Gas Chromatography–Mass Spectrometry Assessment of Amines in Port Wine and Grape Juice after Fast Chloroformate Extraction/Derivatization

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ABSTRACT: A simple, reliable, and sensitive gas chromatography–mass spectrometry method for the quantification of volatile and nonvolatile biogenic amines in Port wines and grape juices was developed and evaluated. The method was based on a previously reported two-phase derivatization procedure with isobutyl chloroformate in a toluene medium, which provides a quantitative reaction in 10 min. Following the derivatization step, the excess of reagent was eliminated by treatment with alkaline methanol. The derivatization procedure was performed directly on 1 mL of sample, avoiding any fastidious and time-consuming cleanup extraction steps. The method allows the simultaneous quantification of 22 amines, which can be found in wines: methylamine, dimethylamine, ethylamine, diethylamine, propylamine, isopropylamine, butylamine, isobutylamine, amylamine, isoamylamine, 2-methylbutyl-amine, hexylamine, pyrrolidine, piperidine, morpholine, 1,3-diaminopropane, putrescine, cadaverine, 1,6-diaminohexane, 2-phenyl-ethylamine, histamine, and tyramine. Because of the fact that histamine and tyramine derivatives are degraded during the isobutyl chloroformate elimination step, the corresponding determination was made after removal of the excess of derivatizing reagent by evaporating an aliquot of the toluene layer obtained after the reaction. The presented method showed excellent analytical characteristics in what linearity, recovery, repeatability, and limit of detections were respected. It was used to assess the concentration of biogenic amines in juice grapes and Tawny and Vintage Port wines with different aging times. On the whole, the total content of amines in Port wines was low. Most of the amines found in wines have their origin in the raw material used for their elaboration, so the Port winemaking process is not prone to the production of this kind of compounds. Total biogenic amine contents have shown a decrease with the aging of both types of Port wines.

KEYWORDS: Port wines, grape juice, GC-MS, isobutyl chloroformate, biogenic amines

■ INTRODUCTION

The presence of biogenic amines in food and wine remains a problem that deserves cautious attention due to its human potential toxicity, widely reported in the literature.[1−4] In wines, the toxic effects of some amines are enhanced by ethanol, by direct or indirect inhibition of amine oxidases, responsible for amine catabolism in the gut.[5]

Despite the lack of legal limits, some countries have introduced recommended upper limits for histamine,[6−8] and there are ongoing discussions in the European Union (EU) regarding the need to regulate biogenic amines levels toward imported wines, being the prevailing outlook that similar regulations to those proposed for allergens should be introduced.[9]

Biogenic amines present in wine can be originated from two different sources, raw material and fermentation processes, usually as a result of decarboxylation of amino acids.[10] Thus, a wide variety of viticultural and enological factors may have an impact on the nature and levels found in wine. While some factors may influence the amount of amino acids precursors available in the grape and wine (grape variety, geographic region, vintage, extent of grape skin maceration, nutrients addition, and aging practices), other factors (such as nutrition status, pH, temperature, and SO_2 level) exert their influence mainly in the gene expression in microorganisms affecting decarboxylase activity.[11]

Despite the large number of authors who have studied the formation of biogenic amines in wines, many questions remain unanswered, and this is even more evident with regard to Port wine, given the scarcity of studies that have been committed. Port wines are sweet fortified wines of great prestige throughout the world, produced from selected grape varieties grown and processed in the demarcated Douro region on the North of Portugal, by a particular winemaking procedure. Shortly after the start of alcoholic fermentation, usually past 36−48 h, when the Baumé degree reaches 6.2, the bulk solids are removed and the partially fermented must is fortified by adding a neutral grape spirit (77°) known as "aguardente" (100 L to each 440 L of must) to stop fermentation, leaving residual sugar in wine and boosting the alcohol content until 18−20°. Then, the wine is maintained for 2 years in small oak barrels and finally aged in the bottle in the case of Vintage Port, which is made only in special years of exceptional quality. Tawny Ports are stored in oak barrels during several years, subjected to an oxidative aging process being bottled only at the time of commercialization. Up to now, only few authors have reported the presence of biogenic amines in Port wine,[12] and there is a notorious lack of studies about their formation/degradation during wine aging.

The quantification of biogenic amines in grape juices and Port wines is hampered by the complexity of the matrixes and the heterogeneity of the compounds involved, including primary and secondary monoamines, di- and polyamines, aromatic, and heterocyclic amines, some of them with a strong volatile nature, unlike others. Indeed, sensitive and selective methods must be developed to ensure a reliable determination of the compounds.

High-performance liquid chromatographic (HPLC) methods with spectrophotometric or fluorimetric detection are frequently used,[6,11,13] although they typically imply lengthy sample cleanup and preconcentration procedures to improve selectivity and sensitivity. The hyphenation of HPLC to mass spectrometry (MS) has also been used to increase method performance.[14] Contrarily to the usual practice in other analytical fields, as biochemistry or environmental analysis, the use of quantitative gas chromatographic methods to analyze biogenic amines in food matrices, and particularly in alcoholic beverages, has been restricted to a few authors. After the pioneering work of the group of Ough, who analyzed volatile biogenic amines in wines and grape juices by gas chromatography (GC)-NPD as their trifluoroacetyl derivatives,[15,16] Pfundstein et al.[17] developed a method for the determination of volatile biogenic amines based in its derivatization with benzene sulfonylchloride, and further detection by a thermal energy analyzer operated in nitrogen mode. In 2000, our group proposed two different GC-MS methods, one of them for the determination of di- and poly-amines and aromatic amines after derivatization with heptafluoro-butyric anhydride, in Port wine and grape juice,[12] and the other one for the single quantification of histamine as tris-pentafluo-robenzyl-histamine, in the same matrices.[18] In both methods, a previous ion pair extraction procedure was successfully used to extract the amines from the samples, characterized for high concentrations of sugars, among other interferents. Ngim et al.[19] determined primary alkylamines in wines with GC-MS after derivatization with pentafluorobenzaldehyde to the correspond-ing pentafluorobenzylimines. More recently, Paik et al.[20] used an ethoxycarbonyl reaction (with ethyl chloroformate) conducted in a two-phase mode with a pH shift, combined with a penta-fluorpropionyl acylation, followed by GC-MS analysis, for the simultaneous assay of alkylphenols and aliphatic and aromatic amines.

In the study here reported, a sensitive and accurate GC-MS method that enables the determination of all of the amines of interest in alcoholic beverages, volatile amines included, was developed and validated. The method was adapted from a proposal of Lundh and Akesson[21] for the determination of volatile amines, taking use of isobutyl chloroformate (IBCF) as a derivatizing reagent in a two-phase reaction system, and allowed the accurate identification and quantification of 22 amines, in a simple and quick way, excluding the need for a previous extractive procedure. The method was applied to the evaluation of biogenic amine levels in samples representative of the two main types of Port wines, Tawnies and Vintages, with different aging times, and in grape juices from the same varieties used for Port wine production.

■ EXPERIMENTAL SECTION

Reagents and Materials. The amine standards were obtained, mostly as hydrochloride salts, from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), and Fluka (Buchs, Switzerland). The deuterated internal standards (IS), ethyl[$^2H_5$]amine HCl, $\alpha,\alpha,\beta,\beta$-[$^2H_4$]histamine·2HCl,

methyl[$^2H_3$]amine·HCl, 1,4-butane[$^2H_8$]diamine·2HCl (or [$^2H_8$]-putrescine·2HCl), and 2,2,3,3,4,4,5,5[$^2H_8$]pyrrolidine were supplied by CDN isotopes (Québec, Canada) through Regie (Montlugon, France). The ISs, heptylamine, amphetamine and hydroxyamphetamine sulfate, were from Sigma. Stock standard solutions (2.0 mg/mL) of each free compound were prepared by weighing and dissolving in 0.1 M HCl; the solutions were stored at 4 °C in silanized screw-capped vials with solid PTFE-lined caps (Supelco, Bellefonte, PA). Working standard solutions were prepared by dilution and mixing of these solutions with 0.1 M HCl.

The derivatizing reagent IBCF was supplied by Sigma. Methanol and toluene of high purity were obtained from Fluka. A 0.1M HCl and 10 M NaOH were also obtained from Fluka; the latter was diluted with water at the time of analysis. Buffer solution pH 12 was from Riedel-de Haen (Seelze, Germany). All other chemicals were of analytical grade. The solution of alkaline methanol was prepared by dissolving KOH in methanol until saturation, followed by filtration through a 0.45 μm filter. Ultrahigh purity He (helium) for GC-MS and N_2 (nitrogen) for solvent evapora-tion were obtained from Gasin (Maia, Portugal).

Sampling. A total of 40 Port wine samples were analyzed. Twenty-five "Tawny" Port wine samples produced in the same winery from 2 to 26 years old were kindly offered by a Port wine company. These samples were kept in 550 L barrels in the same local until the analysis when 0.5 L was collected for dark glass bottles. Fifteen "Vintage" Ports dated from 3 to 30 years old were purchased in the market.

Twenty-four samples of grape juices from the most representative varieties used in Port wine production (Touriga Nacional, Touriga Franca, Tinta Barroca, Tinta Roriz, and Tinto Cão) were obtained in three consecutive years, from a vineyard located in the Douro valley. In the first 2 years, different locations of the vineyard were sampled. Grape juices were obtained from manual crushing of 5 kg of grapes of each sample and stored in 500 mL flasks filled to completion. Sodium azide was added as a preservative, and samples were kept at −20 °C until analysis.

Sample Preparation. Wines and grape juices were centrifuged (5 min at 3500 rpm), and then, a 5 mL sample of the clear supernatant was added with IS (100 μL of an HCl 0.1 M solution containing all of the standards at 50 μg/L). After thorough mixing, an 1 mL aliquot was transferred to a 4 mL silanized screw-capped glass vial containing 1 mL of toluene, the mixture was made alkaline with 1 mL of phosphate buffer 0.5 M (pH 12), and 25 μL of IBCF was added. The vial was shaken for 10 min and centrifuged (5 min at 3500 rpm), and the toluene (upper) layer was split into two portions. The main portion of the toluene layer (500 μL) was transferred to another vial, and 500 μL of alkaline methanol was added. The tube was shaken by hand 5 min, then 1.5 mL of 5 M NaOH was added, and the mixture was shaken for another 5 min. After the mixture was centrifuged, the toluene layer was used for analyzing all amines except tyramine and histamine.

A smaller portion of the toluene layer (250 μL) was transferred to an identical vial and evaporated to dryness under a stream of nitrogen. The dry residue was redissolved in 100 μL of toluene, and the solution was used for analysis of tyramine and histamine.

GC-MS Equipment and Conditions. The gas chromatograph 6890 (Agilent, Little Falls, DE) equipped with an electronically con-trolled split/splitless injection port was interfaced to a single quadropule inert mass selective detector (5973N, Agilent) with an electron impact ionization chamber.

GC separation was performed on HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, (J&W Scientific, Folsom, CA) preceded by a 2 m guard column of the same inner diameter connected to the column by a press frit glass union. Helium was the carrier gas with a constant pressure of 30 psi. The injection was made in pulsed splitless mode (injection pulse pressure, 32 psi; purge-off time, 45 s) at 280 °C. The oven temperature program was as follows: 100 °C held for 1.0 min,

Table 1. MS Conditions for GC-MS Analysis of Derivatized Biogenic Amines and IS (Time Windows and Ions Selected in SIM Mode, Quantification Ions Are Indicated in Bold)

injection	compounds	window time (min)	t_R (min)	$[M^+]^a$	m/z SIM ions (% relative abundance)
	[$^2H_3$] methylamine (IS)	3.2–5.0	3.62	134	**79** (92), 61 (100)
	methylamine		3.63	131	**76** (100), 58 (99), 88 (8)
	dimethylamine		3.78	145	**72** (86), 90 (100), 145 (2)
	[$^2H_5$] ethylamine (IS)		4.16	150	**95** (100), 77 (78)
	ethylamine		4.20	145	**90** (100), 72 (74), 130 (6)
	isopropylamine		4.50	159	**144** (100), 86 (57), 104 (60)
	diethylamine	5.0–8.0	5.21	173	**118** (58), 72 (32), 102 (46), 158 (24), 173 (4)
	propylamine		5.41	159	**104** (100), 86 (39), 130 (47)
	isobutylamine		6.26	173	**118** (34), 100 (11), 130 (63), 158 (3), 173 (5)
	butylamine		7.10	173	**118** (70), 100 (20), 130 (41), 158 (1), 173 (3)
	2-methylbutylamine	8.0–11.0	8.53	187	**187** (8), 114 (10), 130 (89), 132 (31)
	[$^2H_8$] pyrrolidine (IS)		8.55	179	**106** (50), 124 (100)
first	isoamylamine		8.65	187	**132** (56), 114 (17), 130 (56), 187 (8)
	pyrrolidine		8.67	171	**98** (53), 114 (48), 116 (100)
	morfoline		9.53	187	**116** (48), 114 (42), 130 (34), 187 (12)
	amylamine		9.67	187	**132** (99), 114 (18), 130 (80), 187 (5)
	piperidine		9.75	185	**128** (100), 112 (30), 130 (56)
	hexylamine	11.0–15.1	11.33	201	**146** (90), 130 (72), 128 (13)
	heptylamine (IS)		12.35	215	**160** (65), 142 (10), 130 (60)
	2-phenylethylamine		13.48	221	**221** (35), 91 (59), 104 (70), 130 (100), 148 (18)
	amphetamine (IS)		13.49	162	**144** (100), 162 (5), 91 (63)
	1,3-diaminopropane		14.87	274	**101** (86), 144 (62), 201 (16), 274 (8)
	[$^2H_8$] putrescine (IS)	15.1–25.0	15.37	296	**176** (36), 296 (9)
	putrescine		15.40	288	**170** (71), 130 (46), 288 (8)
	cadaverine		15.81	302	**130** (76), 84 (78), 129 (72), 302 (9)
	1,6-diaminohexane		16.23	316	**130** (88), 316 (6)
second	[$^2H_4$] histamine (IS)	3.2–25.0	11.93	315	**197** (65), 242 (7), 128 (15)
	histamine		11.99	311	**194** (93), 238 (9), 138 (22)
	hydroxyamphetamine (IS)		12.50	351	**144** (100), 107 (20)
	tyramine		12.62	337	**120** (100), 107 (55), 176 (12), 237 (5), 337 (2)

^{a} Corresponding to the molecular mass of the derivative.

ramped to 160 at 10 °C/min, then ramped to 280 at 25 °C/min, and held for 13.3 min. The total run time was 25 min. The MS transfer line temperature was held at 280 °C.

Mass spectrometric parameters were set as follows: electron impact ionization with 70 eV energy; ion source temperature 230 °C; and MS quadrupole temperature 150 °C. The MS system was routinely set in selective ion monitoring (SIM) mode, and each analyte was quantified based on peak area using one target and one or more qualifier ion (s). Complete SIM parameters and retention times of the analytes are shown in Table 1. Agilent Chemstation was used for data collection/processing and GC-MS control.

RESULTS AND DISCUSSION

Optimization of Extraction and Derivatization Conditions. Alkyl chloroformates constitute a group of derivatizing reagents with very favorable characteristics to the gas chromatographic determination of compounds with amino groups. These reagents convert, easily and quantitatively, the amines into carbamates, which exhibit good chromatographic properties. Furthermore, the derivatives formed usually present interesting mass spectra properties, being an invitation to the use of mass spectrometers detectors.

Among the chloroformates, the IBCF seems adequate for the purposes that oriented this work, that is, the simultaneous determinations of main biogenic amines present in alcoholic beverages and the corresponding raw material. However, during the optimization assays to adapt the procedure to this kind of determination, a rapid deterioration of the column performance occurs with the successive injections of the chloroformate extracts, which definitely hampers the achievement of results supporting the minimum criteria of precision and accuracy. To bypass this issue, the same solution described in a previous paper dealing with determination in biogenic amines in beers was adopted,[22] consisting in the elimination of the excess of IBCF by a solution of alkaline methanol after the derivatization reaction.

Of 22 biogenic amines studied, only both tyramine and histamine produced derivatives that were partially or totally degraded by the alkaline solution used to eliminate excess reagent. Because histamine and tyramine derivatives have a low volatility, they were analyzed after eliminating the excess of IBCF by evaporation, as reported in other studies about nonvolatiles amines. Under these analytical conditions, the behavior of tyramine and histamine derivatives was excellent.

For all other biogenic amines studied, the assessment experiments provided excellent results for all of the performance
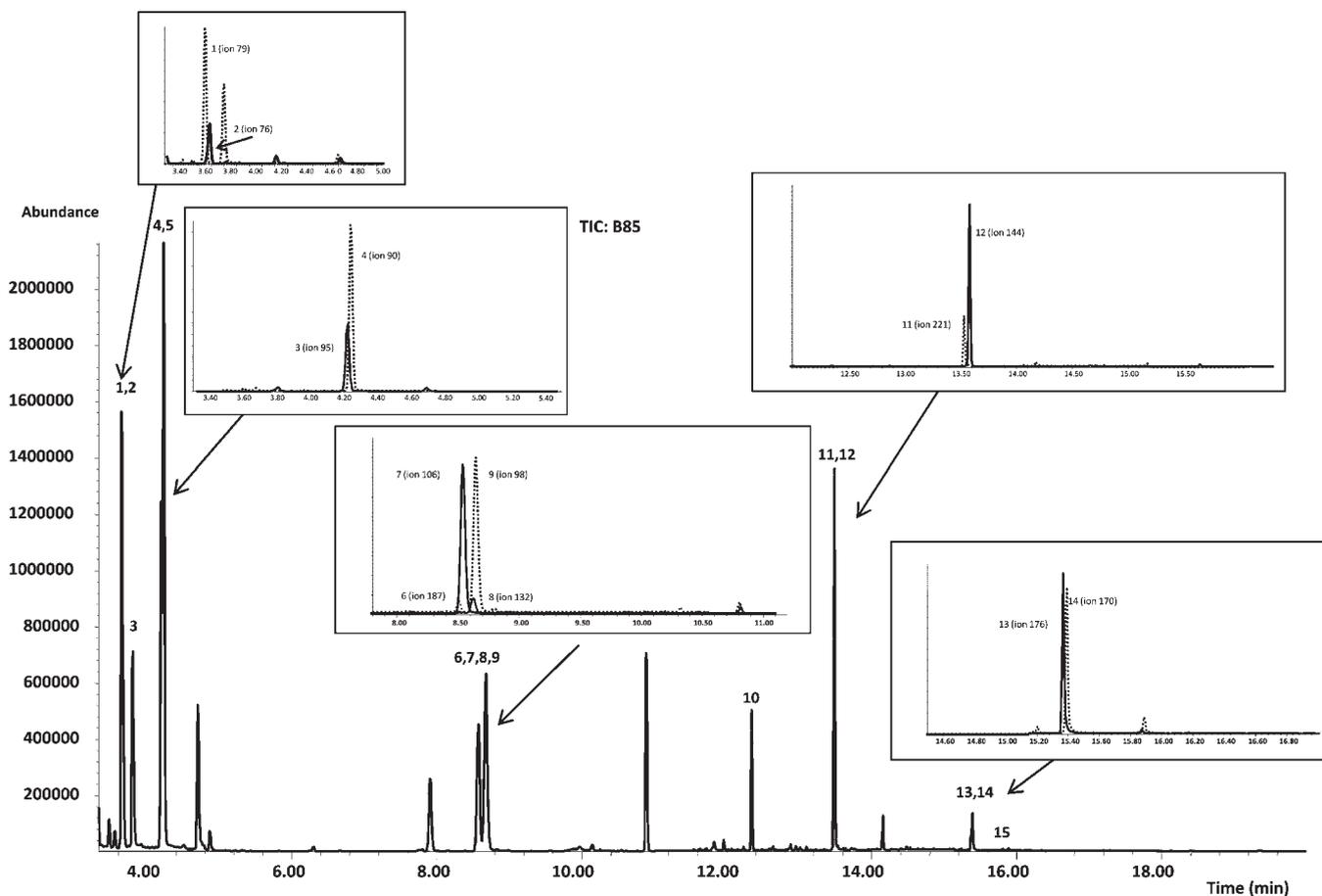
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Figure 1. Total ion chromatogram (TIC) corresponding to a Tawny Port wine sample containing (1) $[^2H_3]$ methylamine (1.00 mg/L), (2) methylamine (1.835 mg/L), (3) diethylamine (0.094 mg/L), (4) $[^2H_5]$ ethylamine (1.00 mg/L), (5) ethylamine (1.835 mg/L), (6) 2-methylbutilamine (0.018 mg/L), (7) $[^2H_8]$ pyrrolidine (1.00 mg/L), (8) isoamylamine (0.378 mg/L), (9) pyrrolidine (0.894 mg/L), (10) heptylamine (1.00 mg/L), (11) 2-phenylethylamine (0.356 mg/L), (12) amphetamine (1.00 mg/L), (13) $[^2H_8]$ putrescine (1.00 mg/L), (14) putrescine (0.773 mg/L), and (15) cadaverine (0.043 mg/L).

parameters. In Figure 1, a total ion chromatogram obtained from Tawny Port wine sample and some examples of the correspondent reconstructed ion chromatograms used for quantification are shown.

Method Performance. *Linearity.* Ten aqueous standards and 10 synthetic matrices, obtained as described by Fernandes and Ferreira,[12] containing all amines under study with concentrations ranging from 0.010 to 10 mg/L, were submitted to the whole analytical procedure. The results showed that linearity was excellent with correlation always higher than 0.9990 for all amines in both matrices. However, higher slopes were obtained for all amines using synthetic matrices; thus, it was used during the routine quantitative work. Amine concentrations were measured using the ratio of the peak areas of the target ion chosen for the amine and those for the corresponding IS. Multiple ISs were used in this study to improve the precision and accuracy of the analysis. Thus, ISs were the following: $[^2H_3]$ methylamine for methylamine and dimethylamine; $[^2H_5]$ ethylamine for ethylamine, diethylamine, propylamine, and isopropylamine; heptylamine for butylamine, isobutylamine, amylamine, isoamylamine, 2-methylbutylamine, and hexaxylamine; 2,2,3,3,4,4,5,5 $[^2H_8]$ pyrrolidine for pyrrolidine, piperidine, and morpholine; amphetamine for 2-phenylethylamine; $[^2H_8]$ putrescine for cadaverine, putrescine, and

1,3-diaminopropane; $\alpha,\alpha,\beta.\beta$-$[^2H_4]$histamine for histamine; and hydroxyamphetamine for tyramine.

Repeatability. The repeatability of the method was evaluated by performing 10 replicate analyses of both Port wine and grape juice samples. As shown in Table 2, the average of relative standard deviation (RSD) ranged from 0.7 (pyrrolidine) to 17.5% (isobutylamine) for the Port wine sample. For grape juice sample, the RSD ranged from 1.2 (ethylamine) to 11.9% (pyrrolidine). The results proved that the optimized method guarantees that all amines can be properly quantified.

Recovery. The reliability of the method was confirmed twice by recovery experiments performed in the two types of matrices under study: Port wine and grape juice. The same samples utilized for precision experiments were used spiked at six different levels of each of the studied amines and added with all of the ISs. Each sample was extracted and injected twice. The overall recovery was obtained by pooling all of the data for the same matrix ($n = 12$). Excellent recoveries were obtained for all amines ranging from 92 (2-phenylethylamine) to 111% (1,3-diaminopropane) for the Port Wine and from 92 (butylamine) to 112% (propylamine) for grape juice, as can be seen in Table 3.

Limit of Detection (LOD) and Limit of Quantification (LOQ). Under conditions of ideal performance of the GC-MS system, it was possible to detect (peak heights higher than three times the

Table 2. Analytical Precision of a Port Wine Sample and a Grape Juice Sample (n = 10)[a]

	Port wine		Grape juice	
compounds	average (mg/L)	RSD (%)	average (mg/L)	RSD (%)
methylamine	0.318	1.7	0.664	1.9
dimethylamine	0.086	1.5	0.067	5.2
ethylamine	2.426	1.2	1.266	1.2
isopropylamine	0.003	3.6	0.005	1.7
diethylamine				
propylamine				
isobutylamine	0.010	17.5	0.039	4.2
butylamine				
2-methylbutylamine	0.019	3.3	0.117	2.2
isoamylamine	0.430	1.9	1.168	1.4
pyrrolidine	0.635	0.7	0.005	11.9
morfoline				
amylamine				
piperidine				
hexylamine				
2-phenylethylamine	0.281	1.1	0.535	2.1
1,3-diaminopropane	0.027	1.8	0.016	3.4
putrescine	2.297	2.3	1.479	3.0
cadaverine	0.131	4.2	0.064	5.8
1,6-diaminohexane				
histamine	0.019	9.0	0.037	11.2
tyramine	0.508	4.1	0.039	6.7

[a] Blank entries = not detected.

baseline noise level) all of the amines under study at a concentration of 1 μg/L, which was established as the LOD. The LOQ was established as the lowest concentration assayed quantified with acceptable accuracy and precision (less than 15%), which were the lowest calibration levels of the calibration curve about 10 μg/L for all of the analytes. The LOD values are lower than those reported in the previous papers using MS detectors for most of the studied biogenic amines in wines, <10[12,18] and 1.8−36.8 μg/L.[20]

Analysis of Biogenic Amines in Juice Samples. A total of 24 samples of grape juices from the five varieties, 3 years, and different locations were analyzed. Results obtained for the biogenic amines are shown in Table 4.

The total content of amines in the 24 grape juice samples analyzed ranged from a minimum of 1.941 mg/L to a maximum of 13.794 mg/L, with a global average level of 5.939 mg/L. All of the five studied varieties showed similar average levels, from 4.887 mg/L in Touriga Nacional to 6.922 mg/L in Tinta Roriz.

Fifteen out of the 22 biogenic amines studied were found in the grape juice samples. Putrescine was the most abundant, ranging from 1.059 to 7.254 mg/L, with an average level of 3.024 mg/L, usually accounting for around 50% of the total amine content. It was followed by ethylamine (average level of 1.466 mg/L) and methylamine (average level of 0.595 mg/L) that roughly accounts for 25 and 10%, respectively, of the total amine content. Although in lower amounts, 2-phenylethylamine and histamine, two of the most health-concerning amines, were also found in all of the samples with average contents of 0.136 and 0.073 mg/L, respectively, while tyramine was only found in 11 samples, with an average level of 0.030 mg/L.

The presence of putrescine in grape juices, as well as cadaverine and two polyamines not included in this study, spermine and spermidine, was first reported by Desser et al.[23] and further confirmed by other authors.[7,24,25] In a previous work dealing with 11 samples of grape juice,[12] our group reported average levels of 2.118 mg/L for putrescine and 0.160 mg/L for cadaverine, which are in good agreement with the results obtained. More recently, Bover-Cid et al.[26] reported the presence of putrescine and cadaverine in 16 samples of Cabernet Sauvignon grape juices, with an average of 6.81 and 1.16 mg/kg, respectively. Del Prete et al.[27] found an average level of 11.23 mg/L of putrescine for 14 grape juices representative of seven different cultivars from the 2004 and 2005 vintages. Increased concentrations of putrescine in plants could be linked to potassium deficiencies in the soil.[28] The low levels found for 2-phenylethylamine, histamine, and tyramine are in agreement with the few references found in the literature.[8,12,23,25]

Regarding the most relevant volatile amines, ethylamine and methylamine, the reports about their presence in grape juices are scarce. In an original work of the group of Ough,[29] grape levels ranging from 0.150 to 4.900 mg/L were reported for ethylamine, while the same samples showed levels of methylamine ranging from 0.145 to 0.850 mg/L. The authors had observed an important influence of the degree of ripeness of grapes in the levels of these two compounds. The levels of methylamine tend to fall during the final stages of maturation, while by contrast the levels of ethylamine tend to rise in the same period. Del Prete et al.[27] found an average level of 9.83 mg/L of ethylamine for 14 grape juices above referred.

The presence of other volatile amines in grape juices was solely reported by the group of Ough,[12,29] which found traces of dimethylamine, diethylamine, n-propylamine, isobutylamine, n-amylamine, isoamylamine, and 2-methylbutylamine. In this study, we have confirmed the presence of all of the amines quoted, with the exception of n-propylamine and n-amylamine, while isopropylamine and pyrrolidine were for the first time found at trace levels in grape juices.

The presence of volatile amines in vegetable products could be explained by transamination of the correspondent amino acids in the presence of aldehydes[30] or, alternatively, by nonenzymatic decarboxylation of amino acids.[31] According to the same authors, the occurrence of enzymatic decarboxylation does not seem to have great expression. Whatever the procedure, methylamine, ethylamine, and isoamylamine may have their origins from glycine, alanine, and leucine, respectively.

Analysis of Biogenic Amines in Tawny and Vintage Port Wines. To evaluate the presence and the evolution of biogenic amines in Port wines, a total of 40 samples of Tawny (n = 25) and Vintage Ports (n = 15) with different aging times were analyzed by the developed method. Results are shown in Table 5.

On the whole, the total content of amines in Port wines was low, being the highest levels of 13.730 mg/L in Tawny Ports (average, 6.172 mg/L) and of 14.705 mg/L in Vintage Ports (average, 6.427 mg/L). Ninety percent of the wines (36/40 samples) had a biogenic amine content of less than 10 mg/L, which is the enological acceptable level.[4] Taking into account the amine contents above-reported for grape juices, it is likely that most of the amines found in wines have their origin in the raw material used for their elaboration, so the Port winemaking process is not prone to the production of this kind of compounds.

The two most abundant amines in both Tawny and Vintage Port wines were ethylamine and putrescine (both also

Table 3. Average Recovery of Biogenic Amines from Spiked Port Wine and Juice Grape Samples[a]

compounds	Port wine			Grape juice		
	initial amount (mg/L)[b]	average recovery (%)	RSD (%)	initial amount average (mg/L)[b]	average recovery (%)	RSD (%)
methylamine[c]	0.318	101	0.8	0.664	103	2.3
dimethylamine[c]	0.086	105	3.0	0.067	100	4.7
ethylamine[c]	2.426	101	4.5	1.266	106	9.9
isopropylamine[d]	0.003	103	1.6	0.005	98	1.1
di ethylamine[d]		105	1.4	0.053	94	1.3
propylamine[d]		99	3.7		112	11.1
isobutylamine[d]	0.010	109	2.6	0.039	96	1.7
butylamine[d]		110	2.3		92	2.8
2-methylbutylamine[d]	0.019	98	5.1	0.117	104	5.8
isoamylamine[c]	0.430	99	4.9	1.168	95	3.5
pyrrolidine[c]	0.635	97	3.4	0.005	98	2.6
morfoline[d]		108	2.1		98	6.4
amylamine[d]		102	2.1		98	2.8
piperidine[d]		103	1.5		98	1.7
hexylamine[d]		97	3.5		106	3.6
2-phenylethylamine[c]	0.281	92	3.6	0.535	105	2.9
1,3-diaminopropane[d]	0.006	111	7.9	0.016	94	5.2
putrescine[e]	2.297	110	8.9	1.479	104	4.1
cadaverine[c]	0.131	97	4.2	0.064	97	2.5
1,6-diaminohexane[d]		105	7.9		101	2.3
histamine[d]		85	2.8		95	0.9
tyramine[c]	0.508	99	5.2	0.039	96	4.8

[a] Blank entries = not detected. [b] Average of 10 replicate analyses. [c] Amounts added (mg/L): 0.100, 0.250, 0.500, 1.00, and 2.500. [d] Amounts added (mg/L): 0.05, 0.10, 0.250, 0.500, and 1.00. [e] Amounts added (mg/L): 0.250, 0.500, 0.500, 1.00, and 2.500.

predominant in grape juices), followed by isoamylamine, pyrrolidine, and 2-phenylethylamine. With few exceptions, these five compounds correspond to about 90% of the total amines found in each wine sample. Methylamine, dimethylamine, isobutylamine, 2-methylbutylamine, 1,3-diaminopropane, cadaverine, histamine, and tyramine were also detected. Two of the amines that were found at trace levels in grape juices, diethylamine and isopropylamine, were not detected in any of the wine samples.

Concerning nonvolatile amines, putrescine has proved to be the most abundant compound, ranging from 0.162 to 4.720 mg/L (average, 1.431 mg/L) in Tawny Ports and from 0.560 to 6.011 mg/L (average, 2.298 mg/L) in Vintage Ports. It was followed by 2-phenylethylamine, ranging from 0.182 to 2.338 mg/L (average, 0.502 mg/L) in Tawny Ports and from 0.039 to 0.935 mg/L (average, 0.307 mg/L) in Vintage Ports. Although in smaller quantities, cadaverine and tyramine were also detected in all of the samples. Histamine was found in only 15 of the 25 Tawny Port samples studied, and 1,3-diaminopropane was found in 14 Tawny Port samples and in all of the Vintage Port samples.

These results were in good agreement with results previously reported for Port wines by Fernandes and Ferreira,[12] which have shown average contents (n = 12) of 3.047, 0.553, 0.209, and 0.089 mg/L for putrescine, 2-phenylethylamine, cadaverine, and tyramine, respectively. Similar results are also found by Zee et al.[32] in Port type wines from Canada, which found average contents (n = 17) of 3.30, 2.17, and 0.023 mg/L for putrescine, tyramine, and cadaverine, respectively. In the case of putrescine, the results confirm that levels found in Port wines are significantly lower than those usually reported for table wines. As example, red Portuguese wines from different regions showed

average contents of 8.0 (Dão), 10.9 (Douro), and 17.3 mg/L (Alentejo).[33] For histamine, one of the amines of more toxicological concern together with tyramine the highest level found was 0.432 mg/L, in line with the reported by Fernandes and Ferreira,[18] considerably lower than the recommendations issued from many European countries whose guideline limits are in the range of 3−10 mg/L.[6] These findings are likely due to the specific features of Port winemaking, such as short and incomplete fermentation time, absence of malolactic fermentation, and alcohol fortification.

In what concerns volatile amines, as a whole, they typically account for more than 50% of the total biogenic amine content, being their presence more relevant in Tawny Ports, which show a mean relative proportion of 72.8%, against 55.6 in Vintage Ports. The most prominent are ethylamine (ranging from 0.895 to 3.954 mg/L in Tawny Ports and from 1.208 to 6.006 mg/L in Vintage Ports), isoamylamine (ranging from 0.101 to 5.393 mg/L in Tawny Ports and from 0.046 to 0.907 mg/L in Vintage Ports), pyrrolidine (ranging from 0.167 to 1.276 mg/L in Tawny Ports and from 0.053 to 0.181 mg/L in Vintage Ports), and methylamine (ranging from 0.147 to 0.447 mg/L in Tawny Ports and from 0.065 to 0.457 mg/L in Vintage Ports). Dimethylamine, isobutylamine, and 2-methylbutylamine were also found in almost all of the samples studied, although at levels considerably lower.

The prevalence of ethylamine and isoamylamine is in accordance with the literature, where they are described as the more constant and significant volatile amines in wines and grape juices.[11,34] Concerning pyrrolidine, it is the first time that its presence in Port wines is reported as far as we know. Its presence

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Table 4. Biogenic Amine Content (mg/L) in Grape Juices (n = 2)[a]

Grape Juices	Age and Location	Biogenic Amines (mg/L)															total
		methylamine	dimethylamine	ethylamine	diethylamine	isopropylamine	isobutylamine	2-methylbutylamine	isoamylamine	pyrrolidine	2-phenylethylamine	1,3-diaminopropane	putrescine	cadaverine	histamine	tyramine	
Touriga Nacional	1a	0.997	0.090	0.989	0.057	0.013		0.020	0.147	0.008	0.068	0.014	2.210	0.100	0.138	0.039	4.890
	1b	0.783	0.076	0.653	0.012	0.010		0.020	0.142	0.006	0.044	0.013	1.582	0.092	0.054		3.487
	1c	0.438	0.080	0.441	0.059	0.003		0.016	0.005	0.006	0.023	0.012	1.059	0.071	0.113		2.326
	1d	0.412	0.070	0.662	0.012	0.003		0.017	0.033	0.004	0.028	0.030	2.152	0.109	0.086		3.618
	2a	0.774	0.106	2.250	0.010	0.007	0.034	0.081	0.473	0.011	0.281	0.036	3.141	0.115	0.082	0.023	7.424
	2b	0.706	0.067	1.597	0.015	0.005	0.032	0.076	0.097	0.007	0.327	0.029	2.834	0.133	0.067	0.016	6.008
	3	0.362	0.116	1.249	0.072	0.002	0.002	0.051	0.291	0.008	0.102	0.032	3.867	0.262	0.043		6.459
	mean	**0.639**	**0.086**	**1.120**	**0.034**	**0.006**	**0.023**	**0.040**	**0.170**	**0.007**	**0.125**	**0.024**	**2.406**	**0.126**	**0.083**	**0.026**	**4.887**
Touriga Franca	1a	0.507	0.102	1.565	0.022	0.022	0.006	0.040	0.543	0.006	0.059	0.011	2.601	0.460	0.121		6.065
	1b	0.451	0.086	1.140	0.022	0.022	0.003	0.024	0.063	0.006	0.043	0.009	2.832	0.440	0.140		5.281
	2a	0.512	0.077	1.143	0.010	0.010	0.021	0.066	0.139	0.005	0.375	0.014	2.194	0.219	0.109	0.011	4.905
	2b	0.586	0.103	0.795	0.011	0.011	0.008	0.030	0.293	0.008	0.087	0.015	2.707	0.723	0.105		5.482
	2c	0.798	0.091	3.071	0.011	0.011	0.004	0.037	0.329	0.006	0.312	0.010	2.089	0.276	0.036	0.038	7.119
	3	0.768	0.090	2.410	0.008	0.008	0.014	0.044	0.333	0.008	0.189	0.020	2.896	0.234	0.059		7.081
	mean	**0.604**	**0.092**	**1.687**	**0.014**	**0.014**	**0.009**	**0.6040**	**0.283**	**0.007**	**0.178**	**0.013**	**2.553**	**0.392**	**0.095**	**0.025**	**5.989**
Tinta Barroca	1	0.349	0.094	1.177	0.030	0.024		0.022	0.090	0.006	0.071	0.011	3.310	0.587	0.102	0.018	5.891
	2	0.664	0.067	1.266	0.053	0.005	0.039	0.117	1.168	0.005	0.486	0.014	1.485	0.069	0.131	0.015	5.584
	3	0.388	0.061	1.100	0.011	0.002		0.016	0.025	0.005	0.020	0.017	4.150	0.070	0.077		5.942
	mean	**0.467**	**0.074**	**1.181**	**0.031**	**0.010**	**0.039**	**0.052**	**0.428**	**0.005**	**0.192**	**0.014**	**2.982**	**0.242**	**0.103**	**0.017**	**5.806**
Tinta Roriz	1	0.246	0.041	0.322	0.009	0.002		0.016	0.022	0.003	0.103	0.011	1.075	0.074	0.017	nd	1.941
	2a	0.596	0.072	3.211	0.011	0.002	0.038	0.092	0.508	0.009	0.271	0.038	3.695	0.236	0.028	0.013	8.820
	2b	0.943	0.058	4.547	0.011	0.003	0.010	0.032	0.465	0.006	0.094	0.084	7.254	0.263	0.024		13.794
	3	0.580	0.048	0.337	0.009	0.001		0.016	0.006	0.005	0.030	0.023	1.979	0.073	0.026		3.133
	mean	**0.591**	**0.055**	**2.104**	**0.010**	**0.002**	**0.024**	**0.039**	**0.250**	**0.006**	**0.125**	**0.039**	**3.501**	**0.162**	**0.024**	**0.013**	**6.922**
Tinto Cão	1a	0.600	0.071	1.674	0.010	0.002	0.009	0.032	0.153	0.005	0.102	0.019	4.671	0.030	0.054	0.066	7.498
	1b	0.602	0.091	0.576	0.008	0.002		0.018	0.027	0.003	0.022	0.011	1.700	0.030	0.047	0.071	3.208
	2	0.732	0.090	1.523	0.009	0.002	0.004	0.032	0.456	0.005	0.110	0.016	3.900	0.034	0.078	0.067	7.058
	3	0.764	0.058	1.182	0.009	0.009		0.018	0.009	0.003	0.014	0.024	4.433	0.025	0.054		6.602
	mean	**0.675**	**0.078**	**1.239**	**0.009**	**0.004**	**0.007**	**0.025**	**0.061**	**0.004**	**0.062**	**0.018**	**3.676**	**0.030**	**0.058**	**0.068**	**6.092**

[a] Blank entries = not detected.

Table 5. Biogenic Amine Content (mg/L) in Tawny and Vintage Port Wines ($n = 2$)[a]

Wines	Age								Biogenic Amines (mg/L)						
		methylamine	dimethylamine	ethylamine	isobutylamine	2-methylbutylamine	isoamylamine	pyrrolidine	2-phenylethylamine	1,3-diaminopropane	putrescine	cadaverine	histamine	tyramine	total
Tawny	26	0.147	0.067	0.963	0.021	0.076	1.271	1.109	0.607	0.009	0.203	0.001		0.060	4.544
	25	0.161	0.056	0.895	0.014	0.047	1.106	0.968	0.498	0.007	0.162	0.007		0.037	3.958
	24	0.160	0.088	1.484	0.010	0.025	0.591	1.233	0.580	0.010	0.207	0.010		0.041	4.439
	23	0.174	0.063	1.469	0.010	0.022	0.572	1.276	0.462	0.008	0.372	0.021		0.036	4.485
	22	0.184	0.067	1.740	0.007	0.011	0.275	1.052	0.340	0.011	0.317	0.012		0.035	4.051
	21	0.179	0.066	1.178	0.006	0.009	0.308	0.708	0.254	0.007	0.247	0.006		0.038	3.006
	20	0.204	0.079	1.519	0.009	0.021	0.478	1.012	0.388	0.011	0.381	0.018		0.056	4.176
	19	0.215	0.024	1.216	0.005	0.017	0.353	0.963	0.305		0.244	0.021		0.064	3.427
	18	0.276	0.095	3.339	0.002	0.012	0.249	1.202	0.349		0.617	0.027	0.050	0.035	6.253
	17	0.184	0.093	1.588	0.005	0.017	0.375	0.758	0.485		0.532	0.027		0.044	4.108
	16	0.245	0.094	1.623	0.003	0.016	0.371	1.064	0.376		0.674	0.053	0.005	0.026	4.550
	15	0.258	0.094	1.835	0.002	0.018	0.378	0.894	0.339		0.711	0.035	0.012	0.116	4.692
	14	0.180	0.095	1.552	0.004	0.020	0.440	0.591	0.448		0.429	0.068		0.049	3.833
	13	0.314	0.105	3.057		0.009	0.180	0.733	0.316		1.438	0.053	0.011	0.049	6.265
	12	0.302	0.091	2.397	0.001	0.009	0.127	0.701	0.278		1.049	0.068	0.005	0.018	5.046
	11	0.278	0.098	1.628	0.003	0.003	0.101	0.452	0.182		1.070	0.053	0.012	0.378	4.258
	10	0.322	0.099	3.742	0.002	0.035	0.169	0.444	0.220		1.705	0.076	0.019	0.309	7.142
	9	0.327	0.083	3.954		0.014	0.351	0.422	0.434	0.004	2.817	0.100	0.018	0.034	8.558
	8	0.318	0.086	2.426	0.010	0.019	0.375	0.635	0.287	0.007	2.420	0.116	0.026	0.451	7.176
	7	0.269	0.066	1.043	0.032	0.289	5.393	0.466	2.338		1.545	0.193	0.078	0.032	11.744
	6	0.189	0.068	1.899	0.006	0.008	0.155	0.585	0.385	0.005	2.217	0.113	0.032	0.032	5.694
	5	0.269	0.109	3.735		0.005	0.513	0.492	0.434	0.009	3.752	0.196	0.034	0.041	9.589
	4	0.275	0.091	1.836		0.032	1.134	0.193	0.610	0.016	3.300	0.302	0.074	0.068	7.931
	3	0.447	0.087	4.151		0.128	2.605	0.266	0.914	0.017	4.638	0.287	0.432	0.205	13.730
	2	0.384	0.084	3.237		0.083	2.124	0.167	0.718	0.033	4.720	0.240	0.183	0.050	11.639
	mean	**0.236**	**0.082**	**2.140**	**0.008**	**0.038**	**0.800**	**0.735**	**0.502**	**0.011**	**1.431**	**0.083**	**0.066**	**0.092**	**5.305**

Table 5. Continued

Wines	Age	Biogenic Amines (mg/L)													
		methylamine	dimethylamine	ethylamine	isobutylamine	2-methylbutylamine	isoamylamine	pyrrolidine	2-phenylethylamine	1,3-diaminopropane	putrescine	cadaverine	histamine	tyramine	total
Vintage	30	0.065	0.090	2.653	0.002	0.010	0.079	0.181	0.118	0.008	0.560	3.857		0.011	3.857
	25	0.158	0.086	1.305	0.009	0.049	0.907	0.113	0.697	0.070	1.349	0.106		0.420	5.269
	23	0.106	0.081	2.122	0.003	0.010	0.099	0.126	0.159	0.011	1.315	0.094		0.028	4.454
	22	0.190	0.087	2.878	0.006	0.025	0.200	0.143	0.262	0.010	2.122	0.144		0.025	6.062
	21	0.153	0.097	1.349	0.004	0.025	0.353	0.113	0.295	0.034	1.742	0.115		0.046	4.326
	20	0.165	0.087	1.738	0.003	0.012	0.181	0.103	0.114	0.020	1.739	0.129		0.012	4.303
	18	0.151	0.088	2.004	0.005	0.011	0.143	0.097	0.086	0.021	2.049	0.111		0.010	4.773
	17	0.266	0.079	2.967	0.008	0.038	0.667	0.137	0.935	0.017	1.709	0.131		0.010	7.054
	15	0.144	0.085	1.208		0.006	0.017	0.053	0.039	0.022	2.025	0.148		0.013	3.760
	13	0.307	0.122	4.854	0.002	0.007	0.063	0.064	0.121	0.021	2.055	0.110		0.047	7.773
	11	0.251	0.081	1.829		0.009	0.046	0.079	0.096	0.041	1.857	0.133		0.013	4.435
	9	0.285	0.105	4.712	0.004	0.016	0.266	0.085	0.183	0.082	3.915	0.159		0.168	9.980
	6	0.336	0.093	2.928	0.008	0.049	0.870	0.092	0.650	0.246	3.710	0.226		0.075	9.283
	5	0.457	0.101	6.006	0.011	0.043	0.833	0.085	0.529	0.293	6.011	0.215		0.121	14.705
	3	0.294	0.078	3.164	0.005	0.019	0.209	0.040	0.319	0.043	2.305	0.174		0.021	6.671
	mean	**0.222**	**0.091**	**2.781**	**0.005**	**0.022**	**0.329**	**0.101**	**0.307**	**0.063**	**2.298**	**0.136**		**0.074**	**6.427**

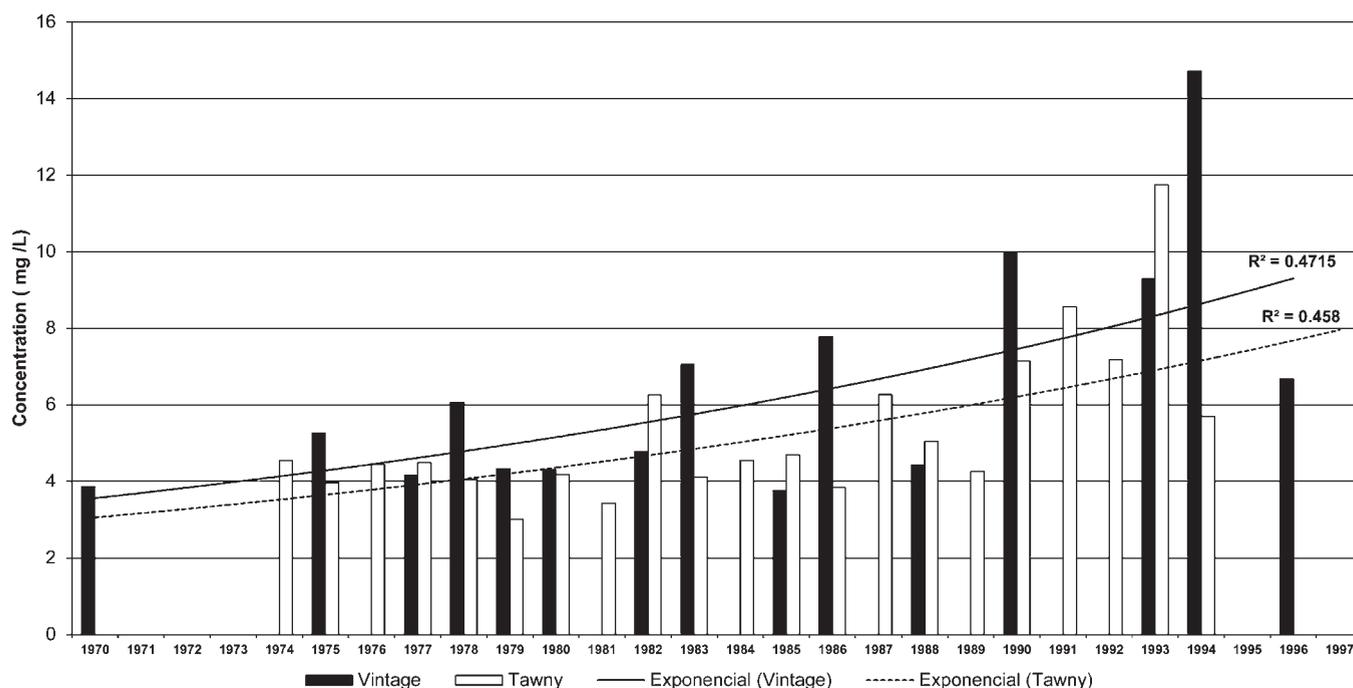[a] Blank entries = not detected.

Figure 2. Total content (mg/L) of biogenic amines in Tawny and Vintage Port wine samples.

in table wines was reported by Pfundstein et al.,[17] who found an average level of 0.2 mg/L in nine wine samples, with a maximum of 0.4 mg/L, and by Ibe et al.,[35] who found the compound in 16 of 32 red wine samples, with an average level of 0.070 mg/L and a maximum of 0.180 mg/L. More recently, Moreno et al.[36] and Marco and Azpilicueta[37] reported pyrrolidine levels of 0.9 and 0.5 mg/L, respectively, in a red and a white monovarietal wines used for storage experiments at different temperatures.

Pyrrolidine corresponds to the decarboxylation product of proline. Given that proline is usually the most abundant amino acid in wines, representing 35−80% of the total amino acid content,[6] because it is not usually metabolized by yeast during the fermentation, there is a strong possibility to be a source of most of the pyrrolidine found. The presence of pyrrolidine, as well as dimethyl- and diethylamine, can be a cause of concern due to its role in the formation of nitrosamine compounds, although the presence of the latter in wines was not detected to date.[38]

An overall evaluation of the results obtained has allowed the recognition of some characteristic patterns correlated to each of the two types of Port wines studied and to their aging time.

Generally, the two most abundant amines found, ethylamine and putrescine, were present in higher amounts in Vintage Ports than in Tawny Ports. By contrast, the contents of pyrrolidine and also of isoamylamine are considerably higher in Tawny Ports. These findings may be explained by the different conditions, to which the two types of wines are subjected, a reducing atmosphere in the case of Vintage samples, or an oxidizing environment, typical of Tawnies aging.

More outstandingly, it was possible to observe for the first time clear evidence about the behavior of some biogenic amines throughout the aging of Port wines. Considering the total content of amines, a trend toward the decline of the levels with increasing age of the wine is noticeable, as can be seen by the exponential lines in the Figure 2. This decline is mainly triggered by the levels of putrescine, which show a clear tendency to

diminish over the aging, in both Tawny and Vintage Ports. A similar trend was also observed for cadaverine, although at levels 15−20 times lower, which is likely due to their structural similarity; thus, the chemical pathways leading to their degradation are probably the same. In a less marked way, methylamine and ethylamine also showed a clear bias to decreased levels with increasing age of the wines. Various researcher groups reported a general decrease or stabilization in the concentration of biogenic amines in table wines during the storage, after an initial increase.[11,39] Biogenic amines can be degraded by oxidase enzymes present in some bacteria toward the end of the aging period, even at wine pH.[8,36] Unlike putrescine and the other amines above cited, pyrrolidine showed a clear trend to increased levels over the aging in both Port types, although the levels found in Vintage samples have been well below than those reported for Tawny Ports. As stated before, it is our opinion that there is a strong possibility of being proline, usually the amino acid more abundant in the wines, the source of most of the pyrrolidine formed. The knowledge of pyrrolidine levels could therefore constitute an important contribution, together with other parameters, to institute a dating system of Port wines based on their analytical characterization.

In conclusion, the proposed method was suitable to evaluate the presence of biogenic amines in grape juices and Port wines, providing a good separation and high sensitivity for all of the amines. The performance characteristics obtained within the validation study such as linearity, recovery, repeatability, and LOD were very good as compared to previously reported methods. Overall, the total concentration of amines in Port wines proved to be low when compared with table wines, which is likely to be justified by the specificity of the winemaking procedure. Ethylamine and putrescine were found to be the two most abundant biogenic amines in both types of Port wines, followed by isoamylamine, pyrrolidine, and 2-phenylethylamine. The main differences between Tawny and Vintage Ports were the

higher levels of pyrrolidine and isoamylamine found in Tawny Ports, while ethylamine and putrescine were more abundant in Vintage Ports. The content of some amines such as putrescine, cadaverine, ethylamine, and methylamine was shown to be correlated with the aging, as higher levels were found in younger wines. Inversely, pyrrolidine, which is for the first time reported in Port wines, showed a clear trend to increase with wine aging.

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