Journal of Chromatography A, 1218 (2011) 842–848

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development of a mixed-mode solid phase extraction method and further gas chromatography mass spectrometry for the analysis of 3-alkyl-2-methoxypyrazines in wine

Ricardo López*, Elisa Gracia-Moreno, Juan Cacho, Vicente Ferrreira

Laboratorio de análisis del aroma y enología, Departamento de Química Analítica, Facultad de Ciencias, Instituto de Investigación en Ingeniería de Aragón I3A, Universidad de Zaragoza, C/Pedro Cerbuna, 12, 50009 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 19 July 2010
Received in revised form
25 November 2010
Accepted 13 December 2010
Available online 21 December 2010

Keywords:
3-Alkyl-2-methoxypyrazines
Wine
Aroma
Gas chromatography
Solid phase extraction
Mixed-mode resins
Selective isolation

ABSTRACT

A new method for analysing 3-isopropyl-2-methoxypyrazine, 3-sec-butyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine in wine has been developed and applied to wine. The analytes are extracted from 25 mL of wine in a solid-phase extraction cartridge filled with 60 mg of cation-exchange mixed-mode sorbent. Analytes are recovered with triethylamine in dichloromethane and the organic extract is analysed by GC–SIM–MS using 3-isopropyl-2-ethoxypyrazine as internal standard. The detection limits of the method are in all cases under 1 ng/L, below the olfactory thresholds of the compounds in wine. The repeatability of the method is around 15% for levels in wine of 2 ng/L. Linearity is satisfactory and recoveries are in all cases close to 100% with RSD between 13% and 20%. The method has been applied to the analysis of 12 Chilean white and 8 Spanish red wines. The levels found suggest that 3-alkyl-2-methoxypyrazines can exert a significant sensory contribution to the aroma of Chilean Sauvignon Blanc wines, while most likely they play a nearly negligible role on traditional Ribera and Rioja Spanish red wines.

1. Introduction

Pyrazines are a family of compounds whose presence has been amply reported in several kinds of foods [1]. The importance of 3-alkyl-2-methoxypyrazines (MPs) in the aroma of wine has been widely studied since the first report of 3-isobutyl-2-methoxypyrazine in Cabernet Sauvignon grapes in 1975 [2]. The presence of 3-isobutyl-2-methoxypyrazine (IBMP), 3-sec-butyl-2-methoxypyrazine (SBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) in wines has been related with the green and vegetative aromas characteristic of some wines made with Cabernet Sauvignon, Sauvignon blanc, Merlot or Cabernet Franc grapes [3–8]. Although there is still some controversy about the eventual contribution of these compounds to wine aroma typicality [9] it has been demonstrated that these compounds exert a negative influence on the perception of wine fruitiness [10], they have also been found to take part of negative vectors of quality in premium Spanish red wines [11] and it has even been proposed that it is possible to consider IBMP as a marker for grape unripeness [7]. The origin of these compounds is mostly endogenous as they are part of the chemicals produced in the first stages of grape development, being their levels strongly correlated with vine vigour and shade conditions [12], although the isopropyl isomer can have its origin in the vine infestation by the multicoloured Asian lady beetle, *Harmonia axyridis* [13,14].

MPs have extremely low sensory detection thresholds. In the case of IBMP, detection thresholds of just 0.5 ng/L in water and of 10 ng/L in red wine have been reported [15], and its recognition threshold in red wine has been found to be 15 ng/L [7]. IPMP still can be more powerful since its thresholds can be as low as 0.3 ng/L in white wine or just 2.3 ng/L in red [16]. In addition, these compounds can act additively [10], or even can interact with some other compounds with green aroma [3]. All this makes that these compounds can exert a relevant effect on wine quality at very low concentration levels and that the development of reliable quantitative methods for their determination at those low levels is a peremptory need. Several approaches have been essayed to achieve this goal in the last decades. The first attempts in the nineties were all methods using relatively large volumes of sample and quite complex and work intensive sample enrichment strategies. The key isolation step of all these strategies was the selective retention of the 3-alkyl-2-methoxypyrazines on a cation-exchange resin, but the methods required wine distillation, several pH adjustments and several liquid–liquid or liquid–solid partitions [4,15,17–19] which

* Corresponding author. Tel.: +34 976761290; fax: +34 976761292.
E-mail address: riclopez@unizar.es (R. López).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.12.048

made the analyses extremely laborious and time-consuming. In the last decade, different automated or semi-automated techniques have been tried such as headspace solid-phase microextraction (HS-SPME) [14,20–22] and solid-phase extraction (SPE) [23,24] but yet most of the procedures are complicated or do not reach adequate method detection limits for all the MPs, and in fact most of them are only able to get quantitative data for the most abundant IBMP.

As for the detection system, gas chromatography coupled with mass spectrometry has been used in most of the cases [4,5,7,14,15,17–19,25], although some authors have used nitrogen-phosphorous detection (NPD) [13,20,26]. More recently, comprehensive two dimensional-gas chromatography (GC × GC) with NPD [22] and mass spectrometry as detection systems [22,27,28] and a dual GC–GC with ion trap mass spectrometry [24] have been proposed. Such multidimensional techniques have been proved useful for its capacity to resolve co-elutions that usually affect MPs analyses [27,28]. The possibility of using liquid chromatography as separation technique for MPs has been also recently reported [29]. From the point of view of selectivity, researchers very soon realized that the key property of MPs is their ability to form a cation at acid pHs, which a priori provides the best way to separate MPs from other aroma matrix compounds. As aforementioned, this property was and yet is the base for many analytical procedures, but the poor chromatographic characteristics and solvent limitations of the most often used cation-exchange sorbents cause that most of the procedures are work intensive, complex and that recoveries are non-quantitative and often matrix-dependent. From this point of view, the use of mixed mode sorbents combining hydrophobic and cation-exchange extraction properties could provide a powerful and relative simple way to concentrate, isolate and elute MPs in a single separation device, as has been previously suggested [30]. This possibility is further explored in this paper whose goal is the optimization of a simple isolation strategy for the quantitative determination of the three most important MPs at ng/L levels.

2. Materials and methods

2.1. Reagents and solutions

IPMP, SBMP and IBMP were from Sigma–Aldrich (Steinheim, Germany) and 3-isopropyl-2-ethoxypyrazine (IPEP) was from Pyrazine Specialties Inc. (Atlanta, USA). Dichloromethane and methanol (both of them Suprasolv quality) and ethanol (LiChrosolv quality) were purchased from Merck (Darmstad, Germany), *n*-pentane (99% purity) was from Fluka (Buchs, Suiza) and hydrochloric acid (37% weight), sodium hydroxide and tartaric acid (both of them for analysis quality) were provided by Panreac (Barcelona, Spain). Ethyl acetate was from Sigma–Aldrich, diethylether from Fisher Chemicals (Loughborough, UK) and isopropanol from Scharlau (Barcelona, Spain), all were HPLC quality. Triethylamine (TEA) 95% was purchased from Aldrich. Pure water was obtained from a milli-Q purification system (Millipore, Bedford, MA, USA). SPE was performed with the help of a Vac Elut 20 system from Varian. SPE mixed-mode sorbents were: Bond Elut Certify from Varian Inc. (Walnut Creek, USA), OASIS MCX from Waters (Milford, USA), Strata XC from Phenomenex (Torrace, CA, USA) and Bond Elut Plexa PCX from Varian Inc. In addition, pre-packaged 200 mg LiChrolut EN resins (3 mL reservoirs) from Merck were used to carry out the analytical method described in [31].

Standard solutions of 2000 mg/L of MPs were prepared in ethanol and stored in the dark at low temperature (−25 °C). Two intermediate dilutions of these solutions were prepared for wine spiking depending on the final concentration required. To carry out wine spiking volumes larger than 50 μL of the standard solutions were used always controlled by weight. Furthermore, wines were spiked with the standards just prior to use to avoid evaporation and photodecomposition of the MPs [32].

2.2. Wine samples

A Rioja D.O. red wine was used for validation of the method because of its low content of MPs (below 0.5 ng/L, analysed following the method described in [24]). Twelve Chilean wines and eight Spanish aged red wines were analysed with the proposed method.

2.3. Chromatographic conditions

For the initial steps of method development, the gas chromatographic analysis was performed with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass-spectrometric detection system from Varian (Sunnyvale, CA, USA). A DB-WAXETR capillary column (J&W Scientific, Folsom, CA, USA) (60 m × 0.25 mm i.d., film thickness 0.25 μm) preceded by a 3 m × 0.25 mm uncoated (deactivated, intermediate polarity) precolumn from Supelco (Bellefonte, USA) was used. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature programme was 5 min at 40 °C, then increasing by 4 °C min^{-1} up to 94 °C, with a second ramp at 8 °C min^{-1} up to 120 °C and a third one at 50 °C min^{-1} up to 220 °C and finally held at this temperature for 20 min. The MS-parameters were: both MS transfer line and chamber ionization temperature 200 °C, and trap emission current 80 μA.

For the fine tuning of the method and subsequent validation and analysis of samples, a QP-2010 gas chromatograph, coupled with a quadrupole mass spectrometer, with an electron impact ionization source, from Shimadzu was used with a Factor Four VF-5 MS capillary column (20 m × 0.15 mm × 0.15 μm) provided by Varian preceded by a 3 metres long uncoated pre-column from Agilent. The carrier gas was helium at a constant flow of 1 mL min^{-1}.

The oven temperature was programmed as follows: 35 °C for 4.5 min, then raised at 4 °C min^{-1} up to 94 °C, with a second ramp at 8 °C min^{-1} up to 120 °C and a third one at 50 °C min^{-1} up to 260 °C, maintained for 10 min. Automatic injection of 8 μL of sample was accomplished with the help of a CombiPAL (CTC Analytics AG, Zwingen, Switzerland). An Optic 3 injector from ATAS-GL (Veldhoven, The Netherlands) was used, with a liner from ATAS and a carbofrit insert from Restek inside it. The injection was done in the splitless mode for 4.5 min, after which the split ratio was 1/10, with the following injection temperature program: 45 °C during 3 min, then increased at 16 °C s^{-1} up to 250 °C, remaining at this temperature until the end of the analysis. A pressure pulse of 400 kPa was used during the splitless time.

Quadrupole mass detector was operated in selected ion monitoring (SIM) mode with EI ionization at 70 eV with a dwell time of 0.2 s. Quantifier ions were 137, 138, 124 and 123 m/z for IPMP, SBMP, IBMP and IPEP, respectively, while qualifier ions were 152 and 124 *m*/*z* for IPMP, 124 and 151 for SBMP, 94 and 151 for IBMP, and 151 and 166 for IPEP.

2.4. SPE method development

2.4.1. Sorbent selection and breakthrough volumes

A comparison of different sorbents and wine pHs was carried out to obtain the optimum breakthrough volumes (V_B) for the MPs. Several sorbents (see Section 2.1) were tested in order to ascertain which one could provide the largest V_B. This comparison was conducted at two different pHs.

A young red wine, in which the MPs were not detected (levels below 0.5 ng/L), was used for this experiment. It was spiked with

50 μg/L of the analytes and then pH was adjusted to 0.5 or 2.0 with concentrated hydrochloric acid.

Mixed-mode sorbents were used (500 mg in 6 mL reservoirs). First of all they were conditioned with 20 mL dichloromethane, 20 mL methanol and 35 mL of milli-Q water. Then the spiked wine was loaded at a rate of 2 mL min^{-1} and the percolated solution was collected in 20 mL fractions. Vacuum suction was not applied in this particular experiment to avoid losses of the non-retained analytes due to their volatility. The collected fractions were analysed as described in [31] with one modification: prior to the loading of the samples, pH was re-adjusted to 5.0 with sodium hydroxide to be sure that all the MPs were in their neutral form. After conditioning the 200 mg LichrolutEN cartridges (4 mL dichloromethane, 4 mL methanol and 4 mL 12% ethanol/water) the wine was loaded at 2 mL min^{-1}; the sorbents were vacuum dried and then eluted with 1.6 mL of dichloromethane and analysed by GC–MS.

2.4.2. Removal of interferences and matrix compounds

Five hundred mg of Plexa PCX pre-packed cartridges were conditioned as described in Section 2.4.1, and loaded with wine spiked with the analytes (50 μg/L), the only difference being that the loading was achieved with the help of the vacuum system. The volume and the pH of the wine were the optimum ones obtained for wine in the experiment described in Section 2.4.1, 200 mL of spiked wine adjusted to pH 2.0.

The sorbent loaded with the MPs was washed with different volumes of milli-Q water acidified to pH 2, then with *n*-pentane after vacuum-drying the sorbents and finally with dichloromethane. This washing was done without the help of a vacuum system and the percolated fractions were collected, then analysed directly or as in [31], depending on the solvent.

In addition, methanol and isopropanol were tested as possible washing solvents. Conditioning of the cartridges, sample-loading and acid washing were done as related before. Then, the sorbents were dried and washed with either methanol or isopropanol collecting 2 mL fractions of the percolated solution and analysing them directly by GC–MS.

2.4.3. Optimization of elution strategy

Conditioning of the sorbents was performed as described in Section 2.4.1. Then, they were loaded with 200 mL of a hydroethanolic (12% EtOH, v/v) solution containing 50 μg/L of the analytes and washed with 20 mL of milli-Q water, vacuum-dried and washed with 4 mL of dichloromethane and then vacuum-dried again. Then, several elution strategies were tested:

Polar solvents: methanol and isopropanol were tested, 2 mL fractions were recovered and analysed.

Previous neutralization and further elution: 45 mL of milli-Q water both with 1% and 25% of methanol, at pH 12 were used to neutralize. Then, the sorbents were kept soaked with this solution during one hour to be certain of the neutralization. Final pH of the percolated solution was checked. After neutralization and drying, dichloromethane followed by dichloromethane/TEA/methanol (5%) were examined as possible eluents. Isopropanol was tested after neutralization, too.

Alkaline aqueous-methanol mixtures: milli-Q water/methanol (1:1, v:v) at pH 12 was used, the percolated was collected in 20 mL fractions and then analysed as described in Section 2.4.2 for the acid washing.

Solvents containing an excess of alkali: dichloromethane, methanol, ethyl acetate and diethylether were used, each of them, in combination with (TEA) (10 g/L). Fractions of the percolated solution were collected (2 mL each) in centrifuge tubes containing 5 mL of milli-Q water with the purpose of eliminating the excess of TEA to avoid damage to the column, except in the case of methanol. Centrifugation at 2500 rpm during 5 min was carried out when needed, 10 mL standard centrifuge tubes and a Centro8-BL centrifuge from Selecta were used.

2.5. Proposed method

Twenty-five mL of wine were spiked with 30 ng/L of internal standard (IPEP). Then, wine pH was adjusted to 2.0. Plexa PCX 60 mg sorbent in 1 mL cartridges was conditioned with: 2.5 mL dichloromethane, 2.5 mL methanol and 4 mL milli-Q water. After that, wine was passed through the SPE cartridge. Then, the sorbent was washed with 1 mL of milli-Q water/methanol (30%) adjusted to pH 2.0 and dried during 10 min with a nitrogen stream. A second washing step with 0.5 mL of dichloromethane was done. Afterwards, elution with TEA in dichloromethane (10 g/L) was carried out: 600 μL of this eluent were added discarding the eluate, and after that, another 200 μL were added but this time collecting the eluate and washing it with 1 mL of milli-Q water adjusted to pH 3.0 with tartaric acid. This mixture was shaken during 2 min and then centrifuged at 2500 rpm during 5 min. The vials used for handling the volumes were micro reaction vessels (1 mL) from Supelco. Finally, the organic phase containing the MPs was recovered with a micro-syringe, transferred into a micro-vial and then stored at −25 °C until GC–MS analysis.

2.6. Method validation

A red wine with undetectable levels of MPs (assessed following the proposed procedure) was fortified with different amounts of the analytes (0.5, 1, 5, 10 and 25 ng/L) and 30 ng/L of IPEP to build the calibration curve. All the calibration points were analysed in duplicate.

Method accuracy was assessed by a standard recovery experiment carried out on 14 different wines. The wines were spiked with around 20 ng/L of IPMP and SBMP and 24 ng/L of IBMP. Spiked and unspiked original samples were analysed following the proposed procedure in order to determine the recovery. This was defined as the ratio (in %) between the amount of analyte determined in the spiked sample minus that found in the corresponding unspiked original sample to the amount effectively added in the spiking process.

Method limits of detection and quantification were determined from the standard addition plots built previously as the concentration of analyte in wine which would give a signal 3 or 10 times higher than the noise, respectively.

3. Results and discussion

3.1. SPE method development

The following SPE parameters have been optimized during method development: mixed-mode sorbent, sample pH, breakthrough volume, cleaning solvent and eluting solvent.

3.1.1. Sorbent selection and breakthrough volumes

The most important parameter when designing a SPE based method is the breakthrough volume (V_B) of the analytes in the sorbent used, since it measures the capacity of a SPE system to isolate the analytes from a given liquid matrix. In this work V_B has been defined as the maximum volume of wine sample that can be loaded into a SPE bed with loses of analyte in the percolated sample below 1% of the total amount of analyte loaded. Four different mixed-mode sorbents have been considered in this study. The main difference between them is the skeleton to which the benzenesulphonic groups are attached, being a siliceous or a polymeric one depending on the brand. Details of the sorbents used can be seen in Table 1.

Table 1
Properties of the sorbents used extracted from the datasheets and user guides from each brand.

Properties	Sorbents			
	Bond Elut Certify	OASIS MCX	Strata XC	Bond Elut Plexa PCX
Composition	Silica base with octyl and benzensulfonic groups	Poli(N-vinilpirrolidone-divinylbenzene)	Styrene divinylbenzene	Hydroxylated styrene divinylbenzene
Specific surface (m^2/g)	460–520	727–889	705–825	450
Ion-exchange equivalent capacity (meq/g)	n.a.	0.80–1.20	0.9–1.2	0.6–1.20
Medium particle size (μm)	47–60	50–65	28–34	40–55
Medium pore size (A)	58–87	73–89	75–91	50–250
Pore volume (mL/g)	n.a.	1.18–1.44	1.57–1.87	n.a.
Washable residue (mg/g)	≤2.0	n.a.	n.a.	≤7.00
Turbidity (NTU)	≤10.0	n.a.	n.a.	≤7.00

n.a. Data not available.

Retention phenomena in these kinds of sorbents and analytes should be pH dependent, with the best extraction conditions expected at pHs at which the analytes are in cationic form. Unfortunately, MPs are very weak alkalis with pK_a around 0.5 [25], and they are fully cationic at very low pHs. At pHs below 1.0 the sorbents could even loss their cation-exchange properties since benzenesulphonic groups could be protonated. Because of this, a comparative study was carried out between the 4 sorbents at two different pHs (0.5 and 2.0). Results of the experiment are shown in Table 2. The table lists the V_B obtained in red wines spiked with the analytes at two different pHs (see Section 2). As shown in the table, and in accordance with a previous report [30] retention properties are highly dependent on both the pH and the type of sorbent. As can be seen, the worst results were obtained in both cases with Bond Elut Certify, which is based on derivatized silica, while the other sorbents are based on organic polymers, as detailed in Table 1.

It can be hypothesized that this result is due to both the lower specific surface area of this sorbent and to the additional π–π interactions that the organic polymers can provide. Anyway, results shown in the table cannot be easily explained in terms of specific surface or ion exchange capacity and, as noted in the mentioned report, the existence of strong secondary interactions relatively specific to a type of polymer make that the specific retention ability is strongly dependent on the type of polymer. In the present case, the best results were obtained with Bond Elut Plexa PCX, in spite of the fact that their specific active surface is not very large.

On the other hand, pH has also a great influence in the retention capacity of these sorbents. In all cases, retention was improved at pH 2.0, in spite of the fact that at this pH the proportion of protonated analytes is relatively low. This result can be attributed to different facts, first to the potential deactivation of the cation-exchangers at low pH and second to the existence of additional compounds in wine that become cations at this pH and compete for the exchangers. It is worth mentioning at this point that analytes are readily retained in cationic form at pH 2.0, since as it will be shown later, they cannot be eluted by neutral organic solvents. This suggests that the acid–base equilibrium is shifted to the acid, protonated forms in the presence of the mixed-mode cation exchanger, which in turn supports the importance of secondary hydrophobic interactions in the extraction in these sorbents. Preliminary research conducted at wine pH (ca. 3.5) showed a poorer retention of MPs in comparison with that obtained at pHs 2.0 or 0.5 (data not shown), which suggests, therefore, that at pH 2 an optimum combination of retention mechanisms is attained.

As shown in Table 2, breakthrough volumes were in all cases smaller for the least non-polar MP, IPMP, but in the best conditions up to 200 mL of wine can be loaded onto a 500 mg Bond Elut Plexa PCX cartridge, which suggests that enrichment factors above 100 will be easily reached.

3.1.2. Removal of interferences and matrix compounds

Different washing-up solvents were investigated in order to eliminate as many potential interferences as possible. First of all, a cleaning step with water at pH 2 was tested. Forty mL of this polar rinsing solvent could be applied to a 500 mg cartridge without appreciable losses of the analytes, which makes it possible to eliminate important wine interferences such as part of fusel alcohols and polyphenols. Later, this cleaning step was reinforced with 30% of methanol in order to eliminate a larger amount of polar compounds, after verifying that the analytes were not eliminated.

After an exhaustive drying of the sorbents, *n*-pentane and dichloromethane were also tested as washing solvents. Results showed that 4 mL of those solvents can be applied to a 500 mg cartridge containing the analytes without significative losses (less than 1%). Dichloromethane was therefore selected as non-polar rinsing solvent, since it has a much greater capacity to eliminate non-polar or medium–polar interferences. Methanol and isopropanol were also tested as potential washing solvents but both of them eluted significant amounts of the MPs, the reason why they were not further considered.

In the final method design (see Section 2.5) a simple washing with 1 mL of the rinsing solvent (30% methanol in water at pH 2.0), followed by drying and a second additional rinsing with just 0.5 of dichloromethane was found enough to eliminate most interferences from the 60 mg cartridge used in the proposed procedure.

Table 2
Breakthrough volume[a] (mL) comparison at pH 0.5 and 2.0, 500 mg sorbent bed.

Sorbents	pH 0.5				pH 2.0			
	Bond Elut® Certify	OASIS® MCX	Strata™ XC	Bond Elut Plexa™ PCX	Bond Elut® Certify	OASIS® MCX	Strata™ XC	Bond Elut Plexa™ PCX
IPMP	<20	20	40	80	20	60	100	200
SBMP	<20	40	100	180	40	160	180	>400
IBMP	<20	40	120	>200	40	160	200	>400
IPEP	n.a.	n.a.	n.a.	>200	n.a.	n.a.	80	>400

n.a. Data not available.
[a] Analyte losses less than 1%.

Table 3
Percent of MPs eluted out of a 500 mg sorbent cartridge in a given volume of eluent.

Elution approach	Neutralization	Elution	IPMP	IPEP	SBMP	IBMP
Previous neutralization and further elution	1% MeOH/water pH 12 (45 mL)	DCM[a]	29	23	27	17
	1% MeOH/water pH 12 (45 mL)	DCM/TEA/5% MeOH[b]	2.7	4.9	3.6	9.2
	25% MeOH/water pH 12 (45 mL)	DCM[b]	34	34	39	39
	25% MeOH/water pH 12 (45 mL)	DCM/TEA/5% MeOH[b]	<0.1	<0.1	<0.1	<0.1
	1% MeOH/water pH 12 (45 mL)	Isopropanol[b]	78	79	91	94
Alkaline aqueous-methanol mixture	50% MeOH/Water pH 12 (100 mL)	50% MeOH/water pH 12 (100 mL)	22[c]	18[c]	19[c]	21[c]
Solvents containing an excess of alkali (10 g/L TEA)	n.a.	DCM/TEA[d]	77	71	79	78
	n.a.	MeOH/TEA[d]	77	76	74	70
	n.a.	Ethyl acetate/TEA[a]	64	63	67	70
	n.a.	Diethyl ether/TEA[a]	76	72	77	75

[a] 6 mL of eluent.
[b] 2 mL of eluent.
[c] First fraction of 20 mL of eluent.
[d] 8 mL of eluent n.a.: not applicable.

Table 4
Figures of merit of the method.

	Calibration curve	R^2	DL (ng/L)	Linear range (ng/L)	Recovery[a] (%)	Repeatability (% RSD)	
						Low level[b]	High level[c]
IPMP	$0.0366x + 0.0654$	0.996	0.4	1.3–25	103 ± 19	17	2.2
SBMP	$0.0628x + 0.0284$	0.998	0.5	1.6–10	98 ± 16	13	1.1
IBMP	$0.0343x + 0.0109$	0.998	0.3	1.0–25	105 ± 13	21	2.1

[a] ±Standard deviation.
[b] Values obtained at 2 ng/L level.
[c] Values obtained at 10–20 ng/L level.

3.1.3. Optimization of the elution strategy

As aforementioned, analytes are retained mostly in cationic form, since only very polar solvents, such as methanol or isopropanol could elute part of the analytes retained in the SPE bed. The quantitative elution of the retained compounds in a solvent system compatible with GC was the most critical methodological step and different approaches were therefore considered:

1. The direct use of polar solvents.
2. The use of a previous neutralization and further elution.
3. The use of alkaline aqueous-methanol mixtures.
4. The use of solvents containing an excess of alkali.

The direct elution of the analytes with polar solvents (methanol or isopropanol) was found impractical, since recoveries were very low (data not shown) and the extracts were difficult to analyse by GC.

Recoveries were better when the analytes were previously neutralized by the addition of aqueous alkaline solutions containing different proportions of methanol (see Section 2), added to improve the wettability of the polymer. Results, shown in Table 3 suggest that neutralization is not complete and that only good recovery results can be obtained if polar solvents are used. By far, the best results were those obtained using isopropanol, for which recoveries between 78% and 94% in the first fraction of 2 mL were

Table 5
Concentration of MPs (ng/L) in Chilean and Spanish wines.

Wine	Grape varieties	Vintage	Sample origins	IPMP	SBMP	IBMP
Chilean wine 1	Sauvignon Blanc	2007	San Antonio, Chile	<DL	<DL	5.7
Chilean wine 2	Sauvignon Blanc	2008	Casablanca Valley, Chile	2.2	<DL	10.5
Chilean wine 3	Sauvignon Blanc	2008	Casablanca Valley, Chile	1.4	<DL	8.8
Chilean wine 4	Sauvignon Blanc	2007	Leyda Valley, Chile	<DL	<DL	6.5
Chilean wine 5	Sauvignon Blanc	2007	Leyda Valley, Chile	1.9	<DL	8.5
Chilean wine 6	Sauvignon Blanc	2008	Casablanca Valley, Chile	4.2	1.1[a]	20.9
Chilean wine 7	Sauvignon Blanc	2007	Leyda Valley, Chile	1.1[a]	<DL	9.3
Chilean wine 8	Sauvignon Blanc	2008	Casablanca Valley, Chile	<DL	<DL	2.9
Chilean wine 9	Sauvignon Blanc	2008	Elqoi, Chile	7.8	<DL	19.8
Chilean wine 10	Sauvignon Blanc	2008	Lontué, Chile	<DL	<DL	1.0
Chilean wine 11	Sauvignon Blanc	2007	Casablanca Valley, Chile	4.9	<DL	14.9
Chilean wine 12	Sauvignon Blanc	2008	Maule, Chile	<DL	<DL	2.2
Spanish wine 1	Garnacha (60%) Tempranillo (40%)	2005	Rioja, Spain	<DL	<DL	1.7
Spanish wine 2	Tempranillo	2004	Rioja, Spain	1.2[a]	<DL	2.7
Spanish wine 3	Tempranillo (90%) Graciano (5%) Garnacha (5%)	2003	Rioja, Spain	<DL	<DL	1.0
Spanish wine 4	Tempranillo (90%) Others (10%)	2003	Rioja, Spain	<DL	<DL	1.1
Spanish wine 5	Garnacha (55%) Tempranillo (40%) Mazuelo (5%)	2005	Rioja, Spain	<DL	<DL	0.8[a]
Spanish wine 6	Tempranillo (80%) Cabernet Sauvignon (20%)	2005	Ribera del Duero, Spain	<DL	<DL	5.9
Spanish wine 7	Tempranillo (80%) Cabernet Sauvignon (10%) Merlot (10%)	2007	Ribera del Duero, Spain	2.6	<DL	3.4
Spanish wine 8	Tempranillo (80%) Cabernet Sauvignon (20%)	2005	Ribera del Duero, Spain	1.1[a]	<DL	6.6

<DL: under detection limit.
[a] Below quantitation limit.

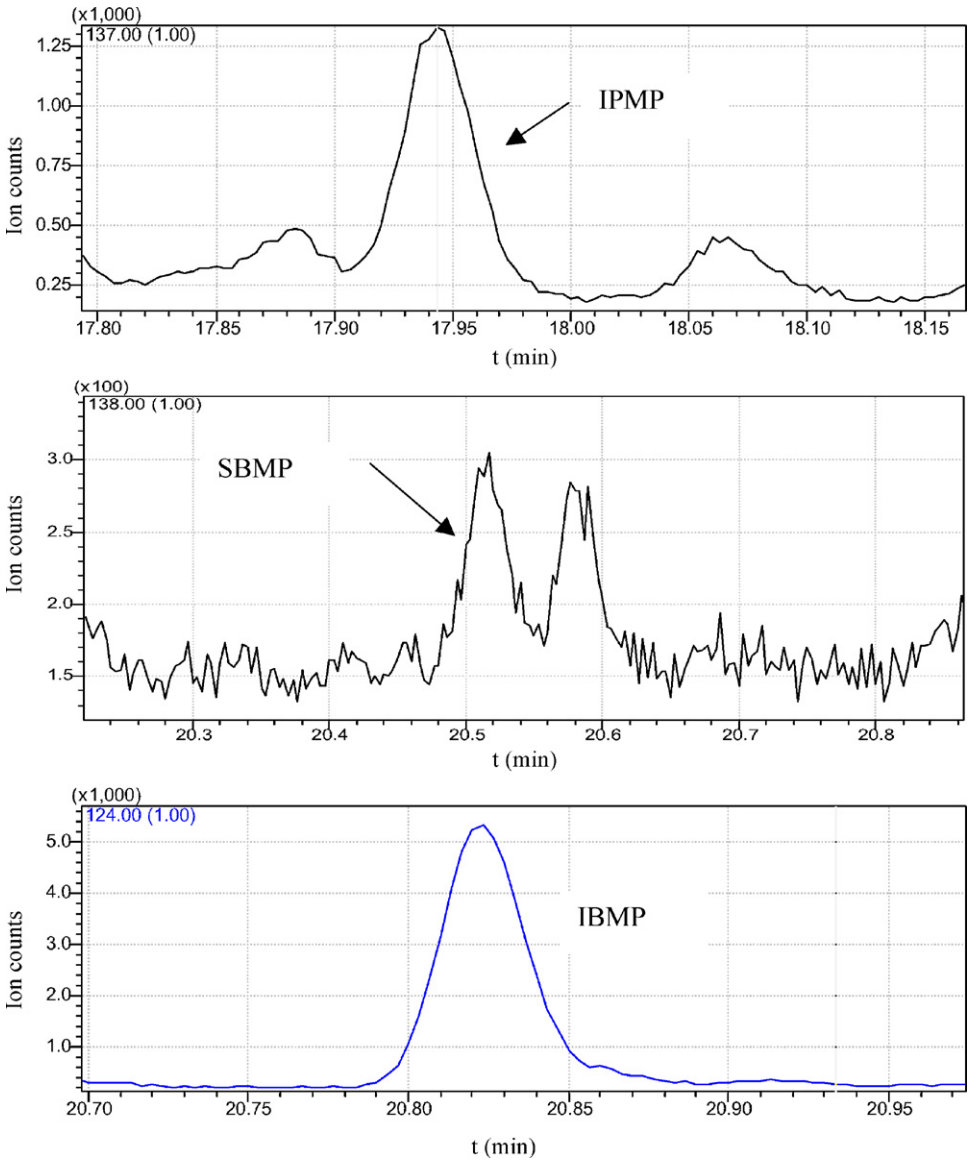

Fig. 1. SPE–GC–MS chromatograms obtained in the analysis following the proposed procedure, of a Chilean white wine containing 4.9 ng/L IPMP (*m*/*z* 137), 1.1 ng/L SBMP (*m*/*z* 138) and 14.9 ng/L IBMP (*m*/*z* 124).

obtained. In spite of this good result, this elution strategy was found inconvenient since the extracts contained considerable amounts of non-volatile compounds (mostly wine polyphenols) not fully eliminated during the previous washing-up steps. Additional cleaning of these solutions was found impractical.

The use of alkaline aqueous-methanol mixtures was also considered, but their elution power was also poor. For instance, a 50% methanol in water pH 12 solution provided a recovery around 20%.

Finally, different solvent systems containing 10 g/L of TEA were considered. Results of these studies are shown in Table 3, they represent total recoveries found in 8 mL of the solvent system. As shown in the table, recoveries were quite similar in all the systems and major differences appear in the distribution of analytes between the different fractions (data not shown). Surprisingly, in the solvent system dichloromethane/TEA there was no recovery at all in the first two fractions. That experiment was repeated 5 times in different days and recoveries were always null. Not surprisingly, the volume of the first two fractions corresponds exactly to the ion-exchange equivalent capacity of the sorbent system taking into account the concentration of TEA present in the solvent. This suggests that TEA dissolved in dichloromethane seems to be very efficient at neutralizing the exchangers and that only after neutralization has been completed and analytes are retained exclusively by non-ionic interactions, the elution takes place. Consequently, this solvent system was selected as the preferred for eluting MPs, although the first fractions should be discarded and considered as an additional rinsing phase. In the final procedure, to a 60 mg cartridge containing the analytes, 600 μL of the dichloromethane/TEA mixture are applied and discarded and analytes are recovered in 200 additional μL of the solvent mixture.

3.2. Proposed method

3.2.1. Method validation

3-Isopropyl-2-ethoxypyrazine (IPEP) was used as internal standard, as proposed in some reports [8,20,26]. As shown in the method optimization, this compound showed a similar behaviour to the targeted MPs in all the procedural steps.

Method linearity, repeatability, detection and quantitation limits were assessed by spiking a wine free of the analytes with levels between 0.5 ng/L and 25 ng/L. All this data are given in Table 4. As shown, in the case of SBMP linearity held only up to 10 ng/L, while in the other two cases, it held up to 25 ng/L. Determination coefficients were in all cases above 0.996, which can be considered satisfactory. Method detection limits were 0.4, 0.5 and 0.3 ng/L for IPMP, SBMP and IBMP, respectively, while method quantification limits were 1.3, 1.6 and 1.0 ng/L.

Method repeatability at 10–20 ng/L levels was excellent, with RSD values below 4% in the three cases. At 2 ng/L, RSD (%) were as expected, slightly worse: 17% for IPMP, 13% for SBMP and 21% for IBMP, but yet satisfactory for those low concentrations.

Method accuracy was finally determined by a standard recovery experiment carried out on 14 different samples spiked with 20–25 ng/L of analytes (see Section 2). Results of this experiment are shown in Table 4. As shown in the table, average recoveries are in all cases close to 100%, which suggests that the method is free from matrix effects. The RSD (%) obtained in the experiment, between 13% and 19%, are good estimates of the overall method accuracy. These values can be considered satisfactory for the low levels at which these compounds are found. Fig. 1 shows a typical chromatogram of one of the white Chilean wines analysed.

3.2.2. Wine analysis

The method was applied to the determination of the three compounds in 12 Chilean Sauvignon Blanc wines and in 8 Spanish aged red wines. Results are shown in Table 5. As shown in the table, only IBMP was detected in all the Chilean samples at concentrations above the method quantification limits, which is in accordance with data reported for this type of wines [6,33]. On the other hand, IPMP was found in 7 out of the 12 Chilean wines reaching a maximum level of 7.8 ng/L in one of the samples. SBMP was detected only in one of them and it was below method quantification limits. Results for Spanish red wines suggest that MPs are not aroma-relevant compounds in the Rioja wines analysed, as expected since wines from this area do not usually show odours related with MPs, while in wines from Ribera del Duero levels are higher as could be expected because of the presence of Cabernet Sauvignon in the blending. In this set of samples SBMP was not even detected.

4. Conclusions

A method to analyse MPs in wine has been developed. The proposed procedure utilizes a mixed-mode sorbent bed combining hydrophobic and cation-exchange extraction properties. The proposed method allows a determination based on a series of optimized steps to concentrate and isolate the analytes in a single separation cartridge. The procedure requires a small volume of sample and provides satisfactory linear range and enough sensitivity to determinate the concentration of 3-alkyl-2-methoxypyrazines in wine. The importance of pH and composition of mixed mode sorbents has been evaluated in the comparisons carried out. The application of the method has confirmed the low levels and likely low sensory relevance of 3-alkyl-2-methoxypyrazines in the aroma of Rioja Spanish wines.

Acknowledgements

This work has been funded by the Spanish Government, project CICYT AGL 2007-65139. E. Gracia-Moreno has received a grant of the FPU program from the Spanish Government. The authors also want to thank Manuel Flores Cabrales from the University of Chile for providing us with the Chilean wine samples.

References

[1] J.A. Maga, C.E. Sizer, J. Agric. Food Chem. 21 (1973) 22.
[2] C. Bayonove, R. Cordonnier, P. Dubois, C.R. Acad, Sci. Paris Ser. D 281 (1975) 75.
[3] A. Escudero, E. Campo, L. Farina, J. Cacho, V. Ferreira, J. Agric. Food Chem. 55 (2007) 4501.
[4] M.S. Allen, M.J. Lacey, R.L.N. Harris, W.V. Brown, Am. J. Enol. Viticult. 42 (1991) 109.
[5] D.M. Chapman, J.H. Thorngate, M.A. Matthews, J.X. Guinard, S.E. Ebeler, J. Agric. Food Chem. 52 (2004) 5431.
[6] L.D. Preston, D.E. Block, H. Heymann, G. Soleas, A.C. Noble, S.E. Ebeler, Am. J. Enol. Viticult. 59 (2008) 137.
[7] D. Roujou de Boubee, C. Van Leeuwen, D. Dubourdieu, J. Agric. Food Chem. 48 (2000) 4830.
[8] C. Sala, O. Busto, J. Guasch, F. Zamora, J. Sci. Food Agric. 85 (2005) 1131.
[9] V. Ferreira, A. Escudero, E. Campo, J. Cacho, in: R.J. Blair, P.J. Williams, I.S. Pretorious (Eds.), Australian Wine Industry Technical Conference Proceedings, Australian Wine Industry Technical Conference Inc., Adelaida, 2007, p. 142.
[10] E. Campo, V. Ferreira, A. Escudero, J. Cacho, J. Agric. Food Chem. 53 (2005) 5682.
[11] V. Ferreira, F. San Juan, A. Escudero, L. Cullere, P. Fernandez-Zurbano, M.P. Saenz-Navajas, J. Cacho, J. Agric. Food Chem. 57 (2009) 7490.
[12] I. Ryona, B.S. Pan, D.S. Intrigliolo, A.N. Lakso, G.L. Sacks, J. Agric. Food Chem. 56 (2008) 10838.
[13] T.L. Galvan, S. Kells, W.D. Hutchison, J. Agric. Food Chem. 56 (2008) 1065.
[14] Y.S. Kotseridis, M. Spink, I.D. Brindle, A.J. Blake, M. Sears, X. Chen, G. Soleas, D. Inglis, G.J. Pickering, J. Chromatogr. A 1190 (2008) 294.
[15] Y. Kotsteridis, A. Anocibar Beloqui, A. Bertrand, J.P. Doazan, Am. J. Enol. Viticult. 49 (1998) 44.
[16] G.J. Pickering, A. Karthik, D. Inglis, M. Sears, K. Ker, J. Food Sci. 72 (2007) S468.
[17] M.J. Lacey, M.S. Allen, R.L.N. Harris, W.V. Brown, Am. J. Enol. Viticult. 42 (1991) 103.
[18] M.S. Allen, M.J. Lacey, S. Boyd, J. Agric. Food Chem. 42 (1994) 1734.
[19] M.S. Allen, M.J. Lacey, S.J. Boyd, J. Agric. Food Chem. 43 (1995) 769.
[20] C. Sala, M. Mestres, M.P. Marti, O. Busto, J. Guasch, J. Chromatogr. A 880 (2000) 93.
[21] C. Prouteau, R. Schneider, Y. Lucchese, F. Nepveu, R. Renard, C. Vaca-Garcia, Anal. Chim. Acta 513 (2004) 223.
[22] D. Ryan, P. Watkins, J. Smith, M. Allen, P. Marriott, J. Sep. Sci. 28 (2005) 1075.
[23] E. Campo, V. Ferreira, A. Escudero, J.C. Marqués, J. Cacho, Anal. Chim. Acta 563 (2006) 180.
[24] L. Culleré, A. Escudero, E. Campo, J. Cacho, V. Ferreira, J. Chromatogr. A 1216 (2009) 4040.
[25] S. Boutou, P. Chatonnet, J. Chromatogr. A 1141 (2007) 1.
[26] C. Sala, M. Mestres, M.P. Marti, O. Busto, J. Guasch, J. Chromatogr. A 953 (2002) 1.
[27] I. Ryona, B.S. Pan, G.L. Sacks, J. Agric. Food Chem. 57 (2009) 8250.
[28] H.G. Schmarr, S. Ganß, S. Koschinski, U. Fischer, C. Riehle, J. Kinnart, T. Potouridis, M. Kutyrev, J. Chromatogr. A 1217 (2010) 6769.
[29] P. Alberts, M.A. Stander, S.O. Paul, A. de Villiers, J. Agric. Food Chem. 57 (2009) 9347.
[30] L. Cullere, M. Bueno, J. Cacho, V. Ferreira, J. Chromatogr. A 1217 (2010) 1557.
[31] R. Lopez, M. Aznar, J. Cacho, V. Ferreira, J. Chromatogr. A 966 (2002) 167.
[32] H. Heymann, A.C. Noble, R.B. Boulton, J. Agric. Food Chem. 34 (1986) 268.
[33] A. Belancic, E. Agosin, Am. J. Enol. Viticult. 58 (2007) 462.