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Headspace solid-phase microextraction analysis of 3-alkyl-2-methoxypyrazines in wines ☆

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Abstract

A procedure to determine 3-alkyl-2-methoxypyrazines in wines is described. It is based on the headspace solid-phase microextraction (HS-SPME) technique after a clean-up of the sample by distillation (previously acidified to pH 0.5) to remove ethanol and other volatile compounds that can interfere in the SPME. Determination is performed by means of capillary gas chromatography using a nitrogen–phosphorus detector. The method allows quantification of 3-isobutyl-2-methoxypyrazine, 3-*sec*-butyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine at their natural concentration levels and below their sensory thresholds in Cabernet Sauvignon and Merlot wines. The method was successfully applied to experimental red wines and the evolution of their pyrazine contents during the winemaking process was monitored. Pyrazine content increased during the first maceration day but did not change significantly during alcoholic and malolactic fermentation. Final contents in wines were 12–27 ng/l of 3-isobutyl-2-methoxypyrazine and 5–10 ng/l of 3-*sec*-butyl-2-methoxypyrazine

Keywords: Headspace analysis; Solid-phase microextraction; Wine; Food analysis; Aroma compounds; Alkylmethoxypyrazines; Pyrazines

1. Introduction

3-Alkyl-2-methoxypyrazines are very potent odorous compounds which occur widely in natural products, such green peas and bell peppers [1–3]. 3-Isobutyl-2-methoxypyrazine (IBMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) have been reported to be present at very low concentrations (ng/l) among the aroma components of Cabernet sauvignon, Merlot noir and Sauvignon blanc wines, where they contribute to the vegetative/herbaceous/bell pepper character of these wines [4–11]. Although the concentrations of these compounds are very low, they can influence the wine aroma because of their extremely low sensory thresholds. Indeed, sensory thresholds for IPMP, SBMP and IBMP are 1–2 ng/l in water [1,12]. It has been reported that the sensory threshold of IBMP in red wines is higher, about 10–16 ng/l [10,11].

Due to the extremely low concentrations at which

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3-alkyl-2-methoxypyrazines occur in wines, their identification and quantification is very difficult. The presence of ibmp in Cabernet Sauvignon grape juice was reported for the first time in 1975 [13] and later was found in Sauvignon blanc wines [4]. Since then, several methods for analysing 3-alkyl-2-methoxypyrazines in wines have been reported [14,15].

Accurate quantification of IBMP in wines, with detection limits below its sensory threshold, was achieved by GC–MS using a deuterium labeled internal standard [5]. Though the stable isotope dilution method is very laborious, because it involves distillation of wine and extraction of IBMP by cation-exchange resin and liquid–liquid extraction, it has been used for the determination of pyrazines in wines [6–8]. This method has been modified to enable the use of a commercial non-labeled internal standard [16] and used in the study of pyrazines in must and wines [17,18].

Also, a simple and quick method, using liquid–liquid extraction and GC–MS has recently been described [10]. Nevertheless, detection limits of the method are very close to the sensory threshold of the analytes and it has been improved by using the stable isotopic dilution assay [19].

Thus, determination of 3-alkyl-2-methoxypyrazines in wines requires very sensitive and selective techniques as well as the ability to greatly concentrate the analytes. Recently, we have developed a method based on headspace solid-phase microextraction (HS-SPME) and gas chromatography with nitrogen–phosphorus detection (GC–NPD) for the analysis of 3-alkyl-2-methoxypyrazines in musts [20]. This method provides high recoveries and detection limits at 0.1 ng/l level for IBMP and SBMP.

HS-SPME is a relatively new technique that allows a solventless extraction together with a concentration of volatile compounds [21–23]. It has been applied for the determination of volatile compounds in foods and beverages [24–26] and particularly for the analysis of wine aroma [27–35]. The technique is based on the use of a polymer-coated silica fiber that is housed in a stainless-steel needle of a modified syringe. This special device allows the exposure of the fiber to the headspace above a sample wherein the volatile compounds are extracted by and concentrated on the polymer phase. Then, the device allows the removal of the fiber from the headspace and its transfer into the injection port of the gas chromatograph, where the compounds extracted are thermally desorbed. A technique based on HS-SPME coupled with GC–MS/flame ionization detection (FID) has been used to analyse pyrazines and other Maillard reaction products, reaching detection limits at the μg/l-level [36]. Because of its sensitivity, low cost and time saving, the SPME technique is better than the classical techniques of sample treatment.

The aim of this study was to set up a method for determining 3-alkyl-2-methoxypyrazines in wines by means of HS-SPME with GC–NPD, taking into account the previously developed method for must analysis [20], which was modified in order to adapt it to the determination of the same compounds in wines.

2. Experimental

2.1. Reagents and solutions

Analyte standards were all supplied by Aldrich-Chemie (Beerse, Belgium). They were the following: 2-methoxypyrazine (MP) [3149-28-8], 3-methyl-2-methoxypyrazine (MEMP) [2847-30-5], 3-ethyl-2-methoxypyrazine (ETMP) [25680-58-4], IPMP [25773-40-4], SBMP [24168-70-5] and IBMP [24683-00-9]. 3-Isopropyl-2-ethoxy-pyrazine (IPEP) [72797-16-1] was used as internal standard and was provided by Pyrazine Specialties (Atlanta, GA, USA). The purity of all standards was above 97%.

Stock solutions of 200 μg/ml of each pyrazine were prepared in HPLC-grade absolute ethanol. They were stored in darkness at −23 °C until use. Stability tests proved that pyrazines stored like this are perfectly stable for more than 20 months. All solutions prepared from these stock solutions were used for a maximum of 1 month and care was taken to avoid photodegradation [14] of the pyrazines.

A global standard solution containing 2 μg/ml of each analyte and a solution containing 2 μg/ml of IPEP were prepared by dilution of the stock solutions in freshly boiled deionized water. A pyrazine-free wine spiked with suitable amounts of these solutions was used to prepare the samples for the study of

recoveries and detection limits of the HS-SPME method for wines. Samples of this wine with 10 ng/l of each standard and IPEP were used to study the recoveries, and samples with progressively lower concentrations of the analytes and IPEP (10 ng/l) were used to find out the detection limits.

2.2. Musts and wines

Experimental wines of the varieties Merlot and Cabernet Sauvignon were made at the experimental cellar of the Facultat d'Enologia de Tarragona (Universitat Rovira i Virgili) at Constantí (Tarragona, Spain). Samples were collected after each stage of the winemaking process. Three replicates of each sample were taken and analysed in all cases.

Sampling times and procedures were the following: (1) On harvest day, grapes of each variety were randomly collected in the vineyards, they were pressed and samples of juices were collected. (2) Samples of musts were collected from the fermentation tanks after 1 day of maceration, together with the solid parts of the grapes. (3) Samples of wine were collected after alcoholic fermentation by the classical red winemaking process. (4) Wine samples were finally collected after malolactic fermentation. NaF (1 g/l) was added to the must samples as a preservative. All samples were stored in dark bottles at 4 °C until analysis.

2.3. SPME fibers

The SPME device and the 65-μm polydimethylsiloxane–divinylbenzene (PDMS–DVB) fibers used in this study were purchased from Supelco (Bellefonte, PA, USA). Each fiber was conditioned before use by inserting it into a GC injector at 260 °C and immediately used to prevent contamination.

2.4. HS-SPME procedures

Samples of musts were analysed according to a previously described method [21]. The procedure for the analysis of wines was an adaptation of this method to prevent ethanol interference. Preliminary experiments had shown that distillation of a synthetic wine spiked with the target pyrazines at pH 0.5 eliminated ethanol and other volatile compounds, without losing the protonated pyrazines. Subsequent neutralisation of the ethanol-free solution allowed the SPME of pyrazines without any interference. Thus, the final procedure for wines was the following: 10 ml of wine was spiked with 1 ml of IPEP (100 ng/l), acidified with HCl and distilled on a rotary evaporator at low pressure (water pump) and room temperature. After reducing the volume of the sample to 50%, the resulting solution was neutralised with NaOH (pH 7) and transferred into a 20-ml vial containing 3 g of NaCl and a magnetic stirrer. The vial was thickly capped with PTFE-faced silicone septum and the SPME extraction was performed al 30 °C during 4 h with constant stirring. After extraction, the analytes were desorbed into the GC injector at 250 °C in splitless mode (1 min). This modification was validated by determining the recoveries and detection limits of the new overall method.

2.5. Equipment and chromatographic conditions

Chromatographic analysis was performed with a Hewlett-Packard 5890 II gas chromatograph equipped with a NPD system. The NPD temperature was 250 °C; the make-up gas was nitrogen flowing at 24 ml/min. Flows of the flame gases were: 4 ml/min of hydrogen and 100 ml/min of air. Carrier gas was helium and head pressure was 180 kPa. The split flow was 52 ml/min and the purge flow was 2 ml/min. A split/splitless injector was used in the splitless injection mode (1 min). Injection was performed with an inlet of 0.75 mm I.D. and with an injector temperature of 250 °C.

Comparison between retention times of two different fused-silica capillary columns allowed identification of the peaks. A CP-Wax 57 CB (50 m×0.25 mm I.D., 0.2 μm film thickness) column was used as the analytical column. A SPB-35 (30 m×0.25 mm I.D., 0.25 μm film thickness) column was used to confirm peak identities.

Carrier gas was flowing through the CP-Wax and SPB-35 columns at 0.8 and 1.3 ml/min, respectively. Oven temperature was programmed as follows: 30 °C (1 min), 25 °C/min, 100 °C (20 min). In order to purge the column, the temperature was finally raised to 180 °C at a rate of 25 °C/min and kept for 20 min. Because a good separation of the analytes

Table 1
Analytical parameters of the HS-SPME method for wines

Pyrazines	Recovery±SD (%)	LOD (ng/l)
MP	36.2±15.1	4.0
MEMP	31.0±12.5	4.0
ETMP	78.3±24.8	1.0
IPMP	80.5±14.2	0.3
SBMP	100.7±12.0	0.3
IBMP	104.7±17.0	0.3

Mean recoveries and standard deviations (SD) for the extraction of 10 ml of a pyrazine-free red wine spiked with 10 ng/l of pyrazines (n=6). Percentages are calculated from the values obtained by the method, using the calibration curves published in Ref. [20]. Limits of detection (LODs) of the overall method.

was achieved under these conditions on both columns, the same oven program was used for both of them.

3. Results and discussion

The HS-SPME parameters (fibre coating, temperature, ionic strength, and extraction time) were optimised for the analysis of 3-alkyl-2-methoxypyrazines in musts in our previous work [20]. A good chromatographic separation of the target pyrazines was obtained in less than 20 min with the CP-Wax 57 CB column. Using IPEP as internal standard (I.S.), the calibration graphs showed a very good correlation (r^2>0.99). These were constructed, as in the previous study [20], with five replicates of six standard solutions in the range 2–100 ng/l of each analyte, all of which contained I.S. at 10 ng/l. Regression, slope and intercept values were calculated by the linear least-squares method.

The HS-SPME method for analysing pyrazines in wines shows very high mean recoveries (Table 1), with acceptable standard deviations. For the most important analytes, SBMP and IBMP, recovery is about 100%. IPMP and ETMP showed mean recoveries from 78 to 80%, MEMP and MP were found to have the lowest mean recoveries (31–36%) and the highest relative standard deviations. These recoveries are good enough for quantification of IBMP, SBMP, IPMP and ETMP in red wines. Besides, the chromatograms obtained with this method show a clean baseline in the region of the analytes, proving that the HS-SPME method provides a good clean-up of the sample, together with a high concentration of the analytes (Figs. 1 and 2).

Limits of detection, with a signal-to-noise ratio of 3, are 0.3 ng/l for IPMP, SBMP and IBMP, and 1 ng/l for ETMP (Table 1). MEMP and MP show higher detection limit levels. The method is suitable for the quantification of 3-alkyl-2-methoxypyrazines at their natural concentration levels in Cabernet sauvignon and Merlot wines.

SBMP and IBMP were identified in both experimental wines (Figs. 1 and 2), and the peak of the

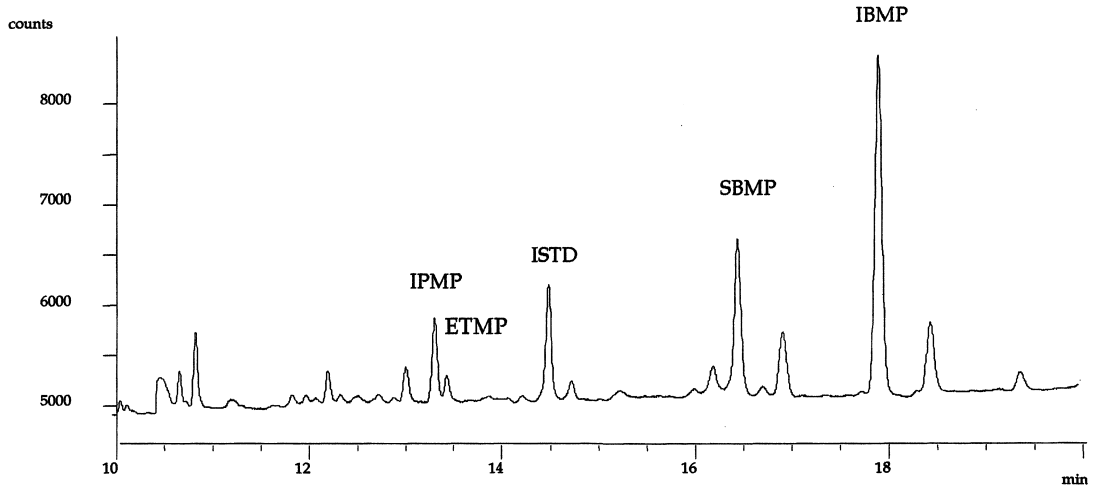

Fig. 1. Chromatogram of a Merlot wine sample analysed using the proposed procedure.

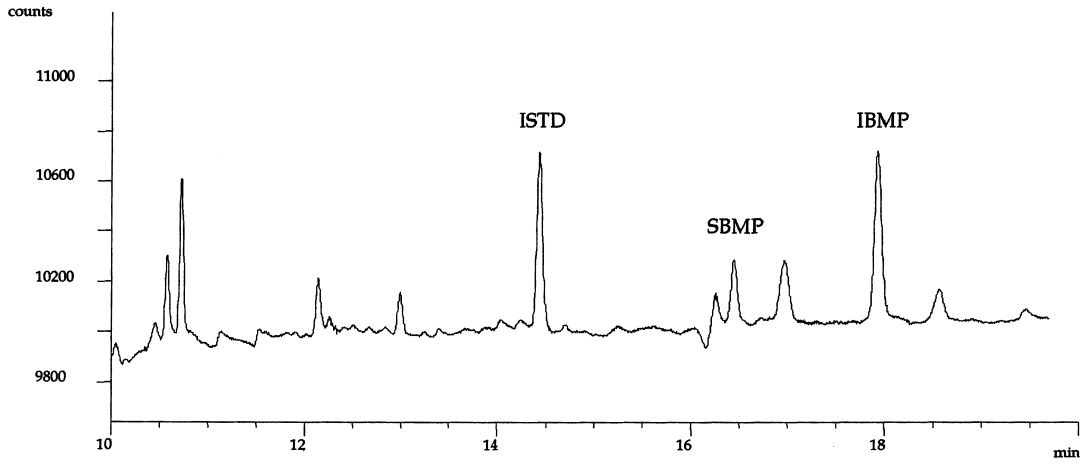

Fig. 2. Chromatogram of a Cabernet sauvignon wine sample analysed using the proposed procedure.

former was in all cases smaller than that of the later. IPMP was clearly identified and quantified in Merlot wines. The concentrations of this compound were clearly lower than those of both SBMP and IBMP. IPMP was not identified in the analysed wines and, although it was identified in some Cabernet sauvignon musts, its contents were generally very close to the detection limits and quantification could not be achieved. ETMP was identified in some Merlot wines and its peak can be observed in Fig. 1. However, the concentration of this compound was always too low to be quantified.

Pyrazine concentrations at the harvest were very low (Table 2). However, there is an important increase in the pyrazine contents throughout the winemaking process, which agrees with data published in the literature [37]. A possible explanation for these results is that 3-alkyl-2-methoxypyrazines occur mainly in the skins of the fruits and they pass to the grape juice during maceration. This hypothesis is supported by the fact that, according to the results presented here, the main increase in the pyrazine content took place during the first maceration day. Moreover, pyrazine contents showed a slight increase or remained constant during alcoholic fermentation, and malolactic fermentation did not alter significantly their concentrations either.

4. Conclusions

A procedure for the analysis of 3-alkyl-2-methoxypyrazines in wines was developed as an adaptation of a previously described HS-SPME method. The method allows the determination of the most relevant pyrazines (IBMP, SBMP and IPMP) in wines, with

Table 2
Ranges of pyrazine contents of the three samples and their evolution throughout the winemaking process of experimental musts and wines

Samples	Pyrazine (ng/l)					
	Merlot			Cabernet sauvignon		
	IBMP	SBMP	IPMP	IBMP	SBMP	IPMP
1	3.4–8.6	1.0–4.4	1.0–3.2	1.7–2.9	–	–
2	13.6–24.1	12.1–22.5	4.7–10.3	8.1–12.4	1.3–4.8	–
3	17.5–35.1	4.5–15.7	2.4–7.4	10.6–15.5	2.4–4.5	–
4	18.8–38.0	4.2–15.1	2.5–6.1	8.2–16.3	3.4–5.8	–

Samples: (1) musts collected on harvest day; (2) musts collected after 1 day of maceration with the solid parts; (3) wines at the end of alcoholic fermentation; (4) wines at the end of malolactic fermentation. All samples were analysed in duplicate.

detection limits at the 0.3 ng/l level, and recoveries higher than 80%. The presented HS-SPME method allows quantification of these pyrazines in red wines at their natural concentration levels, below their sensory thresholds. This method can be suitable for oenological laboratory work because of its simplicity and rapidity.

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