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# Combined ion-pair extraction and gas chromatography–mass spectrometry for the simultaneous determination of diamines, polyamines and aromatic amines in Port wine and grape juice

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**Abstract**

An accurate and very sensitive method which allows for the simultaneous determination of the diamines (1,3-diaminopropane, putrescine and cadaverine), of the polyamines (spermidine and spermine), and of the aromatic amines (β-phenylethylamine and tyramine) found in Port wines and corresponding grape juices is presented. Sample clean-up consisted of the extraction of the amines with the ion-pairing reagent bis-2-ethylhexylphosphate dissolved in chloroform followed by a back-extraction with 0.1 *M* HCl. The hydrochloric extract obtained was dried and the amines were further derivatized with heptafluorobutyric anhydride and analyzed by GC–MS in the selected ion-monitoring mode, with a total run time of 18 min. Under the adopted conditions, the extraction of all the studied compounds was almost complete and the obtained extracts were free of potential interferents present in the samples, namely sugars, and most of the aminoacids and polyphenols. Via the use of a set of five selected internal standards (amphetamine, [$^{2}H_{8}$]putrescine, 1,7-diaminoheptane, norspermidine and norspermine), the data obtained from the linearity, repeatability and recovery experiments were very good for all the compounds assayed. The corresponding limits of detection were invariably below 10 $\mu g\ l^{-1}$. The method was successfully applied to measure the content of biogenic amines in twelve young and five aged Port wine samples, eleven grape juice samples as well as in ten Portuguese red and white table wines. Results are presented and briefly discussed.
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## 1. Introduction

Biogenic amines are a group of biologically active compounds widespread in nature. They can be found in both raw and processed foods, formed by the natural metabolic pathways of the raw material or, more commonly, resulting from the decarboxylation of the corresponding aminoacids by action of spoilage and also fermentation microorganisms [1–5]. Many of these compounds are reported to be psychoactive and vasoactive and, when ingested in large quantities, they may be the cause of serious problems related to food poisoning [6–8].

Among the most important biogenic amines often present in foods, the diamines putrescine and cadaverine attracted considerable attention. Some authors suggested a synergistic relationship between these diamines and histamine, responsible for some cases of food intoxication [7]. In addition, putrescine and cadaverine might be converted by heating or

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cooking to pyrrolidine and piperidine, respectively [9]. These secondary amines, as well as the natural occurring polyamines spermidine and spermine, may undergo nitrosation and form nitrosamines which are considered highly carcinogenic [1,9]. In turn, the aromatic amines, tyramine and β-phenylethylamine are the principal agents responsible for dietary-induced migraine and a number of other adverse effects, including hypertensive crisis, which may be particularly serious in patients with a reduced activity of the monoamine oxidase [6,10].

The presence of biogenic amines in wines was first described in 1954 by Tarantola [11]. Since then, knowledge on this subject has increased greatly as a result of much work performed on biogenic amines, regarded as compounds directly or indirectly toxic. Data available in literature were excellently reviewed by Radler and Fäth [12] in a comprehensive paper, yet, the number of research studies have meanwhile drastically increased [13–21]. Nevertheless, scientifically sound information on the presence of biogenic amines in Port wines and corresponding musts used for their elaboration, remain scarce.

Irrespective of the type of sample, the determination of biogenic amines present at low concentration is difficult to achieve due to two main reasons: the polar character of the compounds which makes their isolation from aqueous matrices difficult to achieve [22] and the absence of intrinsic physicochemical properties, prohibits direct detection (i.e. detection without derivatization) via spectrophotometric or fluorimetric methods.

The analytical methods used in the analysis of biogenic amines found in wines were reviewed by Busto et al. [23] and by Lehtonen [24]. The determination is generally accomplished by high-performance liquid chromatographic (HPLC) methods, which includes pre- or post-column derivatization and the fluorimetric detection of the corresponding derivatives. Among the several fluorescence labelling reagents used, *o*-phthalaldehyde (OPA), generally in the presence of mercaptoethanol, is the one preferred by most of the authors [13–21,25–31]. OPA reacts with amines quickly and gives rise to highly fluorescent derivatives. However, the procedure has some drawbacks. OPA reacts with primary amines only, which prevents the determination of polyamines such as spermine and spermidine, and some of the derivatives formed reveal a limited stability. Although a clean-up procedure is often undertaken prior to chromatography, it is usually the presence of some interferents (e.g. amino acids) in the obtained chromatograms which prevent a well-defined resolution of some amine derivatives from being obtained, especially when present in low amounts.

It is common practice in analytical biochemistry or environmental analysis, to use GC methods for the determination of amines in the various matrices [32,33]. In contrast, in wines, the use of these type of methodologies is practically restricted to the group of Ough et al. [34,35], who analyzed volatile biogenic amines in the form of their trifluoroacetyl derivatives. However, the high resolution, the great sensitivity and the opportunity to develop very accurate isotope dilution methods make the GC–MS methods very attractive for such analysis.

In the present study, we described a very sensitive and accurate method for the determination of diamines, polyamines and aromatic amines in Port wines and corresponding grape juices. The amines were extracted from previously dealcoholised and buffered samples by the ion-pairing reagent bis-2-ethylhexylphosphate dissolved in chloroform, followed by a back-extraction with 0.1 *M* HCl and then converted to the corresponding heptafluorobutyric derivatives. Gas chromatography – electron impact mass spectrometry in the selected ion-monitoring mode (SIM) was finally used in the analysis. Amphetamine, [$^{2}H_{8}$]putrescine, norspermidine and norspermine were used as internal standards for the quantification of β-phenylethylamine, putrescine, spermidine and spermine, respectively, while 1,7-diaminoheptane (or alternatively, [$^{2}H_{8}$]putrescine) was used as the general internal standard for the other amines studied. Data in support of method reliability, is provided in terms of accuracy, reproducibility and linearity.

## 2. Experimental

### 2.1. Chemicals

Chemical standards of the various amines in the form of hydrochloric salts, were purchased from

Sigma [St. Louis, MO, USA; 1,3-diaminopropane (Dap), putrescine (Put), cadaverine (Cad) and tyramine (Tyr)] and from Aldrich [Milwaukee, WI, USA; β-phenylethylamine (β-Phe), 1,6-diaminohexane (Dah), spermidine (Spd) and spermine (Sp)]. The internal standards, 1,7-diaminoheptane, *N*-(3-aminopropyl)-1,3-propanediamine (norspermidine) and *N*,*N′*-bis(-(3-aminopropyl)-1,3-propanediamine (norspermine) were obtained from Aldrich and amphetamine was provided by Sigma. The deuterated analogue, ([$^{2}H_{8}$]Putrescine) dihydrochloride, was supplied by CDN Isotopes (Québec, Canada). Stock standard solutions of each compound at concentration of 2.0 mg $ml^{-1}$ (free compound) were prepared in 0.1 *M* HCl and stored at 4°C in silanized PTFE lined screw capped vials (Supelco, Bellefonte, PA, USA). Working standard solutions were prepared by dilution and mixing of these solutions with 0.1 *M* HCl.

The ion-pair reagent bis-2-ethylhexylphosphate (BEHPA) and the derivatizing agent heptafluorobutyric anhydride (HFBA) were obtained from Aldrich. Acetonitrile, chloroform, dichloromethane, ethyl acetate and methanol (all of them stored over a molecular sieve and with purity >99.5%) were obtained from Fluka (Buchs, Switzerland). All the other reagents were analytical grade. Water was prepared by purifying demineralized water in a ''Seral'' system (Seralpur Pro 90 CN).

## 2.2. Samples

Port wine samples corresponded to young and aged Ports, were kindly offered by a firm of the wine sector. Such samples had been stored in a cellar in 550 l oak casks generally used for the elaboration of blending Tawny Ports. Upon arrival at the laboratory, those samples were stored in 500 ml bottles at room temperature, protected from sunlight and were opened only on the moment of analysis. Table wines were commercial Portuguese DOC wines available in 750 ml bottles and purchased from the local retail market. The samples were stored at the laboratory in a similar manner to that of Port wines. The grape juices were obtained from manual crushing of grapes used in the production of Port wine, stored in 500 ml bottles filled to completion, with sodium azide (100 mg $l^{-1}$) added as preservative, and kept at −20°C until analysis.

## 2.3. Standard solutions

Standard solutions used for constructing the calibration curves were prepared by dissolving the required amount of the various amines in synthetic matrices of known composition similar to the composition of authentic wines and juices. Three different synthetic solutions were used pertaining to the type of sample to be analyzed, namely Port wines, table wines or grape juices, respectively (Table 1).

## 2.4. Ion-pair extraction

### 2.4.1. Wines

The wine samples (10.0 ml) were added with 100 μl of a solution of the five internal standards ([$^{2}H_{8}$]putrescine 200 μg $ml^{-1}$, amphetamine and 1,7-diaminoheptane 100 μg $ml^{-1}$ each, norspermidine and norspermine 20 μg $ml^{-1}$ each) and acidified with 1.0 ml of 2 *M* HCl. After standing overnight at 4°C (in order to promote the precipitation of some proteinaceous and polyphenolic material), the samples were centrifuged and decanted. In order to eliminate the ethanol, the supernatant was further evaporated under nitrogen to 8–8.5 ml (Port wines) or 9–9.5 ml (table wines). The pH was then adjusted to 7.4 by dropwise addition of concentrated potassium hydroxide solution and, finally, 2.0 ml of 0.2 *M* phosphate buffer (pH 7.4) were added. A 5-ml aliquot of the buffered and dealcoholised sample was extracted with 5 ml of 0.1 *M* BEHPA solution in chloroform by thorough hand-mixing for 5 min and subsequent stirring for 30 s on a vortex in a 15 ml centrifuge tube; the mixture was subsequently centrifuged at 3500 *g* for 5 min. A 4 ml portion of the chloroformic phase (bottom layer) was taken up to a second centrifuge tube which contained the same volume of 0.1 *M* HCl and, by shaking as previously described, the amines were back-extracted into the aqueous phase. Upon separation by centrifugation, the resulting aqueous phase (upper layer) was ready for derivatization and subsequent chromatographic analysis.

Table 1
Composition of the synthetic solutions used as matrices of the standard (calibrating) solutions of amines

| | Port wine | Grape juice | Table wine |
|---|---|---|---|
| Ethanol | 200 ml | – | 120 ml |
| Glycerol | 6 g | – | 6 g |
| Fructose | 70 g | 125 g | 0.2 g |
| Glucose | 40 g | 125 g | – |
| Tartaric acid | 1.75 g | 3 g | 2 g |
| Malic acid | 1 g | 1.5 g | 0.5 g |
| Lactic acid | 0.25 g | – | 1 g |
| Citric acid | 0.4 g | 0.4 g | 0.4 g |
| Acetic acid | 0.5 g | – | 0.5 g |
| Succinic acid | 0.5 g | – | 1 g |
| Phosphate | 0.2 g | 0.2 g | 0.2 g |
| Tanic acid | 1.8 g | 1.8 g | 1.8 g |
| Acetaldehyde | 0.1 g | – | 0.1 g |
| Sodium chloride | 0.1 g | 0.1 g | 0.1 g |
| pH | adjusted to 3.5 | adjusted to 3.8 | adjusted to 3.4 |
| Water | Q.s. 1000 ml[a] | Q.s. 1000 ml | Q.s. 1000 ml |

[a] Q.s., sufficient quantity.

### *2.4.2. Grape juices*

The grape juice samples were unfrozen and were initially centrifuged for separation of the solid portions. An 8-ml aliquot of the supernatant was added with 100 μl of a solution of the five internal standards (see above) and acidified with 1.0 ml of 2 *M* HCl. After standing overnight at 4°C, the samples were centrifuged and decanted. The pH was then adjusted to 7.4 and 2.0 ml of 0.2 *M* phosphate buffer (pH 7.4) was added. The remaining process is similar to that previously described for wines.

### *2.4.3. Standard solutions*

The appropriate volumes of the respective synthetic matrices (10 ml for wine analysis and 8 ml for grape juice analysis) were added with 50 μl of a solution containing the required amount of each studied amine, and after their addition with the same quantity of internal standards as that in the samples, were treated exactly in the same way as the samples.

## *2.5. Derivatization*

A 100-μl aliquot of the aqueous hydrochloric acid extract was evaporated to dryness at 70°C, under a stream of nitrogen; the compounds were then derivatized with a mixture of acetonitrile and HFBA (200 μl each) by heating at 80°C for 60 min. After cooling, 0.5 ml of methanol was added, and the solution was evaporated to dryness at room temperature, under a stream of nitrogen. The residue was dissolved in 1 ml of 0.5 *M* phosphate buffer (pH 7.0) followed by extraction with 3 ml of dichloromethane. The dichloromethane layer was collected, dehydrated with a small amount of anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen at room temperature. The residue was finally dissolved in 100 μl of ethyl acetate containing 0.2% (m/v) of Carbowax 1000 M and stored at −20°C until injection.

## *2.6. Instrumentation and chromatographic conditions*

The analyses were performed on a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970B mass-selective detector and equipped with a split/splitless injection port. The system was controlled by an HP Vectra XM 5/120 PC using the MS Chemstation software-G1034. A DB-5MS capillary column (J&W Scientific, Folsom, CA, USA) (30 m× 0.25 mm I.D., 0.25 μm film thickness) was used. The column was preceded by a 2 m guard column of the same inner diameter connected to the column via a press fit glass union (J&W Scientific).

A 1–1.5 μl aliquot of each derivatized extract was injected in the splitless mode using a doubled tapped glass insert (HP). The inlet temperature and the interface were set at 280°C and the purge-off time was 1 min. The oven temperature program was: initial temperature 80°C maintained for 1 min, increased at 15°C $min^{-1}$ to 210°C, then increased at 20°C $min^{-1}$ to 290°C and held constant at 290°C for 5 min (total time: 18 min). Ultra-high purity helium was employed as carrier gas with a column head pressure of 80 kPa.

In order to establish the retention time of each compound and the characteristic spectrum of its HFBA derivative, individual amine standards in 0.1 *M* HCl, at 20 μg $ml^{-1}$ each, were prepared, derivatized and analyzed with the instrument on full-scan mode, from 50 to 800 u, at 70 ev. Quantification was carried out by ion-monitoring of characteristic ions of each amine derivative. For each compound at least, a target ion and two qualifying ions were chosen on the basis of their abundance, freedom from interferences and specificity toward the compound. The molecular ion ($M^+$) was preferred when found in appreciable abundance. The acquisition program was divided into three groups of ions, each group containing the ions of two or more compounds, internal standards included (Table 2). The ''solvent delay'' was 5 min and the ''dwell time'' was set at 30 ms. The detector was optimized under standard tuning conditions with perfluorotributylamine.

Quantification was accomplished by measuring the ratio of the peak areas of the target ion peak of each amine and of the corresponding internal standard and by further interpolation of those values on the calibration curve. A new calibration curve was obtained for each batch analysed.

## 3. Results and discussion

### 3.1. Ion-pair extraction

The ion-pair extraction method was introduced many years ago by Schill and co-workers [36]. In this method, ionizable compounds can be extracted as ion-pairs into an organic phase containing a suitable ion with opposite charge. The degree of extraction can be improved by using as extracting agents, such as BHEPA, which act as both ion-pairing and adduct-forming agents in the organic phase [37–39]. Compared to simple liquid–liquid extractions procedures, the application of the ion-pairing technique on a preparative scale may result in increased recovery and enhanced purification of the extracts obtained from complex matrices, an essential prerequisite for their chromatographic analysis.

BHEPA has proved to be a powerful and very efficient agent for the extraction of different organic bases when used as an initial separation step of a chromatographic method. It was successfully applied in the extraction of 4-methylimidazole from caramel colors [40] and of histamine from blood and plasma [41], preceding the HPLC determination of the analytes, and in the extraction of catecholamines from rat brain [42], 3-*O*-methylated catecholamines from urine [43] and several sympathomimetics from biological materials [44] preceding their GC analysis.

In our laboratory we recently developed a slightly improved method for the extraction of the 4-methylimidazole and other imidazolic compounds from ammonia caramel samples being in mind its subsequent derivatization with isobutyryl-chloroformate and GC–MS analysis [45]; based on a similar goal

Table 2
Selected ion-monitoring (SIM) acquisition program

| | *m*/*z* values |
|---|---|
| Group 1 (5 min–8.3 min) | 91, 104, 118, 226, 228, 240, 254, 466, 480, 488 |
| Group 2 (8.3 min–10 min) | 107, 120, 226, 280, 316, 339, 353, 494, 508 |
| Group 3 (10 min–18 min) | 130, 226, 254, 266, 326, 339, 356, 536, 564, 576 |

we were also able to extract amphetamine and some analogues from urine with excellent results [46].

The application of the above mentioned method, with some minor modifications, to the extraction of biogenic amines from such complex samples as Port wine and grape juice proved to be very effective for the extraction of most of the assayed compounds including all the diamines, polyamines and aromatic amines that are subject of this study.

The efficiency of the extraction method was tested at various values of pH by comparing the results obtained from the direct derivatization of a solution of the amines and the internal standards in 0.1 *M* HCl, 1 mg $l^{-1}$ each, with the results obtained from the derivatization of hydrochloric extracts obtained after extraction of buffered aqueous solutions and buffered synthetic wine solutions, with the same concentration of the amines, and at which the internal standards were added only after the extraction procedure. Results showed that recoveries of all the amines were quantitative over a large pH range of 6.2–7.8; pH 7.4 was chosen because it provided a good yield for all the amines assayed in this study and simultaneously, was the best for other amines found in wines, namely histamine, which was further quantified by a stable isotope method using pentafluorobenzyl bromide as the derivatizing agent [47].

The obtained extracts were almost totally free of potential interferents present in the samples, namely sugars and polyphenols; such observation was confirmed by HPLC analysis. The levels of the aminoacids in the extracts were quite lower than in the original sample, although appreciable amounts of proline, the most abundant amino acid in the wine samples, still remained. Interestingly, the extracts used for the determination of the amines assayed in this study could also be used for further determination of other amines usually presents in the wines, e.g., histamine, pyrrolidine and aliphatic monoamines via other derivatizing procedures.

### 3.2. Derivatization and GC–MS analysis

The use of the acylating reagent HFBA for the conversion of the diamines and polyamines into volatile derivatives with good chromatographic properties is described by many authors [48–51]. It is known that the reagent also reacts under mild conditions with phenol groups originating the correspondents *O*-heptafluorobutyryl derivatives [52]. Under the conditions of derivatization used, which are basically those used by the research group of Muskiet in the analysis of polyamines in biological fluids [53,54 and references cited herein], it was possible to accurately and simultaneously quantify seven of the most important biogenic amines found in wines: these included the diamines, Put and Cad, always presents in the wines, the polyamines, Spd and Sp, which are quantitatively important in grape juice but which are rarely mentioned in this wine-related works, and the two main aromatic amines of importance in wines and related musts, β-Phe and Tyr.

Fig. 1a and 1b show, respectively, a typical total ion chromatogram obtained from a grape juice sample and the respective reconstructed ion chromatograms used for quantitative work. From the latter we can observe the perfect separation and the good shape of all the peaks corresponding to the compounds of interest.

All the derivatives were stable when stored at −20°C for at least one month, except the Tyr derivative, which gives rise to a second peak with an area that tends to increase with time. The mass spectrometric analysis of the referred peak identified it as a molecule of monoderivatized Tyr (a single HFB group introduced into the side chain α-nitrogen), whereas the main peak corresponds to a molecule of Tyr derivatized with two HFB groups (one group into the side chain α-nitrogen and the other one into the phenolic function).

### 3.3. Choice of internal standards

One of the major drawbacks when tailoring a multi-residue GC methodology for the analysis of complex matrices, is the appropriate choice of internal standards capable of behavior similar to those of the analytes throughout the whole analytical procedure. In the absence of a good internal standard, the accurate quantitative determination of each compound to be analyzed is made practically impossible, irrespective of the efficiency of the adopted method.

When a mass spectrometer is used as a detection system, the most effective internal standards to be

used are stable isotopically labelled analogues of the substances that are being analyzed. These compounds, which have the same chemical and physical properties as the corresponding analytes, actually compensate for losses that occur in isolation and evaporation steps and for variation in injection volumes and detection efficiency [55]. Availability and cost were the main obstacles to its common employment.

Due to the commercial unavailability of adequate isotopically labelled analogues of most of the substances assayed in this study, we began the development of this method by using two internal standards, namely amphetamine and 1,7-diaminoheptane. The former was used for quantifying β-Phe, the derivative of which was more volatile than the other compounds and therefore, exhibited a behavior greatly dependent on the conditions of the evaporation steps that follows the derivatization procedure, whereas the latter was used for the quantification of all other studied amines. Amphetamine, which has a molecular structure identical to that of β-Phe, except for the presence of a methyl group at the α-carbon, showed excellent properties for monitoring β-Phe and gave results similar in quality to those generally obtained with stable isotopic analogues, whereas 1,7-diaminoheptane exhibited good results for monitoring the homologues diamines, Dap, Cad and Put, and satisfactory results for monitoring Tyr. Poorer results were obtained for the quantification of the polyamines because of their substantially different chromatographic behavior. The results were significantly improved with the use of a triamine (norspermidine) and of a tetramine (norspermine) as internal standards for quantification of Spd and Sp, respectively. In addition, in order to increase precision and

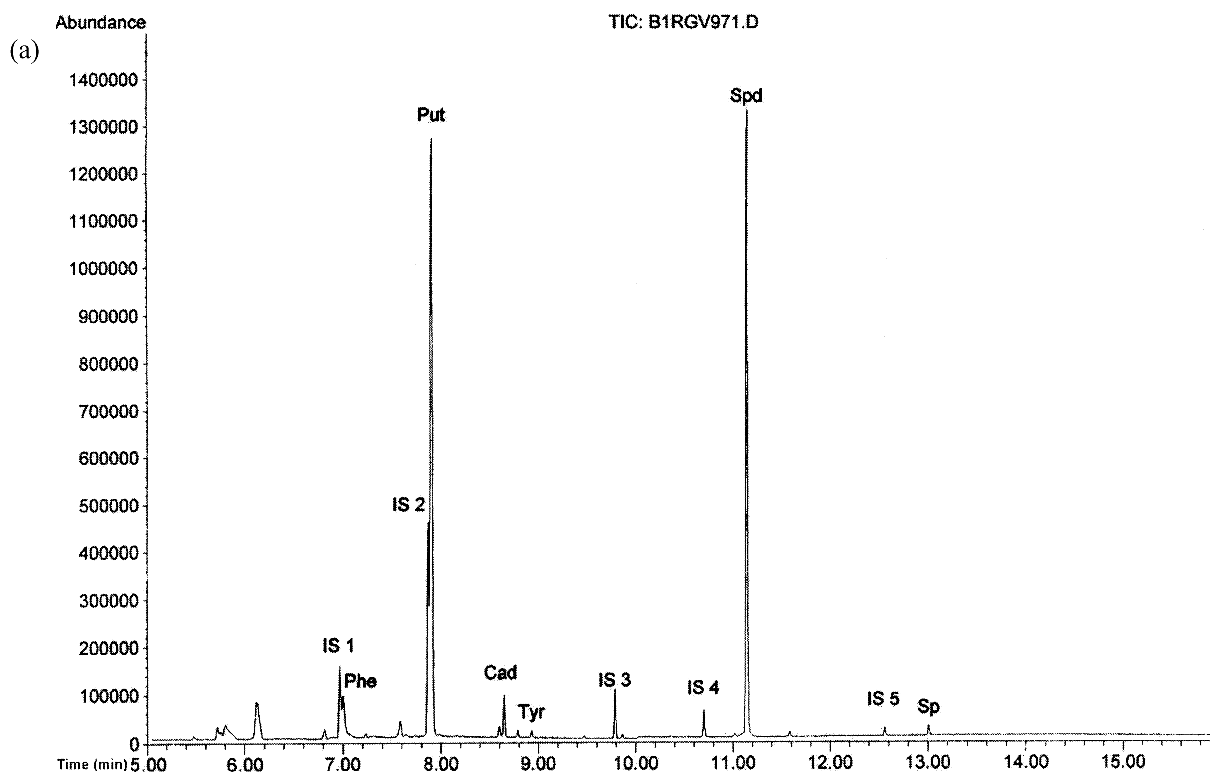


Fig. 1. (a) Total ion chromatogram obtained under the conditions used for quantification (selected ion-monitoring of three different groups of ions) from a grape juice sample with β-phenylethylamine (0.271 mg $l^{-1}$), putrescine (3.096 mg $l^{-1}$), cadaverine (0.232 mg $l^{-1}$), tyramine (0.013 mg $l^{-1}$), spermidine (3.036 mg $l^{-1}$) and spermine (0.232 mg $l^{-1}$). (b) Reconstructed ion chromatograms of the ions $m/z$ 104, $m/z$ 118, $m/z$ 480, $m/z$ 488, $m/z$ 494, $m/z$ 353, $m/z$ 316 and $m/z$ 254 from the chromatogram above, used for quantification of the six referred biogenic amines and the corresponding internal standards.

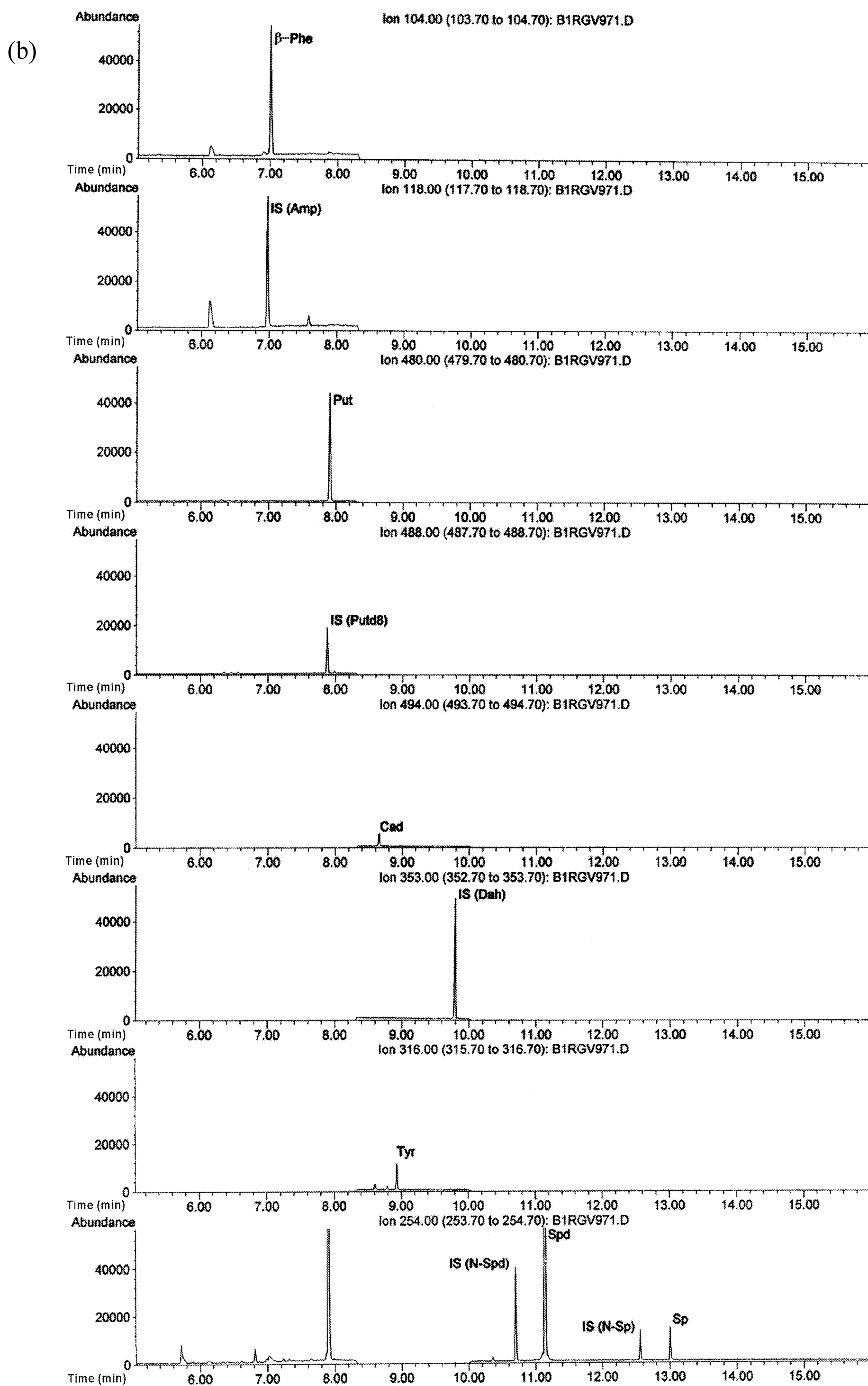


Fig. 1. (*continued*)

accuracy of the method regarding quantification of Put, which was the most abundant amine in most of the samples, the commercially available deuterated isotopic analogue of the referred amine — [$^{2}H_{8}$]putrescine — was used for its monitorization. Such a compound may also be used, as alternative to 1,7-diaminoheptane, in the quantification of the remaining amines assayed. Nevertheless, the ideal solution would, undoubtedly, be the use of the respective deuterated isotopes.

### 3.4. Method assessment

#### 3.4.1. Linearity

The linearity of the method for each compound assayed was tested several times using standard (calibrating) solutions made with synthetic wines and juices, as described in the Experimental section. For each batch of samples to be analyzed (including the samples used for precision and recovery experiments), a new set of standards, usually five or six, was simultaneously prepared and treated in parallel with the samples. The range of concentrations varied according to the expected levels of each amine present in the samples to be analyzed. Typical ranges were 0–1000 μg $l^{-1}$ for Dap, Dah, Spd and Sp, 0–2000 (or 5000) μg $l^{-1}$ for Cad, Phe and Tyr and 0–10 000 (or 15 000) μg $l^{-1}$ for Put. Calibration curves were constructed by plotting the amine/I.S. ratio obtained against the concentration of the respective amine. The correlation coefficients obtained were usually >0.9995 for Cad, Put, Dah, Spd and Phe, >0.9990 for Dah and Tyr and >0.9950 for Sp.

#### 3.4.2. Precision

To study the precision of the method, six replicate analyses of two Port wines, one table wine and one grape juice, with different levels of the different amines, were performed. In all cases, six aliquots of the same sample were added with internal standards,

Table 3
Precision of determination in six replicate analyses of two Port wines, a red table wine and a grape juice samples

| | | Port wine 1 | Port wine 2 | Red table wine | Grape juice |
|---|---|---|---|---|---|
| β-Phenylethylamine | Mean (mg $l^{-1}$) | 0.186 | 0.721 | 1.386 | 0.350 |
| | SD (mg $l^{-1}$) | 0.006 | 0.020 | 0.038 | 0.005 |
| | RSD (%) | 3.2 | 2.8 | 2.7 | 1.4 |
| Tyramine | Mean (mg $l^{-1}$) | 0.309 | 0.037 | 2.354 | 0.038 |
| | SD (mg $l^{-1}$) | 0.017 | 0.006 | 0.090 | 0.004 |
| | RSD (%) | 5.5 | 16.2 | 3.8 | 10.5 |
| 1,3-diaminopropane | Mean (mg $l^{-1}$) | – | 0.025 | – | – |
| | SD (mg $l^{-1}$) | – | 0.002 | – | – |
| | RSD (%) | – | 8.0 | – | – |
| Putrescine | Mean (mg $l^{-1}$) | 1.639 | 4.456 | 11.952 | 2.239 |
| | SD (mg $l^{-1}$) | 0.011 | 0.060 | 0.228 | 0.062 |
| | RSD (%) | 0.7 | 1.4 | 1.9 | 2.8 |
| Cadaverine | Mean (mg $l^{-1}$) | 0.073 | 0.200 | 0.234 | 0.263 |
| | SD (mg $l^{-1}$) | 0.002 | 0.007 | 0.005 | 0.004 |
| | RSD (%) | 2.7 | 3.5 | 2.1 | 1.5 |
| Spermidine | Mean (mg $l^{-1}$) | 0.023 | 0.058 | 0.469 | 2.011 |
| | SD (mg $l^{-1}$) | 0.002 | 0.005 | 0.020 | 0.069 |
| | RSD (%) | 8.7 | 8.6 | 4.3 | 3.4 |
| Spermine | Mean (mg $l^{-1}$) | – | – | – | 0.206 |
| | SD (mg $l^{-1}$) | – | – | – | 0.007 |
| | RSD (%) | – | – | – | 3.4 |

extracted, derivatized and injected twice. The results are presented in Table 3.

### 3.4.3. Recovery

This was evaluated twice for each constituent by adding four or five different known amounts of each amine to aliquots of a Port wine and of a grape juice whose amine content had been determined for six times. After addition, the samples were subject to the whole analytical procedure and the concentration of each sample was measured in duplicate. The results obtained are shown in Table 4. When the added

Table 4
Recoveries of the amines from spiked Port wine and grape juice samples

| | Port wine | | | | Grape juice | | | |
|---|---|---|---|---|---|---|---|---|
| | Initial amount[a] (mg $l^{-1}$) | Added (mg $l^{-1}$) | Found (mg $l^{-1}$) | Recovery (%) | Initial amount[a] (mg $l^{-1}$) | Added (mg $l^{-1}$) | Found mg $l^{-1}$) | Recovery (%) |
| β-Phenylethylamine | 0.186 | 0.100 | 0.275 | 89.0 | | 0.025 | 0.375 | 100.0 |
| | | 0.250 | 0.442 | 102.4 | | 0.050 | 0.389 | 78.0 |
| | | 0.500 | 0.675 | 97.8 | 0.350 | 0.100 | 0.469 | 119.0 |
| | | 1.000 | 1.184 | 99.8 | | 0.250 | 0.604 | 101.6 |
| | | | | | | 0.500 | 0.872 | 104.4 |
| Tyramine | 0.309 | 0.100 | 0.413 | 104.0 | | 0.025 | 0.058 | 80.0 |
| | | 0.250 | 0.522 | 85.2 | | 0.050 | 0.098 | 120.0 |
| | | 0.500 | 0.822 | 102.6 | 0.038 | 0.100 | 0.118 | 80.0 |
| | | 1.000 | 1.425 | 111.6 | | 0.250 | 0.225 | 74.8 |
| | | | | | | 0.500 | 0.463 | 85.0 |
| 1,3-diaminopropane | 0 | 0.100 | 0.091 | 91.0 | | 0.025 | 0.021 | 84.0 |
| | | 0.250 | 0.225 | 90.0 | | 0.050 | 0.041 | 82.0 |
| | | 0.500 | 0.487 | 97.4 | 0 | 0.100 | 0.076 | 76.0 |
| | | 1.000 | 0.992 | 99.2 | | 0.250 | 0.190 | 76.0 |
| | | | | | | 0.500 | 0.403 | 80.6 |
| Putrescine | 1.639 | 0.250 | 1.889 | 100.0 | | 0.200 | 2.429 | 95.0 |
| | | 0.500 | 2.155 | 103.2 | | 0.500 | 2.684 | 89.0 |
| | | 1.000 | 2.629 | 99.0 | 2.239 | 1.000 | 3.200 | 96.1 |
| | | 2.000 | 3.646 | 100.4 | | 2.000 | 4.179 | 97.0 |
| | | | | | | 5.000 | 7.461 | 104.4 |
| Cadaverine | 0.073 | 0.100 | 0.187 | 114.2 | | 0.025 | 0.287 | 96.0 |
| | | 0.250 | 0.347 | 109.7 | | 0.050 | 0.312 | 98.0 |
| | | 0.500 | 0.598 | 105.1 | 0.263 | 0.100 | 0.374 | 111.0 |
| | | 1.000 | 1.143 | 107.0 | | 0.250 | 0.524 | 104.4 |
| | | | | | | 0.500 | 0.826 | 112.6 |
| Spermidine | 0.023 | 0.100 | 0.115 | 92.0 | | 0.025 | 2.033 | 88.0 |
| | | 0.250 | 0.290 | 106.8 | | 0.050 | 2.048 | 74.0 |
| | | 0.500 | 0.542 | 103.8 | 2.011 | 0.100 | 2.093 | 82.0 |
| | | 1.000 | 1.203 | 118.0 | | 0.250 | 2.228 | 86.8 |
| | | | | | | 0.500 | 2.484 | 94.6 |
| Spermine | 0 | 0.100 | 0.117 | 117.0 | | 0.025 | 0.231 | 100.0 |
| | | 0.250 | 0.252 | 100.8 | | 0.050 | 0.249 | 86.0 |
| | | 0.500 | 0.512 | 102.4 | 0.206 | 0.100 | 0.294 | 88.0 |
| | | 1.000 | 0.912 | 91.2 | | 0.250 | 0.412 | 82.4 |
| | | | | | | 0.500 | 0.655 | 89.8 |

[a] Mean of six replicate analyses.

amount was plotted against the recovered amount, quite linear plots were obtained with correlation coefficients usually higher than 0.995.

### *3.4.4. Limit of detection and limit of quantification*

No exhaustive studies for determining the limit of detection were made. However, standards (made from synthetic matrices) containing 10 μg $l^{-1}$ of each amine, were sometimes used for the construction of the calibration curves. Under conditions of ideal performance of the MS detector, it was possible to detect and integrate the corresponding peaks for all the studied amines. Replicate analysis (extraction procedure included) of these standards showed that the values obtained for the ratio area of amine/area of I.S. presented a R.S.D. less than 15%, exception made for Tyr and for Sp, which gave somewhat higher R.S.D. values. This fact was confirmed when samples containing very low levels of some of the amines were analyzed. Usually, it was possible to determine β-Phe, Dap, Cad and Spd at levels of about 5–10 μg $l^{-1}$ (no sample showed so low concentrations of Put) with a R.S.D. less than 15%. For Tyr and Sp, an R.S.D. of about 15% was obtained when samples containing 20–30 μg $l^{-1}$ of these amines were analyzed. Below this level of concentration the values obtained registered an error above 15%.

### *3.5. Method application*

The method developed herein was applied to determine the content of amines in twelve samples of young Port wines (corresponding to the 1996–1998 vintages), five samples of aged Port wines (older than 5 years), ten samples of table wine (six red and four white) and eleven samples of grape juice made from grapes used in the Port wine making. The results are reported in Table 5.

Meanwhile, other samples of Port wines and the corresponding musts are being analyzed as part of a more ample research study based on the formation and evolution of biogenic amines in this type of wines. A first glimpse of the results obtained until

Table 5
Biogenic amine content of some Port wines, grape juices and red and white table wines determined by the developed GC–MS method

| | | Port wines | | Grape juices, $n$=11 | Table wines | |
|---|---|---|---|---|---|---|
| | | Young (96–97 vintages) $n$=12 | Aged (>5 yr) $n$=5 | | Red, $n$=6 | White, $n$=4 |
| β-Phenylethylamine | Range (mg $l^{-1}$) | 0.113–1.321 | 0.186–0.557 | 0.016–0.106 | 0.355–1.461 | 0.142–0.601 |
| | Average (mg $l^{-1}$) | 0.625 | 0.380 | 0.053 | 0.853 | 0.384 |
| Tyramine | Range (mg $l^{-1}$) | 0.031–0.309 | 0.032–0.309 | <0.010 (6)–0.071 | 0.149–2.354 | 0.019–0.137 |
| | Average (mg $l^{-1}$) | 0.089 | 0.097 | 0.052[a] | 1.443 | 0.057 |
| 1,3-diaminopropane | Range (mg $l^{-1}$) | nd (3)–14.7[b] | nd (4)–0.005 | 0.008–0.031 | nd (6) | nd (4) |
| | Average (mg $l^{-1}$) | 0.010[a] | 0.005[a] | 0.013 | – | – |
| Putrescine | Range (mg $l^{-1}$) | 1.940–5.704 | 0.147–2.523 | 0.807–4.036 | 2.423–11.952 | 1.433–2.597 |
| | Average (mg $l^{-1}$) | 3.905 | 1.095 | 2.118 | 6.905 | 2.081 |
| Cadaverine | Range (mg $l^{-1}$) | 0.070–0.988 | 0.006–0.125 | 0.013–0.500 | 0.059–0.385 | 0.049–0.097 |
| | Average (mg $l^{-1}$) | 0.274 | 0.053 | 0.160 | 0.190 | 0.068 |
| Spermidine | Range (mg $l^{-1}$) | 0.006–0.079 | nd (1)–0.023 | 0.688–2.633 | 0.086–0.704 | 0.014–0.236 |
| | Average (mg $l^{-1}$) | 0.020 | 0.016[a] | 1.679 | 0.341 | 0.075 |
| Spermine | Range (mg $l^{-1}$) | nd (12) | nd (5) | 0.035–0.856 | nd (4)–0.044 | nd (4) |
| | Average (mg $l^{-1}$) | – | – | 0.311 | 0.031[a] | – |

[a] Average of the samples containing detectable amounts of the respective amine.
[b] nd — no detection. The number in parentheses is the number of samples for which there was no detection.

the present moment allows us to point out some original conclusions. In a similar manner to table wines, Put is the most abundant amine but its concentration levels tend to be at a minimum during aging of the Port wines. Cad behaves similarly, although its concentration levels are substantially lower. Tyr is present in Port wines at concentrations lower than those normally encountered in red wines, whereas levels of β-Phe are practically of the same order of magnitude. Overall, the level of amines in Port wines is substantially lower than that of red table wines.

With respect to grape juices, besides Put and Cad which are present in concentration levels similar to those found in young Port wines, it is important to note the presence of Spd at concentration levels that are sometimes higher than 2.0 mg $l^{-1}$ and of Sp at some hundreds of μg $l^{-1}$. Interestingly, in Port wines, Spd is found only at trace levels, and Sp is not found at all. β-Phe is found in grape juice at concentration levels lower than those encountered subsequently in young wines, thereby indicating its possible formation during the fermentation process. More definitive results will be presented in the future.

## 4. Conclusions

Until the present moment, HPLC has been the most widely used analytical approach to assay biogenic amines in wines. Although GC analysis has been used in the past to measure some volatile amines, the method presented herein is the first GC–MS method developed to permit simultaneous quantitative determination of the most relevant diamines, polyamines and aromatic amines found in wines and grape juices.

This method combines a simple ion-pair extraction procedure using BEHPA with the GC–MS analysis of the HFB derivatives of the amines. It has proved to be very useful for the simultaneous quantification of seven of the most important amines (β-Phe, Tyr, Dap, Put, Cad, Spd and Sp), in such complex samples as Port wines and grape juices.

The high levels of sensitivity, accuracy and reproducibility achieved recommend the use of the proposed method for the quantification of biogenic amines in these type of matrices, whenever the need for results of high analytical quality is warranted. Preliminary findings from the application of the technique to other foodstuffs, such as beer and meat products, appear promising.

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