 = hexylamine; ◇ = peak corresponding to the excess of AQC. Experimental conditions are given in the text.

OPA, mainly for important amines such as cadaverine and putrescine. On the other hand, there is a change in the elution order for some amines. This change enables histamine to be determined more easily as it clearly elutes before the amino acid compounds, which is not observed when OPA is used.

The precision and linearity of the pre-column derivatization method were then examined. Data obtained from three replicates with ten different concentrations (0.1–10 mg l^{-1}) of biogenic amine standards were used to establish calibration graphs for each amine. As in other studies [19,29], heptylamine was used as the I.S. and was added at a level of 2 ppm. The calibration graphs were calculated by using either the peak area or the peak area adjusted for the incorporation of the I.S. as the dependent variable and the concentration of the amines as the independent variable. Both sets of data gave good r^2 values. Five replicates of standards at concentrations between 0.5 and 5 mg l^{-1} were then injected and quantified by using either the internal or the external standard quantification methods. Comparison of the results shows that there is no significant difference between them (less than 5% at all the concentration levels). As the use of an I.S. did not improve the precision of the calibration graphs and the concentration results did not differ significantly, the external standard quantification method was adopted as it takes less analytical time.

As can be observed, the slopes, and therefore the sensitivity which mainly corresponded to the least polar compounds, increase with respect to those obtained with OPA derivatives [30].

The detection limit (see also Table 2) was calculated from the amount of amine required to give $S/N = 3$. As is shown, the detection limits improve considerably with respect to other methods [19,29,30], mainly for putrescine, iso- and *n*-amylamine, phenethylamine and hexylamine because they reached 5 μg l^{-1}, as could be expected from their higher response factors. The highest detection limits belong to ammonia (50 μg l^{-1}), methylamine, histamine and pyrroldine

Table 2
Linearity of the fluorimetric detector response at 250 and 395 nm as excitation and emission wavelengths

Amine	Slope	Error	Intercept	Error	r^2	LOD[a] (μg l^{-1})
Ammonia	67 788	3330	10 807	633	0.991	50
Ethanolamine	54 458	913	12 664	866	0.999	10
Methylamine	54 285	1240	23 689	325	0.998	20
Histamine	25 717	951	6841	477	0.995	20
Ethylamine	54 817	1540	5634	490	0.998	15
Pyrrolidine	23 828	964	1897	163	0.999	20
Isopropylamine	51 761	964	2451	221	0.999	15
Propylamine	79 244	2770	2288	153	0.996	10
Tyramine	37 323	661	3620	318	0.999	15
Isobutylamine	129 359	4370	558	30	0.996	10
Putrescine	125 027	503	6511	592	0.995	5
Cadaverine	91 394	4600	977	109	0.993	10
Phenethylamine	125 874	4950	23 158	2350	0.998	5
3-Methylbutylamine	113 398	9800	9838	1000	0.999	5
Amylamine	200 695	14 800	46 527	4420	0.988	5
Hexylamine	98 594	1620	5968	614	0.999	5

Detection limit calculated from the amount of the standard amine required to obtain $S/N = 3$. The errors of the slope and intercept were calculated with the ULC computer program [36]. Range of linearity: 0.05–0.10 to 10–20 mg l^{-1}. Detection limits of the amines in wines according to the $S/N = 3$ rule: 100–500 μg l^{-1}.

[a] Limit of detection.

(20 $\mu g\ l^{-1}$), although they were also improved with respect to other previous methods. As could be expected, the detection limits increased for wines. This could be ascribed to the matrix effect and not to the derivatization reaction, because the reaction was tested and the yields obtained were high (90–100%). These derivatization yields were determined by adding amine standards at 0.05, 1 and 10 $mg\ l^{-1}$ to the wine before injection.

The method was used to determine biogenic amines in several wines from Tarragona. The samples were filtered through a 0.22-μm membrane filter before being injected into the chromatograph. Fig. 2 shows the chromatogram of a sample of red wine analysed under the conditions discussed above. Although there are only a few compounds in wines that have native fluorescence, there are many amino acids that can react, like amines, with AQC. As they are present in very high concentrations in wine, massive peaks are observed on the chromatogram, although the response of the amine derivatives is not affected. Further, the amino acids eluted during the first 5 min and, for this reason, they could not interfere with the analytes of interest.

The chromatogram of a typical sample of red wine shows that the amines were usually well resolved because of the selectivity of the fluorescence detection. The method was used to determine the amine contents of red, rosé and white wines from different D.O. ("mark of origin") in Tarragona (Table 3). As expected, the amine content was considerably lower in white than in red wines. Histamine and putrescine were the most abundant amines, a finding which has also been reported in previous studies.

4. Conclusions

AQC reagent has been shown to be a good alternative for determining biogenic amines in wines. It enables primary and secondary amines to be determined simultaneously by giving stable derivatives for a manual derivatization to be carried out, if necessary, with no analyte loss. In this work these amines were analysed by a fully automated method which gave better sensitivities

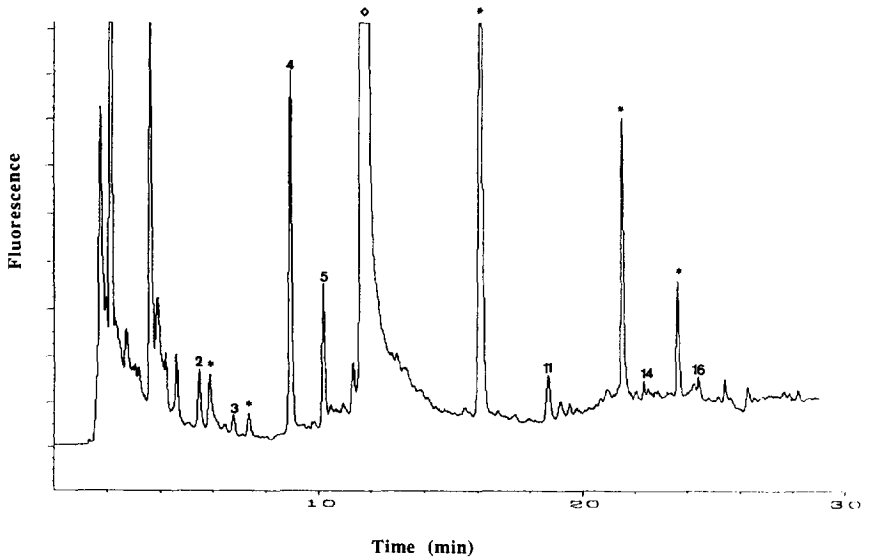

Fig. 2. Example of a red wine processed with the analytical procedure described. Peaks: 2 = ethanolamine; 3 = methylamine; 4 = histamine; 5 = ethylamine; 11 = putrescine; 14 = 3-methylbutylamine (closer to its LOD); 16 = hexylamine (closer to its LOD); ◇ = peak corresponding to the excess of AQC; * = unknown. Experimental conditions are given in the text.

Table 3
Amine contents (mg l^{-1}) in wines from the Tarragona region: results from triplicate direct injections of the samples

Amine	Red wines ($n = 44$)		Rosé wines ($n = 8$)		White wines ($n = 14$)	
	Range (mg l^{-1})	Median	Range (mg l^{-1})	Median	Range (mg l^{-1})	Median
Ethanolamine	3.24–ND[a]	2.12	2.01–ND	1.89	0.15–ND	0.10
Methylamine	1.17–0.41	0.69	0.92–0.25	0.59	1.21–ND	0.50
Histamine	13.5–0.66	5.13	5.18–0.46	2.48	3.46–ND	1.15
Ethylamine	5.68–ND	0.66	2.15–ND	ND	0.60–ND	ND
Isopropylamine	0.19–ND	ND	0.28–ND	ND	0.54–ND	0.10
Propylamine	0.28–ND	ND	0.11–ND	ND	0.17–ND	ND
Tyramine	ND	ND	ND	ND	0.33–ND	ND
Putrescine	5.04–ND	3.76	4.01–2.64	3.65	3.88–1.93	3.04
Cadaverine	0.71–ND	ND	0.34–ND	ND	1.43–ND	ND
Isoamylamine	0.79–ND	0.24	0.68–0.39	0.51	0.82–0.26	0.47
Hexylamine	0.96–ND	ND	0.21–ND	ND	0.40–ND	ND

[a] ND means that amine was not detected under the conditions described in the text.

for direct injection than those reported previously using OPA.

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