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Pitfalls encountered during quantitative determination of 3-alkyl-2-methoxypyrazines in grape must and wine using gas chromatography–mass spectrometry with stable isotope dilution analysis. Comprehensive two-dimensional gas chromatography–mass spectrometry and on-line liquid chromatography-multidimensional gas chromatography–mass spectrometry as potential loopholes

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ABSTRACT

The analysis of 3-alkyl-2-methoxypyrazines in *Vitis vinifera* grape must or wine at the low nanogram per liter level failed in several situations when applying a one-dimensional gas chromatographic analysis with mass spectrometric detection (GC–MS). Sample preparation methods such as headspace solid phase microextraction or solid phase extraction were convenient procedures, however lacking extraction selectivity for complex matrices. Analysis by comprehensive two-dimensional gas chromatography with mass spectrometric detection clearly demonstrated the potential for co-elution in such matrices and the risk for erroneous results when applying one-dimensional GC–MS. In one example, matrix problems would have been a challenge even for a comprehensive two-dimensional chromatographic approach with MS detection (GC × GC–MS). A solution to matrix problems was found by protonating the 3-alkyl-2-methoxypyrazines in acidic pH and sample clean-up using solid phase extraction with a mixed-mode polymeric cation-exchange sorbent. Quantification was performed by a stable isotope dilution assay, following analysis by on-line coupled high performance liquid chromatography with multidimensional gas chromatography and detection with mass spectrometry (on-line LC–MDGC–MS). This new approach allowed trace-level analysis of 3-alkyl-2-methoxypyrazines in grape musts and wines and is described for *V. vinifera* Sauvignon blanc, following 3-alkyl-2-methoxypyrazines concentrations during ripening and in the processed wines.

1. Introduction

A number of 3-alkyl-2-methoxypyrazines (MPs) have been identified in foodstuffs of plant origin as aroma relevant compounds, contributing significantly to the characteristic sensory impression of the raw material or the final products made thereof [1–3]. Three MPs, 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP), are of particular interest to analytical studies in such foodstuffs. Often, all three MPs are present, with one compound usually clearly dominating. IBMP is the dominating MP found in bell peppers and *Vitis vinifera* grapes, while SBMP dominates in beetroot or carrot, and IPMP in peas and cucumber [2].

In *V. vinifera* variety Sauvignon blanc, sensory attributes of the corresponding wines are often described by green, grassy and herbaceous notes, resembling green bell pepper or green asparagus notes [4]. In Sauvignon blanc wine, IBMP represents approximately 80% of the total MP content, with lesser amounts of SBMP and IPMP [5]. With their extremely low sensory detection thresholds, which were found to be in the low ng L^{-1} range (1–2 ng L^{-1} of IBMP in a

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white wine [4], or about 15 ng L^{-1} in red wine [6]), MPs are very important wine flavor components. Since wine quality is strongly influenced by MP concentration, it is not surprising that it has been of long concern to study viticultural and enological parameters influencing their final concentration in MP containing wine varieties [7,8].

During the last decade, winemakers had to face a new problem related to MPs, originating from the multicolored Asian lady beetle *Harmonia axyridis*. Complex off-odors and flavors may be found in affected juice and wine, which is known as "ladybug taint" (LBT) [9].

Due to their importance for the wine quality, the determination of MP concentrations in grape juice or during the wine making process is therefore of particular interest to the wine industry. Such importance puts compounds like MPs also into some focus for food adulteration [10] and also into the focus of food control authorities [11].

Considering the low abundance of MPs and the complexity of wine matrix, it is not astonishing to find a considerable variety of methods published for MP analysis in this field. First reports used a complex sample pretreatment, based on distillation, extraction onto a strong cation exchange (SCX) resin, elution with alkaline buffer, liquid–liquid extraction (LLE) and finally quantification by GC–MS [4,12,13]. Replacement of the tedious distillation and SCX steps with C-18 solid phase extraction (SPE) [14,15] or LiChrolut® EN SPE [16] has been described by other groups. LiChrolut® EN has furthermore been employed in a dynamic headspace-SPE approach by Culleré et al. [17]. Classical LLE and pre-concentration steps for sample preparation have been used as well [18,19]. Since the introduction of solventless extraction and pre-concentration methods like headspace solid phase microextraction (HS-SPME), this technique has also been described for MP analysis [11,20–31], as well as stir bar sorptive extraction (SBSE) with thermal desorption [32]. Often, choice of conditions for HS-SPME needs careful optimization as was investigated in detail by, *e.g.* Hartmann [23]. The quantification of MPs benefits from the use of isotopologous internal standards, and the approach of stable isotope dilution analysis (SIDA) [33–35] has therefore found early applications for quantitative MP analysis in wine [12] and by many authors thereafter.

Due to their volatility, most analytical methodologies developed for the measurement of MPs on trace levels comprise gas chromatography (GC), either in conjunction with mass spectrometric (MS), also with MS/MS [11,31], or nitrogen–phosphorus selective detection (NPD) [20–22,27,31]. Usually, minimum detection levels described by GC vary between some 0.2 and 2 ng L^{-1} for IBMP which is sufficient for most wines, although the natural levels found for IPMP and SBMP are often below the described detection limit of most methods.

Methods based on liquid chromatography (HPLC) are not often found in literature for MP analysis. Heymann et al. reported an HPLC–UV method following extraction by steam distillation and C_{18}-SPE extraction [36]. However, with a minimum detection level of about 1 μg L^{-1}, such a method is entirely unsuitable for wine analysis. Recently, a method has been published, based on HPLC hyphenated to mass spectrometry (LC–MS) as an alternative analytical technique for MPs in grape juice or wine [37] with detection limits for all three relevant MPs being well below 1 ng L^{-1}.

The authors combined their LC–MS/MS method with a sample preparation utilizing distillation in combination with LLE, as this procedure offered quantitative pre-concentration of analytes and allowed processing of a large sample number in their hands.

Potentially interfering matrix might have driven authors to describe the use of coupled chromatographic techniques for MP analysis, like multidimensional GC (MDGC) or comprehensive two-dimensional GC (GC × GC). Cai et al. had used MDGC-olfactometry for determining the characteristic odorants from *H. axyridis* [38], and Culleré et al. [17] used it for quantification of MPs with very low detection limits (about 0.09–0.15 ng L^{-1}) as described by these authors. Today, GC × GC has become popular for its very high separation efficiency, besides other benefits not described here. So far, system availability has probably restricted its use in MP analysis. In 2005, Ryan et al. successfully described the application of HS-SPME–GC × GC coupled to NPD or time-of-flight MS (TOF-MS) for the first time [27], followed recently by Ryona et al. [30], who investigated MPs in wine and in grape berries. Both groups had used SIDA for quantification in their GC × GC–TOF-MS approach.

For the work described here, we started our own approach to MP analysis in grapes and wines, following a method based on pH adjustment of the samples and HS-SPME–GC–MS with deuterated internal standards (SIDA) for quantification, as had been described recently [24]. However, we soon had to face severe matrix problems with the grape juice and wine samples investigated by us, rendering the targeted analytical method (a one-dimensional GC–MS approach) unacceptable in our situation. Having experienced such matrix problems, we looked into alternatives, such as GC × GC, improved sample preparation selectivity, as well as combining chromatographic methods in an on-line LC–MDGC–MS approach.

2. Experimental

2.1. Chemicals and reagents

Sodium hydroxide, *n*-hexane, dichloromethane, sodium chloride, sulfuric acid, and LiChrolut® EN solid phase extraction cartridges (200 mg, 3 mL) were from VWR (Darmstadt, Germany); IPMP, SBMP, IBMP, methanol, triethylamine, tartaric acid and SPME fiber divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) were from Sigma–Aldrich (Steinheim, Germany); *tert*-butyl methyl ether (MTBE) was from Fluka (Buchs, Switzerland); anhydrous magnesium sulfate was from Riedel de Haën (Seelze, Germany); Bond Elut Plexa™ PCX solid phase extraction cartridges (200 mg, 3 mL) were obtained from Varian (Darmstadt, Germany). A Chromabond® SPE vacuum chamber (Macherey-Nagel, Düren, Germany) station was used for SPE preparation. Commercial chemicals were of analytical grade. Deuterated standards of MPs (3-alkyl-2-[$^2H_3$]methoxypyrazines; d_3-MPs) have been synthesized in-house as described elsewhere [39].

2.2. Must and wine samples

V. vinifera c.v. Sauvignon blanc grapes were harvested as part of a diploma thesis [40] over a period of several weeks at two different locations in the Palatinate region (southwest Germany), differing in microclimate and soil conditions. Each harvest comprised a total of about 75 kg of grapes, and care had been taken to pick healthy grapes only. Grapes were then processed by milling and pressing with a maximum pressure of 180 kPa. The must was stabilized by addition of ascorbic acid (10 g kg^{-1}) and potassium disulfite (5 g 100 kg^{-1}). An aliquot of 1 L was refrigerated at −20 °C and stored until analysis, another aliquot was used for standard wine parameter analysis. The majority of the must underwent microvinification, which was carried out in-house at a temperature of 15 °C using FERMICRU® VB1 wine yeast (20 g hL^{-1}; DSM Food Specialties Beverage Ingredients, Delft, The Netherlands) and LALVIN GO-FERM® (30 g hL^{-1}; Begerow, Langenlonsheim, Germany) as yeast nutrient after prior clarification with diatomaceous earth (0.125 m^2) and Perlite. Three days after start of fermentation, an additional 30 g hL^{-1} of Vitamon® Combi (vitamin B1 and diammonium phosphate; Erbsloeh, Geisenheim, Germany) was added. To compensate for different sugar contents, all wines were enriched by addition of sucrose to a total ethanol content of 96 g L^{-1}. At the end

of fermentation (about 14 days), wines were stabilized by addition of sulfur dioxide and ascorbic acid (40 and 50 mg L^{-1}, respectively). After storage on yeast for about six weeks, the wines were filtered and concentration of free sulfur dioxide was adjusted to 50 mg L^{-1}. To ensure comparable conditions for sensory studies, the residual sugar content was adjusted to 6.5 g L^{-1} with fructose before bottling.

Authentic Riesling grape juice (bag-in-box), as well as commercial Palatinate Sauvignon blanc wine (vintage 2009) were obtained from the Staatsweingut Johannitergut, Neustadt a. d. W., Germany. A commercial Riesling wine (vintage 2008) and a red wine (vintage 2007; cuvée of Cabernet Cubin, Cabernet Sauvignon and Dunkelfelder—about one-third each) were from a local winery. A Sauvignon blanc wine from the Marlborough County (vintage 2007), New Zealand was obtained from a local supermarket.

2.3. HS-SPME–GC–MS

Following a procedure recently described by Kotseridis et al. [24] with some modifications, MPs were extracted from grape juice using HS-SPME with a DVB/CAR/PDMS fiber after adjusting the pH to approximately 6.6 and incubation for 30 min at 45 °C. GC–MS analysis was done with the system described for GC × GC–MS (Section 2.4), without activating the modulation for GC × GC operation.

2.4. HS-SPME–GC × GC–MS

A ThermoFisher Scientific GC × GC system was used, equipped with a programmed temperature vaporizing injector and a double cryo jet modulator (ThermoFisher Scientific, Dreieich, Germany). The GC × GC instrument was upgraded to accomplish a DSQ quadrupole mass spectrometer (MS) as detector (ThermoFisher, Milan, Italy). Data acquisition and control of the GC × GC–MS was done with Xcalibur software version 1.4 and HyperChrom software version 2.4.1 was used to process the two-dimensional data. Desorption of the SPME fiber was performed at 230 °C in the vaporizing injector, using a dedicated small bore SPME liner in splitless mode (BGB Analytik, Adliswil, Switzerland) and a TriPlus autosampler in SPME mode (ThermoFisher). After 2 min, the split valve was opened to allow a split flow of 16 mL min^{-1}. Carrier gas used for chromatographic separation was helium at a constant flow of 1.2 mL min^{-1}, applying a pressure surge of 450 kPa during the first 3 min. The analytical column system consisted of a 30 m × 0.25 mm i.d. fused silica first dimension separation column, coated with 0.25 μm SolGel-Wax (a polyethylene glycol phase) from SGE (Griesheim, Germany) and coupled to the second dimension column set, consisting of a 0.15 m × 0.15 mm i.d. fused silica column coated in-house with OV-1701-vi (a 14% cyanopropylphenyl–86% methyl polysiloxane phase; 0.15 μm film thickness) and 2 m × 0.15 mm i.d. of BPX-5 (SGE; a 5% phenyl polydimethylsiloxane phase with 0.25 μm film thickness). All column connections were made *via* press-fit connectors (BGB Analytik). The temperature was programmed from 50 °C (3 min isothermal) at 30 °C min^{-1} to 100 °C (0.5 min isothermal), then at 3 °C min^{-1} to 130 °C. The modulation period was 5 s with a delay time of 7.5 min. Cryo-modulation took place on the very last centimeter of the first dimension column. Mass spectra were recorded with a delay time of 6 min in the electron impact (EI) positive ion mode applying electron energy of 70 eV, having the source and transfer line temperatures set to 240 °C. Selected ion monitoring (SIM) for quantifier and qualifier ions of MPs occurred with a dwell time of 1 ms, resulting in some 6–8 data points for a 2D peak. In-between two analytical runs, a non-modulated column conditioning run, programmed from 50 °C at 50 °C min^{-1} to 250 °C (10 min isothermal) was applied (split flow at 16 mL min^{-1}).

2.5. SPE using LiChrolut® EN

Adapting a procedure recently described by Culleré et al. [17], 100 mL sample (must or wine, previously spiked with deuterated MPs) was loaded onto 200 mg of the SPE material which had been conditioned with 4 mL of dichloromethane, methanol and 13% aqueous ethanolic solution (v/v), each. The sample was passed through the cartridge at a flow rate of about 2 mL min^{-1} using a large volume adapter. After rinsing with 25 mL of a 50% aqueous methanolic solution (v + v) and 1% aqueous sodium hydrogen carbonate, the sorbent was dried with vacuum applied (300 mbar) to the SPE manifold and letting air passing through the sorbent (90 min) until the sorbent was dry as indicated by the changing color. Desorption of the MP containing fraction was done with 1.5 mL of dichloromethane, which was transferred into a GC vial and stored in a refrigerator until analysis. To avoid sample contamination by the ambient air, an air filter (a reconditioned C18-SPE cartridge) was fitted on top of the LiChrolut® EN cartridge during the loading, drying and desorption steps.

2.6. SPE using Bond Elut Plexa™ PCX

Based on a procedure developed by Ferreira and co-workers (personal communication 2009, Zaragoza, Spain; method validation submitted to publication in *J. Chromatogr. A*), 75 mL of the sample (must or wine, previously spiked with deuterated MPs and adjusted to pH 2 with sulfuric acid) was loaded onto 200 mg of the Bond Elut Plexa™ PCX material (a mixed-mode cation-exchange resin; 3 mL cartridge) which had been conditioned with 7.5 mL of dichloromethane, 7.5 mL methanol and 12 mL deionized water (pH adjusted with sulfuric acid to pH 2). The sample was passed through the cartridge at a flow rate of about 2 mL min^{-1} using a large volume adapter. The first rinsing occurred with 3 mL of a mixture of deionized water and 30% aqueous methanolic solution (v + v), which had been adjusted to pH 2 with sulfuric acid. The sorbent was then dried for about 15 min using an argon flow by mounting the SPE cartridge into a rake-like drying apparatus. A second rinsing was performed with 1.5 mL of dichloromethane. This was followed by rinsing with 0.9 mL of a solution of triethylamine in dichloromethane (10 g L^{-1}), which was also discarded. Final elution was done with the triethylamine solution (1.5 mL), which was recovered in a centrifuge tube and washed with 5 mL of deionized water (acidified with tartaric acid to pH 2). The organic phase was then transferred into a GC vial by means of a Pasteur pipette and stored in a refrigerator until analysis. To avoid sample contamination by the ambient air, an air filter (a reconditioned C18-SPE cartridge) was fitted on top of the Bond Elut Plexa™ PCX cartridge during the loading and desorption steps.

2.7. On-line HPLC coupled to two-dimensional GC (on-line HPLC–MDGC)

HPLC involved a syringe pump (Phoenix 20, C.E. Instruments; today ThermoFisher), Rheodyne injection valve 7125 with a 20 μL injection loop, a UV/vis detector TM 486 (Waters, Eschborn, Germany). LC pre-separation was carried out on a 120 mm × 2 mm i.d. silica gel column (Hypersil Si, 3 μm; Chromatographie-Service, Langerwehe, Germany) using *n*-hexane/MTBE as eluent (70 + 30; v + v). The (classical) on-column interface [41] was used for on-line HPLC–GC and capillaries were mounted in two manual switching valves (C10W and C6W, Valco, Schenkon, Switzerland) as has been described earlier [42]. Fig. 1 gives a schematic overview of the instrumental set-up. In transfer mode, the HPLC eluent flows through a transfer line (some 60 cm of a 0.17 mm i.d. fused silica capillary (BGB Analytik), inserted through a stan-

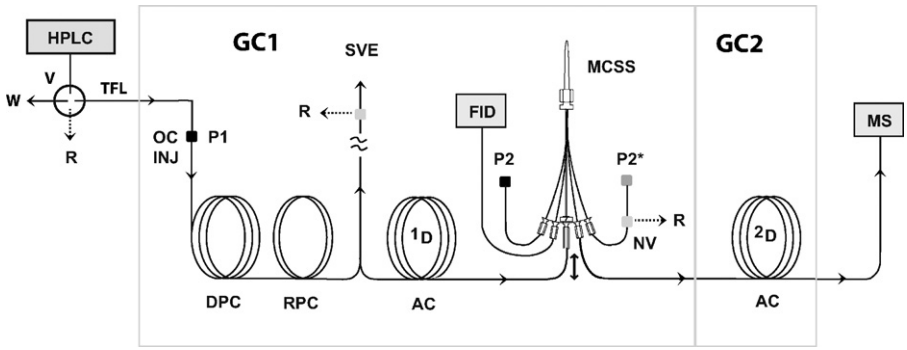

Fig. 1. Schematic drawing of instrument set-up for on-line HPLC–MDGC–MS. AC (analytical column), DPC (deactivated pre-column), FID (flame ionization detector after 1D-separation), RPC (retaining pre-column), MCSS (moving capillary stream switching device), MS (mass spectrometric detector after 2D-separation), NV (needle valve), OC INJ (on-column injector), P1 and P2 (pressure regulator), P2* (manometer with read-out of dome pressure), R (restrictor capillary), SVE (solvent vapor exit), TFL (transfer line on-column interface), V (switching valve), W (HPLC waste).

dard on-column injector of the first GC of the MDGC system (see Fig. 1 and description below). The pre-column system consisted of 10 m × 0.53 mm i.d. uncoated deactivated (OV-1701-OH) pre-column (BGB Analytik), coupled *via* press-fit connection to a 2 m × 0.32 mm i.d. fused silica column, coated in-house with PS-255 (a polydimethyl siloxane, Fluka) to form a film thickness of 1.5 μm (retaining pre-column). Between the retaining pre-column and the first dimension separation column, an early solvent vapor exit was installed *via* a press-fit T-piece. The exit line itself consisted of 30 cm × 0.32 mm i.d. fused silica, which was mounted into a solenoid valve, heated to a somewhat elevated temperature by the unused split/splitless injector body. HPLC eluent transfer occurred under partially concurrent eluent evaporation [43] conditions, allowing solvent trapping of early eluting compounds [44]. Solvent evaporation rate was determined with the flame method as has been described elsewhere [45]. At a transfer temperature of 72 °C and a constant inlet pressure of 100 kPa helium, 171 μL min^{-1} were determined. With a flow rate of 200 μL min^{-1}, and a transfer window for the MP fraction of 2.5 min, the total transfer volume was 0.5 mL. The early solvent vapor exit was closed a few seconds before the end of solvent evaporation to allow sufficient solvent trapping by switching the solenoid valve to a restrictor capillary (30 cm × 75 μm i.d. fused silica). For sensitive detection during method development, the UV adsorption maximum for IBMP was determined with a solution of IBMP (concentration 1 μg mL^{-1} in *n*-hexane) using a Cray 100 UV–visible spectrophotometer (Varian, Darmstadt, Germany) and a 1 cm quartz cuvette. The maximum was found at 277 nm (in good accordance with data previously published by Harris et al. [12]) and was used for establishing the HPLC transfer window with MP standards (for routine analysis, the UV detector was no longer used).

The system for heart-cut two-dimensional GC (MDGC) was based on the "*Moving Capillary Stream Switching*" (MCSS) [46,47] and has been described in detail in an earlier application [48]. Two GC instruments (model 8560, Mega II series (1st dimension GC) and a Vega 6000 series 2 (2nd dimension GC)) from C.E. Instruments were connected *via* a heated transfer line. The 1st dimension GC was equipped with the MCSS device allowing heart-cutting of GC fractions. Modifications of the system described earlier are outlined hereafter.

1st Dimension system (scheme and annotations as in Fig. 1): Carrier gas (helium) was supplied by a pressure regulator P1 (on-column injector), dome pressure (P2) was supplied *via* an additional line from the regulator of the unused split/splitless injector. Inlet pressures were 100 kPa (P1) and 40 kPa (P2), respectively. The actual inlet pressure for the second dimension column was given by the read-out on the pressure gauge P2* and was at 25 kPa. A flame ionization detector (FID) was used as monitor detector. Control of the MCSS system as well as data processing was achieved by the Chromcard data acquisition software, version 2.2 (ThermoFisher). Temperature was programmed from 72 °C (8 min isothermal), at 8° min^{-1} to 250 °C (10 min isothermal). The FID was set to 250 °C. The 1st dimension column configuration consisted of the pre-column system as described above, which was connected *via* a press-fit to a 20 m × 0.25 mm i.d. analytical column, coated with 0.5 μm of a polyethylene glycol phase (ZB-Wax, Phenomenex, Aschaffenburg, Germany). A deactivated fused silica capillary (1.5 m × 0.25 mm i.d., Phenomenex) was guided through a heated transfer line (200 °C), connecting the two GC ovens. Cut intervals were set to 15.08–15.87 min (IPMP), and 16.33–17.66 min (SBMP and IBMP).

2nd Dimension system: The 2nd dimension GC was coupled to a quadrupole mass spectrometer, model MD 800 (Fisons, now ThermoFischer). The transfer capillary from the first oven was connected *via* a press-fit to the 2nd dimension analytical column (15 m × 0.25 mm i.d. fused silica capillary with 0.5 μm of a 35% phenyl methy polysiloxane (ZB-35; Phenomenex). The oven temperature program and MS acquisition were initiated *via* external event activation at the time of the first heart-cut. The oven temperature was raised from 55 °C (3 min hold) at 10° min^{-1} to 250 °C (10 min isothermal). For MS detection, the positive electron ionization mode at 70 eV was used. MPs were monitored with selected ion mode. Mass channels were m/z = 124 (127), **137 (140)** and 152 (155) for IPMP (d_3-IPMP); m/z = 124 (127), **138 (141)** and 151 (154) for SBMP (d_3-SBMP); m/z = 81 (83), **124 (127)** and 151 (154) for IBMP (d_3-IBMP) with 70 ms dwell times (quantifier ions given in bold). Temperature for ion source and MS transfer line was at 230 °C. MS data acquisition was *via* Xcalibur software, version 1.2 (ThermoFisher).

Calibration was done with dichloromethane solutions containing known amounts of the standards d_3-MPs and MPs in varying concentrations, yielding ratios from 0.1 to 2 (IPMP/d_3-IPMP), 0.05 to 2 (SBMP/d_3-SBMP), and 0.025 to 2 (IBMP/d_3-IBMP), respectively. With a sample volume of 75–100 mL used for analysis, a concentration range of 0.4–8 ng L^{-1} (IPMP), 0.2–8 ng L^{-1} (SBMP) or 1–80 ng L^{-1} (IBMP) was calibrated.

3. Results and discussion

3.1. HS-SPME–GC–MS and HS-SPME–GC × GC–MS

For method evaluation we started to analyze spiked Riesling juice, which is supposed to contain no or only minor amounts of MPs. The juice was spiked with about 30 ng L^{-1} IBMP and 3 ng L^{-1} SBMP and IPMP, as well as comparable amounts of the d_3-MPs. Sample preparation was performed on the basis of the method

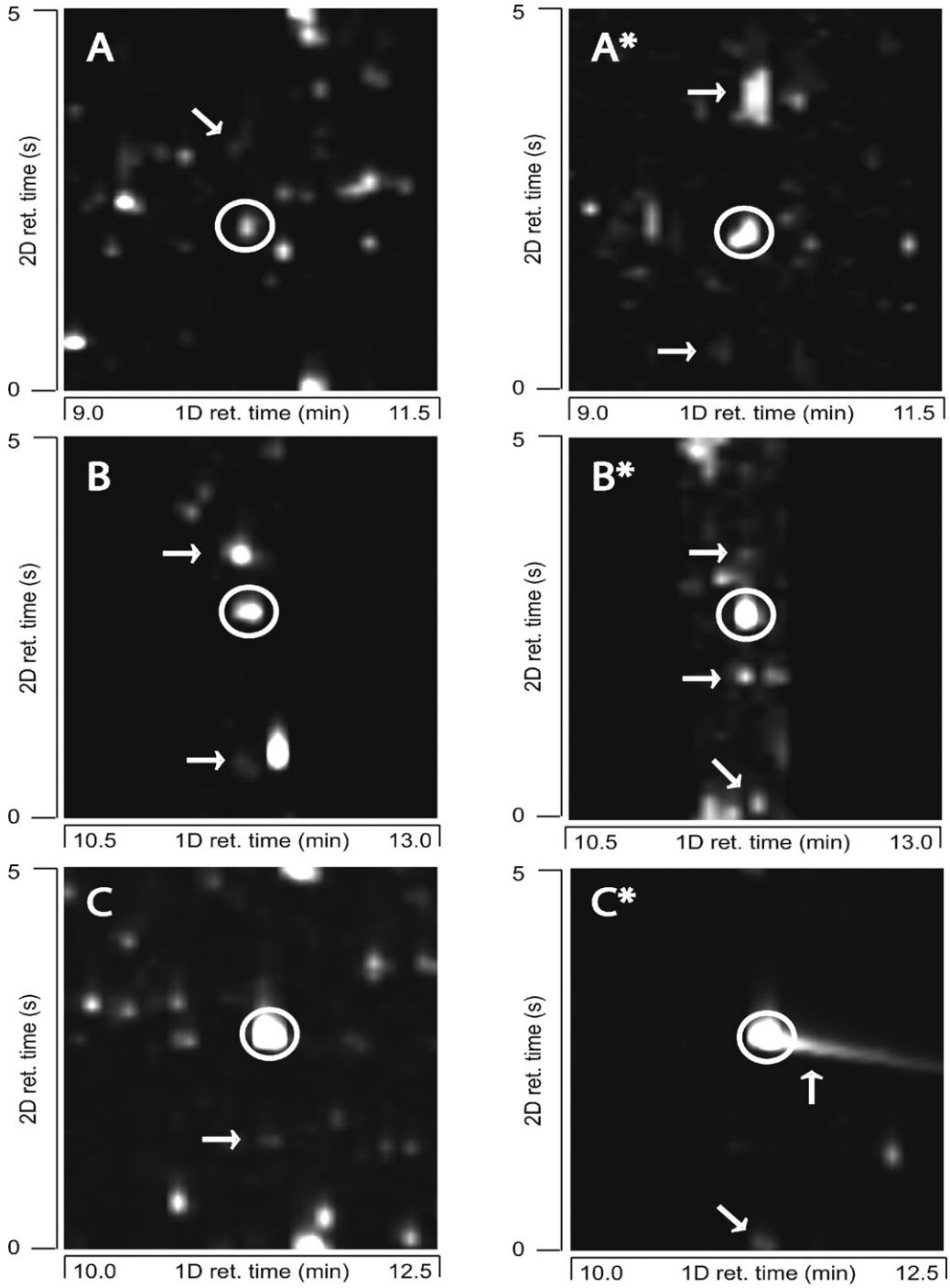

Fig. 2. Contour plots of HS-SPME–GC × GC–MS chromatograms of Riesling juice spiked with about 30 ng L^{-1} (IBMP and d_3-IBMP), or 3 ng L^{-1} (IPMP, SBMP, d_3-IPMP, d_3-SBMP) of MPs. Extracted ion plots for quantifier ions *m/z* 137 (A, IPMP), 140 (A*, d3-IPMP), 138 (B, SBMP), 141 (B*, d3-SBMP), 124 (C, IBMP), and 127 (C*, d_3-IBMP). Circles indicate target compounds and arrows indicate potential for co-eluting regions in a 1D-separation. Note the inexplicable tailing of d3-IBMP in contour plot C*.

described by Kotseridis et al. [24] with some modifications and analysis was done by 1D GC–MS in SIM mode as described in Section 2. However, knowing the spiked amounts for MPs and deuterated MPs, the data obtained after integration of the corresponding MP signals, clearly indicated problems which were thought to be eventually co-eluting substances. Such hypothesis was supported by obscured ion ratios of quantifier and qualifier masses. As an example, ion ratios for the quantifier/qualifier ions *m/z* 124/151 (127/154) are about 100/20 for IBMP and d_3-IBMP, respectively. However, in the sample we found almost no *m/z* 154, indicating a co-elution on mass trace 127. Since our instrument used for 1D GC–MS analysis had previously been set-up with a column combination for comprehensive 2D GC operation and was equipped with a modulator for GC × GC, we were able to re-run our samples in GC × GC–MS mode. Utilizing the higher separation power of comprehensive 2D-separations, our hypothesis for co-elution problems could be verified, as can be seen in Fig. 2, showing the contour plots of the relevant extracted ion chromatograms for quantification of MPs (2A–C) and deuterated MPs (2A*–C*). Peaks separated along the second dimension axis using the GC × GC operation mode, will condense in a non-modulated run to one signal, synonymous to co-elution. Such a co-elution potential can be seen in a pronounced

form in the contour plots at positions indicated by an arrow for Figs. 2A*, 2B and 2B*, and to a minor extent also for the other chromatograms. A closer look at the images in Fig. 2A and A* reveals a more intense signal for d_3-IPMP, although comparable amounts of both compounds had been added. Such observation also suggests a possible co-elution. In the case of the most relevant IBMP, the extracted ion contour plot signal (Fig. 2C) shows almost perfect separation by GC × GC, thus no severe co-elution by a 1D-separation. At first sight, this could also be the information to be extracted from the 2D contour plot of the extracted ion signal of the internal standard d_3-IBMP (Fig. 2C*). However, a closer look reveals the strong tailing of that signal along the 1D axis. This is an alarming indication, as there is no chemical or chromatographic reason why such (or particularly this) isotopologous compound should show other chromatographic properties compared to the non-deuterated target analyte. Furthermore, the chromatographic properties of the synthesized standard per se do not show any noticeable tailing effects on the analytical columns used here. Interestingly, the retention times in GC × GC are perfectly in accordance with the negative isotope effect [49] of the d_3-IBMP, thus showing a somewhat earlier elution on both dimensions for the deuterated compound. The only explanation for this finding can be a substance exhibiting perfect co-elution even after two orthogonal separation modes. Looking at ion ratios for IBMP/d_3-IBMP confirms this co-elution phenomenon. The mass trace for *m/z* 127 is disturbed, and the quantifier/qualifier ratio observed (100/0 instead of 100/20) is violating the requirements described, *e.g.* by EU legislation 2002/657 [50].

Considering the complexity often found in aroma extracts, such co-elution might not be a particular exception. Usually, a selective detection mode such as MS and SIM mode may help solving such problems. Another incidence for such co-elution challenge in quantitative analysis of MPs has recently been described in detail [30]. With IPMP as target analyte, GC × GC–TOF-MS successfully resolved interferences that otherwise co-eluted with the analyte in the first dimension. The IPMP peak co-eluted after the first dimension separation (in their case using a 5% phenyl column) with two interfering compounds. Ryona et al. tentatively identified them as a silylated phenyl compound, eventually derived from either the column or septum bleed and a monoterpene, which was grape derived. The authors could resolve these interferences by the second dimension polyethylene glycol column.

In the very first publication on using GC × GC for MP analysis in wine aroma, Marriott and co-workers applied TOF-MS and the element-specific thermionic detector (in nitrogen selective mode; NPD) as detectors [27]. Using the NPD detector, a somewhat lower detection limit was described for IBMP than with the TOF-MS (0.5 or 1.95 ng L^{-1}). In our case, with the situation found in Fig. 2C*, even the use of such a specific detector might have failed, depending on whether or not the co-eluting substance contains nitrogen in the molecule. TOF-MS might be an alternative, as more qualifier ions can (and should be) be considered for spectral purity. Eventually algorithms for spectral deconvolution might be beneficial in such cases as well. Besides the visual observation of the tailing d_3-IBMP peak in Fig. 2C*, relative ion ratios found for quantifier and qualifier ions deviated from the expected one. In fact, mass *m/z* 127 was the dominating and abundant mass with hardly any abundance found for the qualifier masses. According to a EC commission decision concerning the performance of analytical methods and interpretation of results [50], such practice of verifying the relative ion ratios should be applied in general. However, this is not common practice in scientific publications. Another option for solving the co-elution problem described in Fig. 2C* might have been a high resolution MS, assuming fast enough scan rate suiting GC × GC, but such instrumentation had not been available to us.

Since our quadrupole mass spectrometer used for GC × GC–MS is not fast enough to provide enough data points suiting reliable 2D peak integration, we looked into alternative approaches to solve the co-elution problems encountered.

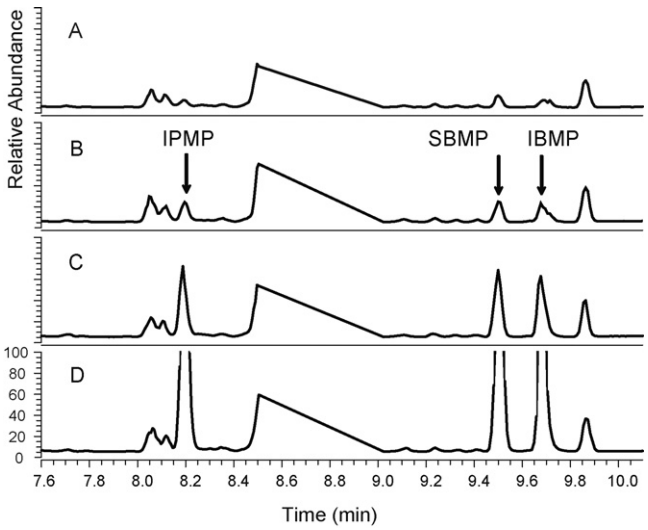

Fig. 3. On-line LC–MDGC–MS of a Riesling juice (A) spiked with 5 (B), 25 (C), or 100 (D) ng L^{-1} IPMP, SBMP and IBMP, respectively (SPE using LiChrolut® EN; conditions given in Section 2.5).

3.2. SPE//on-line LC–MDGC–MS

Starting with sample preparation, we investigated SPE, rather than HS-SPME. Clearly, manual workload increased, but then, an increased variety of available sorbent materials as well as the option to concentrate and re-inject sample extracts under different conditions could be regarded as advantage over SPME. Going back to earlier work [42], we used an on-line HPLC–GC approach as an additional sample preparation step applied on the SPE aroma extract. Considering the complexity of the matrix, we furthermore implemented a two-dimensional rather than a one-dimensional GC system in this instrumental set-up. Such an on-line LC–MDGC–MS approach, has – to the authors' best knowledge – not yet been described before. With the off-line sample preparation by SPE, the method abbreviation is used as follows: SPE//on-line LC–MDGC–MS (the forward slashes representing off-line coupling of techniques according to the suggestion given by Peter Schoenmakers on behalf of the HTC-11 symposium, Bruges, Belgium, 26–29 January, 2010).

3.2.1. SPE using LiChrolut® EN//on-line LC–MDGC–MS

A material often described for extraction of volatiles in the field of wine aroma analysis is LiChrolut® EN [51,52] and has been used successfully in other situations in our lab as well. Adapting to a recent modification of Culleré et al. [17], we started method development with a Riesling juice. First results for the MP analysis of native and spiked Riesling juice can be seen with the chromatograms presented in Fig. 3. Calculated amounts for the native Riesling juice based on standard addition over three spike levels (5, 25, and 100 ng L^{-1}) were 2.5 ng L^{-1} (IPMP), 5.6 ng L^{-1} (SBMP) and 4.2 ng L^{-1} IBMP (with standard deviations of 0.1, 0.3 and 0.2, respectively). This is in some accordance with the rare data previously published for the *V. vinifera* variety Riesling by Hashizume and Samuta [53], who had found some 0.2–6 ng L^{-1} IPMP or 0.3–55 ng L^{-1} IBMP (SBMP had not been determined) in juice or unripe berries, respectively. These results encouraged us to further proceed with this method.

Using LiChrolut® EN for SPE and on-line LC–MDGC–MS, MPs were then determined in grapes of Sauvignon blanc grown in

Table 1
Methoxypyrazine concentration determined for Sauvignon blanc (clone mix) from the Palatinate region, harvested at different stages of maturity (must), and corresponding wines made thereof.

Harvest	Must[a] [ng L^{-1}]			Wine[b] [ng L^{-1}]		
	IPMP	SBMP	IBMP	IPMP	SBMP	IBMP
Series A[c]						
08 August 07	<0.2	nd	9.6	–	–	–
14 August 07	<0.2	nd	6.9	–	–	–
23 August 07	<0.2	nd	4.6	1.0	nd	5.3
28 August 07	<0.2	nd	<1.6	0.5	nd	4.0
09 September 07	<0.2	nd	<1.6	<0.2	nd	3.1
10 September 07[d]	0.6	nd	3.6	0.5	nd	2.9
Series B[c]						
14 August 07	<0.2	nd	5.3	–	–	–
23 August 07	<0.2	nd	3.5	0.7	nd	5.5
28 August 07	<0.2	nd	3.4	0.5	nd	3.6
05 September 07	<0.2	nd	2.4	<0.2	nd	4.6
11 September 07	<0.2	5.7	<1.6	0.7	nd	2.9
17 September 07	<0.2	5.0	1.8	0.6	nd	3.3
11 September 07[e]	<0.2	nd	1.7			
17 September 07[e]	<0.2	nd	1.6			

[a] Must after SPE with LiChrolut® EN.
[b] Wine after SPE with BondElut Plexa™ PCX.
[c] Series A from Weisenheim, series B from Hochstadt, two locations differing in soil and microclimate conditions.
[d] Grape pick before had been on small laboratory scale, this was on the complete vineyard.
[e] Must samples re-evaluated using SPE with BondElut Plexa™ PCX.

the Palatinate region and harvested at different stages of maturity. The results are summarized in Table 1 (must, left part). As expected, IBMP is the dominating MP, with minor amounts found for IPMP. The higher concentrations found at pre-mature harvest (pre-veraison) times are well-known to winemakers, as MP concentrations decrease with increasing ripeness of the fruit and may be influenced by viticultural treatment and microclimate conditions [5,8,53–56] as well as by the enological practice [7]. The increase observed for the latest harvest (one day time difference) within series A can be explained by the different pick, as it was the actual vintage for this vineyard (not a laboratory scale pick), so grapes were under more mechanical stress in the bulk material. This causes more free-flow must and increased extraction of MPs from the stems and skins, where MPs are located primarily [7]. However, there is no explanation for the relatively high amounts of SBMP detected in the late harvest picks for series B, particularly, as SBMP could not be detected at earlier picks as well.

MPs are very characteristic aroma compounds for the green notes in wines made from *V. vinifera* variety Sauvignon blanc, and may contribute significantly to the wine style, as is particularly known for the characteristic New Zealand Sauvignon blanc wines, usually exhibiting increased MP concentration levels [57–59]. This led us to analyze a New Zealand Sauvignon blanc wine from the Marlborough region in New Zealand. To our surprise, we obtained a chromatogram from which we could not get any reliable quantification data for either one of the MPs, as is shown with the example of the quantifier mass traces for IBMP and d_3-IBMP in Figs. 4 and 5 (lower traces). Re-running the aroma extract with MS detection in TIC mode, allowed us to identify one major co-eluting compound as ethyl octanoate, an ester formed in high concentration during vinification of the must by the alcoholic fermentation. Obviously, ethyl octanoate resembles the extraction and chromatographic properties of MPs. On the first separation in MDGC, it co-elutes within the transfer window for IPMP. Linear retention indices determined on the polyethylene glycol stationary phase are 1451 for IPMP and 1447 for ethyl octanoate. Considering the abundance of the ester, a transfer onto the second dimension column occurs, then causing a co-elution with SBMP and IBMP. Although fragment ion *m*/*z* 124 is

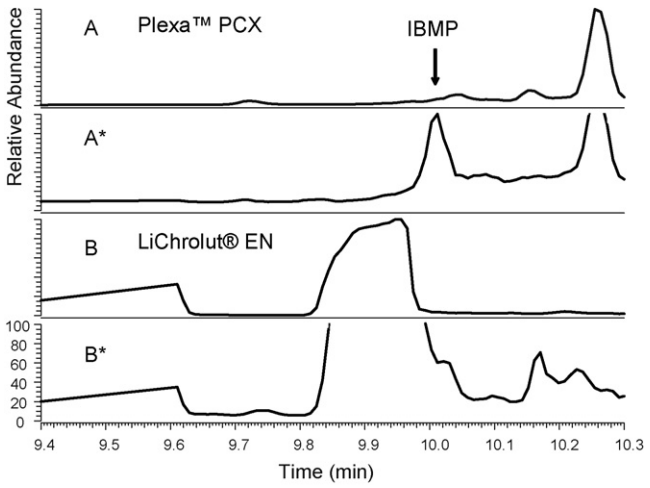

Fig. 4. On-line LC–MDGC–MS of a Sauvignon wine (from New Zealand). Sample preparation was *via* SPE using Bond Elut Plexa™ PCX (A, A*) or LiChrolut® EN (B, B*) as sorbent material (conditions given in Sections 2.5 and 2.6). Total ion chromatograms (SIM masses) for IBMP (A, B) and of quantifier ion *m*/*z* 124 (A*, B*).

not relevant in the mass spectrum of ethyl octanoate, it interferes with the integration of IBMP due to the abundant presence of the ester (Fig. 4B*). The situation found with the quantifier ion for the deuterated standard (*m*/*z* 127), which is a relevant fragment ion for ethyl octanoate, renders the integration of d_3-IBMP impossible (Fig. 5B*).

Using LiChrolut® EN, first results on MP analysis of must or juice samples yielded results which were plausible from the concentration values and knowledge about sample history (besides the above mentioned situation of SBMP values). As the complex wine matrix differs considerably from the must or juice matrix [60], it obviously requires a more selective sample clean-up method for trace level MP analysis.

3.2.2. *SPE using Bond Elut Plexa™ PCX//on-line LC–MDGC–MS*

Going back to the early work of Harris et al. [12], the new strategy considered the basicity of the MPs, allowing protonation at acidic pH and use of a cation-exchange mode for SPE. Based on recent work of Ferreira and co-workers [61,62], we tested sample preparation using BondElut Plexa™ PCX as sorbent. According to the

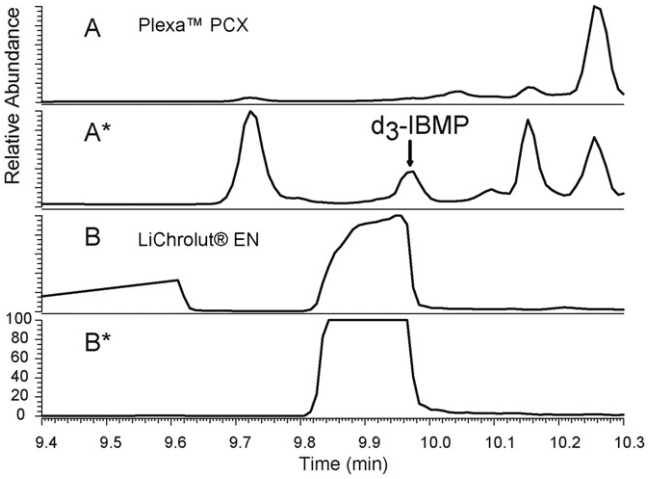

Fig. 5. On-line LC–MDGC–MS of a Sauvignon wine (from New Zealand) spiked with d_3-IBMP. Sample preparation was *via* SPE using Bond Elut Plexa™ PCX (A, A*) or LiChrolut® EN (B, B*) as sorbent material (conditions given in experimental Sections 2.5 and 2.6). Total ion chromatograms (SIM masses) for d_3-IBMP (A, B) and of quantifier ion *m*/*z* 127 (A*, B*).

manufacturers' data sheet, it is a polymeric cation exchange (PCX) resin, allowing a mixed-mode analysis, removing neutral and acidic interferences from the matrix and concentrating basic analytes [63]. Re-analyzing the New Zealand Sauvignon blanc wine using this sorbent and varied SPE conditions (*2.6*) yielded much less disturbed chromatograms as shown with the example of the quantifier mass traces for IBMP and d_3-IBMP in Figs. 4 and 5 (upper traces). The quantitative determination resulted in concentrations of 2.3 ng L^{-1} (IPMP), 0.4 ng L^{-1} (SBMP) and 17.0 ng L^{-1} (IBMP)—values which are perfectly within the range which can be expected for such a wine according to other literature data [11]. Further re-analysis of the Sauvignon blanc must samples in which we had found peculiar levels of SBMP (Table 1, harvested at 11 and 17 September 07, legend e) with the Plexa™ PCX sorbent revealed minor amounts of IBMP and no detectable amounts for SBMP. Such results indicate crucial performance of the LiChrolut® EN sample preparation method even with the less complex must samples.

Since Riesling is an important grape variety for the Palatinate region, we also re-analyzed the juice, for which we had determined preliminary quantitative results during method development, applying standard addition rather than SIDA for quantification. This time, no MPs could be determined. This seems to be more plausible, than the again high amounts of SBMP determined in the first case. MP determination in a local Riesling wine, which had shown noticeable green notes during sensory testing, revealed only a level of 0.6 ng L^{-1} IBMP. Considering the low sensory threshold value of IBMP, such a concentration is at the limit of contribution for the observed green sensory notes, which might otherwise be associated with, *e.g.* C6 alcohols. A local red wine cuvée which also had demonstrated green notes as described by trained wine experts, revealed levels of 0.6 ng L^{-1} IPMP, <0.4 ng L^{-1} SBMP and 2.9 ng L^{-1} IBMP. This may explain the sensory description, also because this cuvée was made with one-third each of Cabernet Sauvignon and Cabernet Cubin, grape varieties likely to contribute to the MP content of this wine.

Analysis of MP in wines made from must samples taken over the whole ripening season are summarized in Table 1 (wine, right column). Again, IBMP is the dominating MP, with minor amounts found for IPMP. The fact of higher MP concentrations at pre-mature harvest (pre-veraison) times has already been discussed above. Although vinification practice (pressing of the must, settling, clarification, *etc.*) has shown to influence MP concentration to some extent, no significant influence has yet been described in literature due to the alcoholic fermentation. The MP content of the wine thus depends mainly on the composition of the grapes [7]. Although the grapes were from two vineyards with different soils and microclimates, no clear difference could be found in MP concentrations amongst the two vineyard sites studied. This might be only due to the moderate climatic difference between the two investigated vineyard locations, as the whole Palatinate region is relatively small in dimension.

3.2.3. Method validation

Calibration graphs for the quantification of MPs in standard mixtures after on-line LC–MDGC–MS analysis (area ratios of non-deuterated over deuterated MPs as a function of concentration of non-deuterated MPs) showed good linearity in the targeted concentration ranges with R^2-values of 0.9991 (IPMP), 0.9944 (SBMP) and 0.9986 (IBMP). The limits of detection were estimated according to previously described procedures [64,65], and were 0.2 ng L^{-1} IPMP, 0.4 ng L^{-1} SBMP and 1.6 ng L^{-1} IBMP.

The repeatability of the SPE//on-line LC–MDGC–MS method (Plexa™ PCX sorbent) was determined ($n = 3$) on a Sauvignon blanc wine from the local region giving the following results: 0.6 ± 0.1 (IPMP), 0.4 ± 0.1 (SBMP) and 5.7 ± 0.1 ng L^{-1} (IBMP). Recoveries were determined for Riesling juice matrix ($n = 5$) with $96 \pm 13\%$ (IPMP), $113 \pm 14\%$ (SBMP) and $97 \pm 5\%$ (IBMP), and for a Sauvignon blanc wine (local origin; $n = 5$) with $76 \pm 7\%$ (IPMP), $104 \pm 5\%$ (SBMP) and $89 \pm 4\%$ (IBMP). Spiking concentrations for recovery studies were 3.8 ng L^{-1} IPMP, 3.9 ng L^{-1} SBMP and 39.2 ng L^{-1} IBMP (both matrices). Validation of the method was done with matrix spike experiments using a Sauvignon blanc wine (previously spiked at the same levels as mentioned for recovery studies) that yielded (after blank subtraction) 3.8 ng L^{-1} IPMP, 3.8 ng L^{-1} SBMP and 38.8 ng L^{-1} IBMP. This data documents good suitability of the described method.

4. Conclusion

Trace level MP analysis was found to be a demanding task, particularly in wine matrix. Besides many analytical approaches described in the past, there is still a demand for a good and reliable method which should preferably include a multidimensional approach and selective detection modes. In our hands, we could show that even data obtained by high resolution GC × GC–MS analysis might be erroneous in one example, as co-elution problems still have to be considered and might be overseen at first inspection. In this respect critical evaluation of peak purity and further consideration of the plausibility of quantitative data obtained is essential for the analyst. Utilizing the advantage that MPs can be protonated in acidic pH conditions, a considerable increase in sample preparation selectivity was found with a mixed-mode cation-exchange resin used for SPE. The combined SPE//on-line LC–MDGC–MS approach added a further dimensionality with respect to separation efficiency, solving the problems encountered with the samples investigated in our studies. However, this success was achieved at the price of a relatively complex instrumental set-up. Further studies will have to show if the on-line LC–(MD)GC approach may be omitted in part, when using the cation-exchange SPE sample preparation mode. Otherwise, such hyphenated chromatographic techniques provide a good portion of a safety margin in critical cases.

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