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Headspace solid-phase microextraction method for determining 3-alkyl-2-methoxypyrazines in musts by means of polydimethylsiloxane–divinylbenzene fibres

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Abstract

A method for determining 2-methoxypyrazine, 3-methyl-, 3-ethyl-, 3-isopropyl-, 3-*sec*.-butyl- and 3-isobutyl-2-methoxypyrazine in musts is described. It involves headspace solid-phase microextraction (SPME) and determination by capillary gas chromatography using nitrogen–phosphorous detection. Pyrazines were satisfactorily separated under isothermal conditions, and quantification was carried out using 3-isopropyl-2-ethoxypyrazine as the internal standard. Ionic strength, time and temperature were studied in order to make SPME as efficient as possible. The developed method enabled detection limits at the 0.1 ng l^{-1} levels for some of the analytes. The method was successfully applied to identify and quantify different 3-alkyl-2-methoxypyrazines in experimental musts of Cabernet Sauvignon and Merlot. Their evolution during the ripening was also monitored.

Keywords: Must; Food analysis; Headspace analysis; Wine; Extraction methods; Alkylmethoxypyrazines; Pyrazines; Aroma compounds

1. Introduction

3-alkyl-2-methoxypyrazines are aromatic compounds present among the flavours of a wide range of foods, such as bell peppers, asparagus, peas and potatoes [1,2]. These compounds have also been found in Cabernet Sauvignon, Sauvignon Blanc, Cabernet Franc and Merlot wines, where they can be important to the variety aroma [3–6]. Although their concentration can vary due to climatic conditions and pruning techniques [7], it has been established that they can be present at ng l^{-1} level in wines [4,7–14].

These concentrations are enough to influence the wine aroma [15]. Indeed, the threshold of 3-isobutyl-2-methoxypyrazine has been established to be between 2 ng l^{-1} in water or white wines [9] and 10 ng l^{-1} in red wines [4], so it can strongly influence the character of those wines even at very low concentrations.

Such low concentrations have made identification and quantification of 3-alkyl-2-methoxypyrazines difficult [16,17], for their analysis requires very sensitive analytical techniques and a great concentration of the analytes. Stable isotope dilution with gas chromatography–mass spectrometry [10,13,14] has been used to determine 3-alkyl-2-methoxypyrazines in wines. However, those techniques are time consuming and expensive.

Solid-phase microextraction (SPME) is a simple rapid solvent-free and inexpensive technique that has been recently developed [18]. Devices for SPME

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consist of a piece of fused-silica fiber of about 1 cm in length coated with a polymer phase. This fibre is placed in contact with the sample, by either immersing it in a liquid or placing it in the headspace of the sample. The polymer is capable of extracting some of the compounds present in the sample, so partitioning and concentrating them. Then the extracted compounds are directly transferred into a GC injector port, where they are thermally desorbed.

SPME has been used for flavour analysis, especially by sampling the headspace of wines (HS-SPME) [19–26]. Recent works have also shown that SPME using fibres of polydimethylsiloxane (PDMS) and Carbowax–divinylbenzene (CW–DVB) were very effective in the analysis of volatile and semivolatile Maillard reaction products, between which there were some 3-alkyl-2-methoxypyrazines [27].

The aim of this study was to develop a method for determining 3-alkyl-2-methoxypyrazines in musts by means of gas chromatography with nitrogen–phosphorous detection (GC–NPD) with HS-SPME.

2. Experimental

2.1. Equipment and chromatographic conditions

Chromatographic analyses were made on a Hewlett-Packard 5890 II gas chromatograph equipped with a nitrogen–phosphorous detector (NPD). The injection was made in the splitless mode for 1 min at 250°C using an inlet of 0.75 mm I.D. that improved the GC resolution [18]. Detector signals were sent to a HP Chemstation, where they were collected, integrated and recorded.

A CP-WAX 57 CB (50 m×0.25 mm I.D. 0.2-μm film thickness) fused-silica capillary column was used as the analytical column. Carrier gas was high-purity helium and was flowing through the column at 0.8 ml min^{-1} (head pressure, 180 kPa; split flow, 52 ml min^{-1}; purge flow, 2 ml min^{-1}). The make-up gas to the detector was nitrogen at 24 ml min^{-1}. The H_2 flow was 4 ml min^{-1} and the air flow, 100 ml min^{-1}. Injector and detector were held at 250°C. The oven temperature was 30°C for 1 min, then it was raised at 25°C min^{-1} to 100°C, and held for 20 min. Finally, the temperature was raised at 25°C min^{-1} to 180°C for 20 min to purge the column. A SPB-35 (30 m×0.25 mm I.D. 0.25-μm film thickness) fused-silica capillary column, operating under the same chromatographic conditions, was used to confirm the pyrazines previously identified with the analytical column.

2.2. Reagents and solutions

Pyrazine compounds were all supplied by Aldrich-Chemie (Beerse, Belgium). They were as follows: 2-methoxypyrazine (MOPI), 3-methyl-2-methoxypyrazine (MEMP), 3-ethyl-2-methoxypyrazine (ETMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*.-butyl-2-methoxypyrazine (SBMP) and 3-isobutyl-2-methoxypyrazine (IBMP). 3-Isopropyl-2-ethoxy-pyrazine (IPEP) was used as internal standard (I.S.), and was provided by Pyrazine Specialties (Atlanta, GA). The purity of all standards was above 97%. HPLC-grade ethanol absolute was provided by Scharlau, (Barcelona, Spain). Freshly boiled deionized water was used to prepare all solutions.

Stock solutions of 200 μg ml^{-1} of each pyrazine and I.S. were prepared in absolute ethanol. They were stored in darkness at −23°C until use. A global standard solution containing 2 μg ml^{-1} of each analyte and a solution containing 2 μg ml^{-1} of the I.S. were prepared by dilution of the stock solutions in water. Finally, six calibration solutions in the range 2–100 ng l^{-1} were prepared by suitable dilution of the global solutions in water. The concentration of I.S. in all calibration solutions was 10 ng l^{-1}. Recovery and detection limits studies were carried out using a pyrazine-free must into which 10 ng l^{-1} of the standards were dissolved.

The manual SPME device and fibers used in this study were purchased from Supelco (Bellefonte, USA). The following fibres were used: PDMS 100 μm, polydimethylsiloxane–divinylbenzene (PDMS–DVB) 65 μm, Carboxen–poly-dimethylsiloxane (Carboxen–PDMS) 75 μm, and CW–DVB 65 μm. They were conditioned and cleaned after use by inserting them into a GC injector at the recommended temperatures. They were immediately used to prevent contamination. Operating temperatures were the same as those used for conditioning.

2.3. Grapes and musts

Samples were collected from the vineyards of Merlot and Cabernet Sauvignon of the experimental fields of the Facultat d'Enologia de Tarragona (Universitat Rovira i Virgili) at Constantí (Tarragona). Samples of grapes were collected during the ripening and the harvest day. They were manually pressed at the laboratory and the juice was analysed. Musts after 1 day of maceration were collected from the fermentation tanks in the experimental cellar of the faculty. Three replicates of each kind of musts were taken in all cases.

Red grapes were used to prepare a pyrazine-free must that was used as control must in the recovery and detection limits experiments.

2.4. HS-SPME of musts

One millilitre of a 100 ng l^{-1} solution of IPEP was added to an aliquot of 10 ml sample of must, which was placed in a 20 ml vial for SPME. Three grams of NaCl and a little magnetic stirrer were added. It was thickly capped with a PTFE faced silicone septum, put in an isothermal bath at 30°C and continuously stirred. By means of the stainless-steel needle of the SPME holder, the PDMS fiber was then introduced into the headspace and allowed contact with it for 4 h. The fibre was fully exposed in the middle of the headspace all the time. As methoxypyrazines are photosensitive [16] the overall microextraction process was done in the darkness. Then, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC for thermal desorption at 250°C for 1 min. All analyses were made in duplicate.

3. Results and discussion

All pyrazines were separated with both columns in less than 20 min. Although some problems in the chromatographic separation of ETMP and IPMP have been described in the literature [28], we have found that they appeared close, but clearly separated, when the 57CB column is used. A chromatogram of a solution containing all the analytes is shown in Fig. 1. IPEP was chosen as I.S. because it belongs to the series of 3-alkyl-2-alcoxypyrazines and it has not been referred to in the literature as present in musts. Furthermore, this compound was absent from the analysed samples.

In the analytical method developed, several SPME variables were studied: SPME fibre, ionic strength, temperature and time of extraction. Experiments were carried out with water buffered at pH 3.5 with tartaric acid and spiked with 10 ng l^{-1} of each pyrazine. IPEP was used as internal standard at a concentration level of 10 ng l^{-1}. As stirring usually improves the extraction, all experiments were done under constant stirring conditions [29].

Satisfactory levels of pyrazine recoveries for the PDMS fibers have been reported in the literature

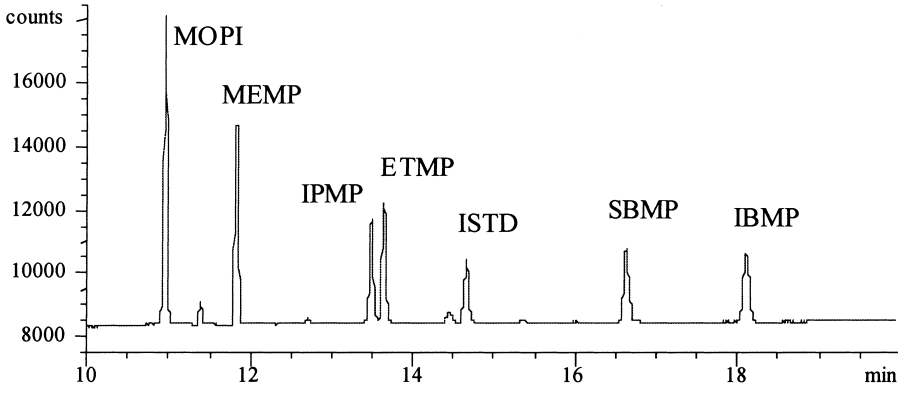

Fig. 1. Standards separated with the 57CB column (direct injection of 1 μl of a 200 μg l^{-1} solution in ethanol). I.S. is 3-isopropyl-2-methoxypyrazine (IPEP).

[27], although among the fibres studied in the present work, the PDMS–DVB fibre showed the best results for the extraction of the pyrazines.

The tendency of the analytes to pass to the headspace and, therefore, to the fiber, can be accelerated by ionic strength [18]. NaCl amounts of 0.1, 0.2, 0.3 and 0.4 g ml^{-1} were tested. It was observed that the chromatographic signal increased as the amount of NaCl increased, but for amounts higher than 0.3 g ml^{-1} the response became constant. So, 0.3 g ml^{-1} was chosen as the optimum value.

3-Alkyl-2-methoxypyrazines do not have a high volatility, so the time for them to reach the equilibrium among the coated-fiber, the headspace and the sample solution was expected to be long. As the diffusion of the analytes through the three system phases is essential in the HS-SPME technique [18], the effect of the time in the extraction of the analytes was optimised. Periods from 30 min to 14 h were tested and compared. Results showed that a minimum of 4 h is required to reach the equilibrium, as shown in Fig. 2, where the influence of the time in the extraction of 3-alkyl-2-methoxypyrazines by the PDMS–DVB fibre can be observed.

Although high temperatures can increase the presence of analytes in the headspace, desorption can also be enhanced at higher temperatures. In order to find the best temperature for extracting the 3-alkyl-2-methoxypyrazines, the effect of temperatures from 20°C to 40°C in the extraction of the analytes was checked. Results showed that the best recoveries were obtained at 30°C. The influence of temperature in the extraction of 3-alkyl-2-methoxypyrazines by the PDMS–DVB fiber can be seen in Fig. 3.

Having studied the extraction parameters, calibration curves, using the internal standard method, were built and the linearity range and the limits of detection and quantification of the method were estimated.

Five replicates of six standard solutions in the range 2–100 ng l^{-1} of each analyte, all of them with a content of I.S. of 10 ng l^{-1} were analysed. The mean values were used to construct the calibration graphs by plotting the peak-area ratios against the concentration ratios. Regression, slope and origin intercept were calculated by linear least-squares regression. Good linearity was obtained in the range 2–100 ng l^{-1} for all the analytes. In all cases, the correlation coefficient was good ($r^2 > 0.99$). Table 1 shows the chromatographic retention times and coefficients of the calibration curves for each analyte.

A pyrazine-free must (pH 3.5) spiked with low levels of pyrazines was used to establish the detection limits of the overall method. Fig. 4 shows a chromatogram of a pyrazine-free must spiked with 10 ng l^{-1} of each standard and I.S. The lowest concentration detectable at a signal-to-noise ratio of

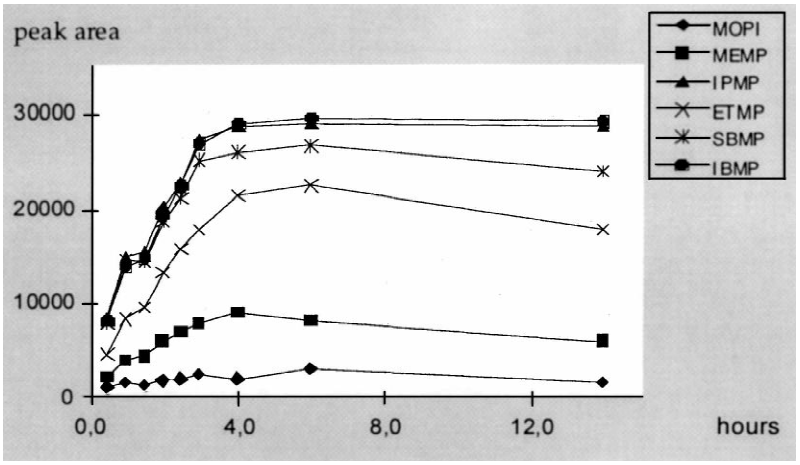

Fig. 2. Influence of time on the extraction of 3-alkyl-2-methoxypyrazines by PDMS–DVB fibres ($n=3$).

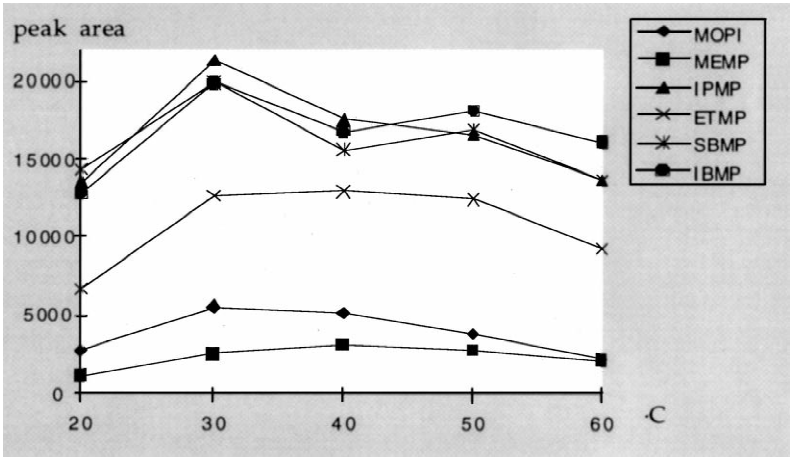

Fig. 3. Influence of temperature on the extraction of 3-alkyl-2-methoxypyrazines by PDMS–DVB fibres ($n=3$).

Table 1
Chromatographic retention times and coefficients of calibration line ($n=6$)[a]

Pyrazines	RRT	Calibration line		
		a	*b*	r^2
MOPI	0.75	0.016	0.061	0.992
MEMP	0.81	0.005	0.285	1.000
IPMP	0.92	0.027	0.830	1.000
ETMP	0.93	0.257	0.619	1.000
SBMP	1.13	0.042	0.814	0.999
IBMP	1.23	0.075	0.801	0.999

[a] RRT, relative retention time=t_R(pyrazine)/t_R(I.S.)×t_R(I.S.)= 15.3 min. Calibration graph: slope (*b*), intercept (*a*) and correlation coefficient (r^2).

3 was 0.1 ng l^{-1} for IPMP, SBMP and IBMP, 0.5 ng l^{-1} for ETMP and 1 ng l^{-1} for MEMP and for MOPI.

Because of the low detection limits and the range of linearity, the method enables the six pyrazines to be determined in musts at the ng l^{-1} level. Such a level is useful because the human olfactory thresholds for the most significant methoxypyrazines are about 2–10 ng l^{-1}[4,9].

The recovery of the overall method was tested with a pyrazine-free must spiked with 10 ng l^{-1} of each pyrazine. Six samples were prepared and extracted, according to the method described. The recoveries were evaluated as the quotient between

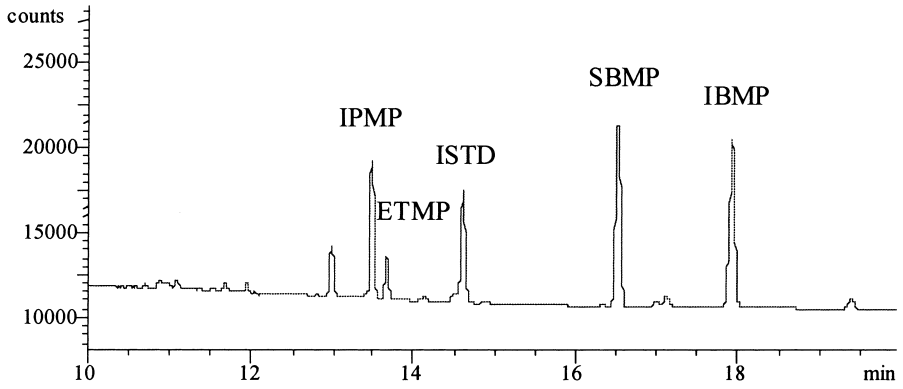

Fig. 4. Chromatogram of a sample of Merlot (must after 1 day of maceration), prepared according to the described method.

Table 2
Mean recoveries and relative standard deviation (RSD) of pyrazines in musts spiked with 10 ng l^{-1} of each pyrazine (n=6)

Pyrazines	Recovery (%)	RSD (%)
MOPI	43.3	35.3
MEMP	56.5	30.8
IPMP	94.1	19.8
ETMP	58.2	29.5
SBMP	109.4	17.2
IBMP	108.2	13.2

the amount of the analyte extracted, determined from the calibration graphs, and the total amount of the analyte spiked in the sample. Table 2 shows the results for recoveries of each analyte. As can be seen, the most important analytes IBMP, SBMP and IPMP showed very good recoveries (90–105%) with standard deviation less than 20%. ETMP showed also good recovery (60–70%). MOPI and MEMP were found to have the lowest recovery, so having the highest standard deviation.

The method proposed was successfully applied to experimental musts. The chromatogram of a sample of Merlot can be seen in Fig. 4. IBMP, SBMP, IPMP and ETMP and 3-ethyl-2-methoxypyrazine were identified in the samples.

Pyrazine contents of the samples are shown in Table 3. These results match those found in the literature. Reported levels of IBMP are 3.6–56.3 ng l^{-1} [4,8–11,13], although, more recently, mean values of 12–13 ng l^{-1} for Cabernet Sauvignon and 4–8 for Merlot were obtained in Bordeaux wines of different vintages [14]. Levels of SBMP in wines that have been reported in the literature (0.05–1.9 ng l^{-1}) [8,10] are lower than the levels of SBMP in musts reported here. Differences in climatic conditions and viticulture techniques [7] may be responsible for this. Reported contents of IPMP are of 0.9–10.1 ng l^{-1} [11,12], which matches the results presented here. Although ETMP was identified in some of the samples, its levels were always below the quantification limits. Any sample showed MEMP and MOPI.

Data show that the content of methoxypyrazines in the juice of the berries decreases with the ripening of the fruit, and the content found on the harvest day are the lowest. But the day after the harvest these contents increase considerably, thus suggesting that maceration of the skins can increase the content of methoxypyrazines in the future wines.

4. Conclusions

The method described allows the determination of 3-alkyl-2-methoxypyrazines in musts at ng l^{-1} levels, which are below their sensory threshold. It provides high recoveries, limits of detection at the 0.1–1 ng l^{-1} level and a range of linearity of 2–100 ng l^{-1}. On account of its simplicity and rapidity, the method seems to be adequate for oenological laboratory work.

Table 3
Pyrazine contents in experimental musts[a]

		Yield (ml g^{-1})	pH	Percentage (v/v) (ap.)	IBMP		SBMP		IPMP	
					(ng l^{-1})	SD	(ng l^{-1})	SD	(ng l^{-1})	SD
Merlot										
10 August 1998	r1	0.466	3.83	9.0	21.7	5.0	15.8	5.3	11.2	4.3
21 August 1998	r2	0.426	3.77	10.7	10.4	3.6	10.7	3.8	6.3	2.3
01 September 1998	h1	0.419	3.59	10.9	5.6	2.7	2.7	1.7	2.1	1.2
02 September 1998	h2	0.419	3.91	11.7	19.4	5.3	18.1	5.4	8.2	3.1
Cabernet Sauvignon										
21 August 1998	r1	0.362	3.38	8.5	18.8	5.4	11.4	4.7	4.5	0.4
08 September 1998	r2	0.361	3.60	10.9	4.1	1.0	2.8	1.8	<q.l.	–
15 September 1998	h1	0.366	3.71	11.1	2.2	0.6	<q.l.	–	<q.l.	–
16 September 1998	h2	0.366	3.83	10.9	10.0	2.2	2.6	1.9	<q.l.	–

[a] SD=standard deviation; r1, first ripening sampling; r2, second ripening sampling; h1, harvest day; h2, must after 1 day of maceration; <q.l., below quantification limits.

The method has been applied to experimental musts of Cabernet Sauvignon and Merlot, and analytes have been monitored along the ripening and harvest. 3-Isobutyl-2-methoxypyrazine, 3-*sec*.-butyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine and 3-ethyl-2-methoxy-pyrazine have been found in the musts. Their contents decrease during the ripening and the lowest contents were found in the harvest day. Maceration seems to enhance concentration levels, suggesting that 3-alkyl-2-methoxypyrazines could be present in the grape skins.

Acknowledgements

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