ISSN 1615-9306 · JSSCCJ 39 (7) 1213–1398 (2016) · Vol. 39 · No. 7 · April 2016 · D 10609

JSS

JOURNAL OF SEPARATION SCIENCE

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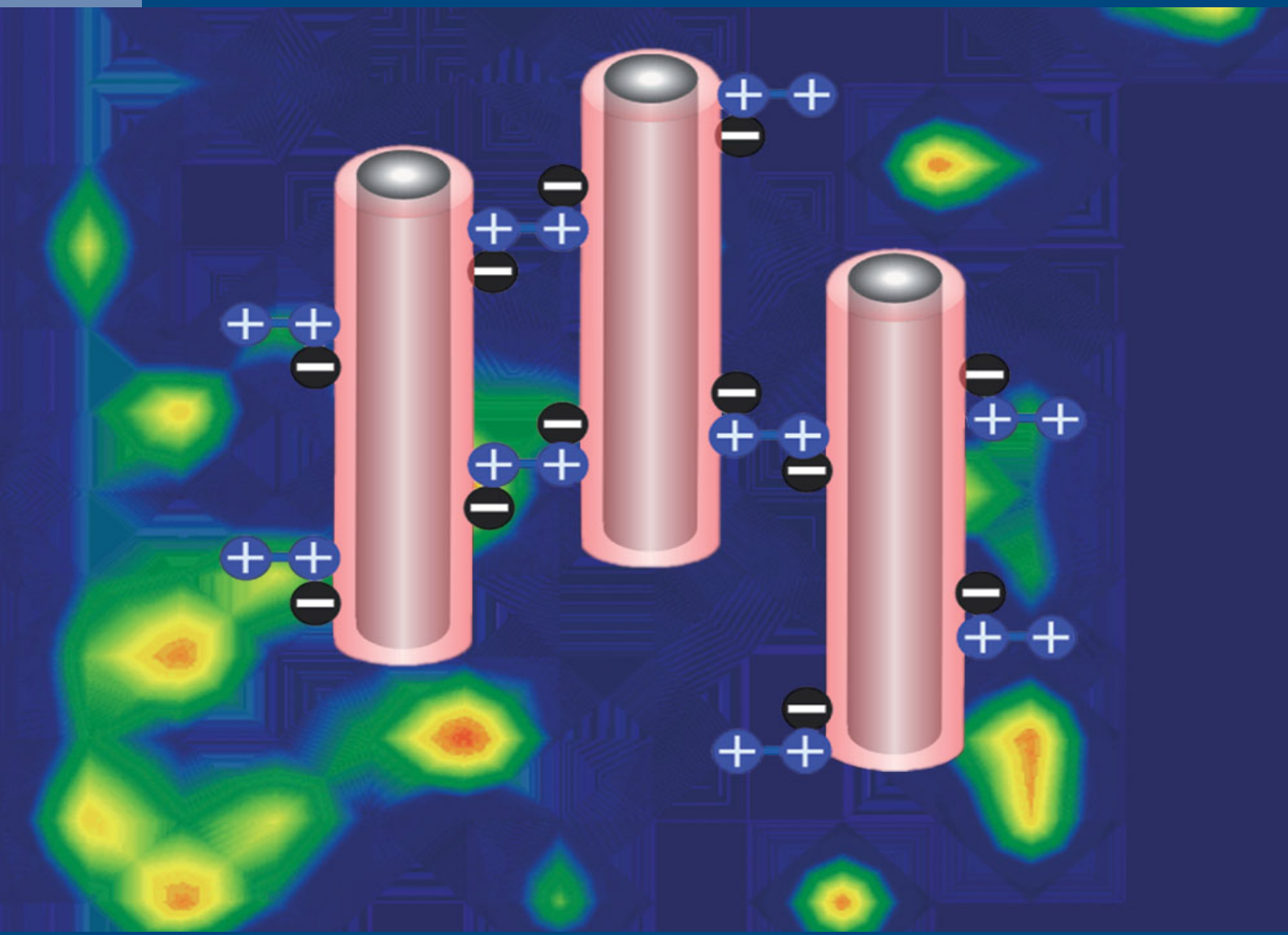

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Received October 1, 2015
Revised January 13, 2016
Accepted January 15, 2016

Research Article

Direct immersion solid-phase microextraction with gas chromatography and mass spectrometry for the determination of specific biomarkers of human sweat in melted snow

To provide a reliable tool for investigating diffusion processes of the specific components of the human odor 3-hydroxy-3-methylhexanoic acid and 3-methyl-3-sulfanylhexan-1-ol through the snowpack, we developed and optimized an analytical method based on direct immersion solid-phase microextraction followed by gas chromatography with mass spectrometry. Direct immersion solid-phase microextraction was performed using polyacrylate fibers placed in aqueous solutions containing 3-hydroxy-3-methylhexanoic acid and 3-methyl-3-sulfanylhexan-1-ol. After optimization, absorption times of 120 min provided a good balance to shorten the analysis time and to obtain suitable amounts of extractable analytes. The extraction efficiency was improved by increasing the ionic strength of the solution. Although the absolute extraction efficiency ranged between 10 and 12% for 3-hydroxy-3-methylhexanoic acid and 2–3% for 3-methyl-3-sulfanylhexan-1-ol, this method was suitable for analyzing 3-hydroxy-3-methylhexanoic acid and 3-methyl-3-sulfanylhexan-1-ol concentrations of at least 0.04 and 0.20 ng/mL, respectively. The precision of the direct immersion solid-phase microextraction method ranged between 8 and 16%. The variability within a batch of six fibers was 10–18%. The accuracy of the method provided values of 88–95 and 86–101% for 3-hydroxy-3-methylhexanoic acid and 3-methyl-3-sulfanylhexan-1-ol, respectively. The limit of detection (and quantification) was 0.01 ng/mL (0.04 ng/mL) for 3-hydroxy-3-methylhexanoic acid and 0.06 ng/mL (0.20 ng/mL) for 3-methyl-3-sulfanylhexan-1-ol. The signal versus concentration was linear for both compounds (r^2 = 0.973–0.979). The stability of these two compounds showed that 3-hydroxy-3-methylhexanoic acid was more stable in water than 3-methyl-3-sulfanylhexan-1-ol. We applied the method to environmental samples in correspondence with an olfactory target buried previously.

Keywords: Biomarkers / Human sweat / Melted snow / Solid-phase microextraction
DOI 10.1002/jssc.201501097

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1 Introduction

The ability of canines to track human subjects based on the latter's odor is well established. Moreover, during rescue operations carried out by avalanche dog teams, search dogs pick up traces of human scent and locate the limited area where the scent is most concentrated [1]. Some correlation (r^2 = 0.637) between search time and burial depth of the avalanche victims was observed [2]. This implies that volatile or semi-volatile organic compounds, specific for human odor, diffuse through the snowpack (up to several meters) until they reach the surface. Moreover, the diffusion through the snowpack also depends on the physical and chemical properties of the compound considered, such as volatility and hydrophilicity, as well as on the density of the snow and on the mean diffusion path length [3, 4].

In human secretions and excreta, a large number of volatile compounds were identified [5–7]. In human sweat,

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Abbreviations: DI, (Direct Immersion); **3MSH**, (3-methyl-3-sulfanylhexan-1-ol); **HMHA**, (3-hydroxy-3-methylhexanoic acid); **HS**, (Headspace); **PA**, (Polyacrylate)

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for instance, various classes of compounds, such as carboxylic acids [7–11], alcohols [7, 9, 12–14], aldehydes [5, 9, 12], esters [5, 9], hydrocarbons [5, 12, 15], ketones [5, 9, 12, 16], sulfur compounds [10, 14, 17–20], and terpenes [9] were detected. Although the majority of these compounds was ubiquitous, some of them, such as 3-hydroxy-3-methylhexanoic acid (HMHA) [10] and 3-methyl-3-sulfanylhexan-1-ol (3MSH) [18, 19] were identified as specific components of the human odor [10, 18, 19]. In particular, HMHA has a very typical axilla-like pungent odor and seems to be both abundant and ubiquitous in human axillary secretions [10]. Gender differences were also observed, as females can liberate significantly higher amounts of sulfur volatiles, such as 3MSH [18].

It cannot be excluded that dog detection might be triggered by other nonspecific volatiles widely excreted by humans, such as isoprene [21]. However, although the diffusion of nonpolar compounds similar to isoprene from the ground to the snowpack was reported [22], the diffusion of such hydrophilic compounds toward the surface of the snowpack was not equally detailed. Hydrophilicity could affect the diffusion dynamics through the snowpack, and, in our view, HMHA and 3MSH represent suitable target molecules also for modeling the diffusion dynamics of polar and slightly polar volatiles. Indeed, since HMHA is more hydrophilic than 3MSH, different diffusion behaviors could also be expected between these two compounds.

In view of elucidating the diffusion dynamics of these human-specific markers through snow that allows, for instance, avalanche dogs to detect buried victims, a fast and reliable analytical method for the determination of these compounds in snow or in aqueous matrices (e.g., melted snow) is required.

Volatile compounds present in human sweat are generally determined by GC–MS, after extraction with organic solvents [10, 11, 20], or by headspace extraction methods [5, 6], directly applied to human secretions. Nevertheless, methods for the identification of these compounds in aqueous systems have never been reported.

SPME is a solvent-free technique that simplifies the sample preparation when compared to conventional approaches: extraction and concentration are included in one single step, and the extracts do not require further clean-up procedures. Moreover, SPME generally provides low detection limits and good reproducibility both in headspace and in direct immersion (DI) mode [23–26]. In DI mode, SPME was successfully tested in aqueous systems for the determination of organic compounds [27–31], proving a promising alternative to conventional extraction methods.

In this paper, we aim to investigate the possibility of using DI-SPME–GC–MS and optimizing the operative condition for the identification of the two main specific human sweat tracers in melted snow. We focused in particular on HMHA, which is the most abundant and non-gender-specific. The analytical performances of the method were evaluated in terms of extraction efficiency, repeatability, reproducibility, linearity, LOD, LOQ, accuracy, and applicability to real cases.

The applicability of the procedure was tested in melted snow obtained from a sub-sampled snow-core collected along the vertical direction of a buried target odorant. This experiment was carried out to evaluate the applicability of the method in real snow samples and the feasibility of future investigations on the diffusion mechanism of these compounds through the snowpack. In fact, although several studies were carried out to investigate the diffusion mechanism of volatiles through the interstitial air of snow from chemicals present in the atmosphere into the snowpack [32–35], the diffusion mechanism of polar volatile compounds that diffuse from the snowpack to the atmosphere, as in the case of avalanche victims, was scarcely ever reported.

2 Materials and methods

2.1 Chemicals and materials

HMHA and 3MSH were prepared by procedures adapted from published protocols [14, 20]. In short, ethyl 2-bromoacetate (130 mL; 1.99 mol) was added to a mixture of pentan-2-one (213 mL; 2.01 mol) and zinc powder (133 g; 2.03 mol) in dry THF (500 mL). The mixture was refluxed overnight and treated with a saturated solution of sodium bicarbonate. After conventional work-up, the crude oil was distilled at reduced pressure (53°C, 0.29 mbars) to yield ethyl 3-hydroxy-3-methylhexanoate (54.2 g). This ester (48.3 g) was subsequently hydrolyzed using a solution of sodium hydroxide (53 g in 460 mL ethanol/water 1:1). The reaction mixture was then washed with petroleum ether, the aqueous phase was evaporated to eliminate ethanol, acidified until pH = 4, and extracted to furnish HMHA as an almost pure slightly yellow oil (42.3 g). An analytical sample was obtained by flash chromatography on silica gel using petroleum ether/ethyl acetate 7:3 for elution. ^{1}H NMR ($CDCl_3$, 200 MHz): $\delta = 0.93$ (t, 3H),1.20–1.65 (m, 4H), 1.27 (s, 3H), 2.53 (dd, 2H), 5.14 ppm (br s, 2H).^{13}C NMR ($CDCl_3$, 50 MHz): $\delta = 14.42, 17.17, 26.33, 44.13, 44.56, 71.85, 176.38$ ppm. MS (EI, 70 eV): *m/z* (%):131 (100), 113 (14), 103 (23), 87 (52), 85 (97), 71 (38), 69 (11), 43 (67).

3MSH was prepared in four steps from pentan-2-one. At first, pentan-2-one was treated with triethylphosphonacetate in the presence of sodium hydride in dry toluene to afford ethyl 3-methylhex-2-enoate as a mixture of isomers (*E*/*Z*, ca. 75:25). This ester mixture was then refluxed for 60 h with benzylthiol (1 equiv.) and piperidine. After evaporation of the excess of piperidine, the crude product was then reduced with $LiAlH_4$ in THF, then treated by sodium in liquid ammonia (Birch conditions) to yield crude 3MSH that was purified by column chromatography on silica gel (petroleum ether/ethyl acetate 8:2) to give pure 3MSH as a pale yellow oil. ^{1}H NMR ($CDCl_3$, 200 MHz): $\delta = 0.93$ (t, 3H), 1.36 (s, 3H), 1.37–1.60 (m, 4H), 1.68 (br. s, 1H), 1.80–1.95 (m, 2H), 3.80–3.90 ppm (m, 2H).^{13}C NMR ($CDCl_3$, 50 MHz): $\delta = 14.50, 18.01, 30.35, 46.30, 46.98, 47.63, 59.98$ ppm. MS (EI, 70 eV): *m/z* (%):

148(5, M^+), 115 (16), 114 (20), 97 (49), 87 (16), 81 (17), 71 (48), 69 (33), 55 (100), 43 (24), 41 (41).

HMHA and 3MSH were dissolved in methyl *tert*-butyl ether (MTBE) (Sigma–Aldrich). Standard solutions were stored at –18°C until use. Sodium chloride of analytical grade was purchased from Sigma–Aldrich. The ultrapure water (18.2 MΩ cm, 0.01 TOC) was produced by a Purelab Ultra system consisting of a Purelab Option R purification plant system coupled to Purelab Ultra Analytical ultra-pure system (Elga, Lab Water, High Wycombe, UK). All experiments were performed at room temperature.

The SPME device for manual extraction consisted in a holder assembly and six replaceable fibers (Supelco) in PA (85 μm). PA fibers were selected because they proved suitable for the absorption of polar and slightly polar compounds such as carboxylic acids and thiols [37, 38]. Before the first use, each fiber was conditioned, as recommended by the manufacturer, by being heated in the injection port of the GC system for 1 h at 270°C. After desorption of the extracts, each fiber was kept at 250°C for 10 min in the injector, to ensure the complete desorption of every compound.

2.2 Melted snow samples

Snow samples were collected at Passo Rolle (Trentino-Alto Adige, Italy, 46°17'48.6"N 11°47'15.6"E) in April 2015, using a home-made snow corer (a detailed description of the snow corer is reported in the Supporting Information). A snow core (0.5 m length) was drilled in correspondence with an olfactory target, consisting of a cotton pad soaked with 500 ng HMHA and 3MSH, previously (1 h) placed at the bottom of the core together with a thermal source consisting of a bottle of warm water (37°C) to simulate the thermal input of avalanche victims. This allowed the human sweat biomarkers to diffuse through the snow. The snow core was sub-sampled collecting five sections (10 cm length). The samples were stored at –18°C for 30 days until analysis.

2.3 Extraction

SPME extraction was carried out by DI-SPME of the PA fiber into 20 mL of aqueous solution, containing 0.05–5.0 ng/mL HMHA and 3MSH, in a 50 mL glass vial under magnetic stirring throughout the time of the extraction. The extraction procedure was optimized evaluating the effect of the extraction time (between 0 and 180 min), NaCl (from free-NaCl solutions to saturated) and analyte concentrations. DI-SPME extraction was carried out at room temperature.

SPME extraction in headspace mode (HS-SPME) was carried out by exposing the PA fiber to the headspace in a 50 mL glass vial over 20 mL of ultrapure water fortified with 1 and 100 ng of HMHA and 3MSH, under magnetic stirring. The PA was exposed for 120 min at 40°C.

In real snow samples, the extraction was carried out following the DI-SPME optimized procedure, where ~20 mL of melted snow was extracted at room temperature. HMHA and 3MSH concentrations were then corrected by the effective volume subsequently measured.

The withdrawal of 50 mL of snow provides melted samples of about 10–20 mL (snow density generally ranges from 0.2 to 0.4 kg m^{-3} [36]). It must be noted that, considering the volume of aqueous solution, higher extraction efficiencies could be obtained in smaller vials. The choice of using 50 mL vials was done to minimize any possible cross-contamination or system perturbation due to phase change.

2.4 GC–MS

HMHA and 3MSH were analyzed with an Agilent GC–MS system composed by a 6890NGC coupled to a quadrupole mass spectrometer 5973N (Agilent Technologies, Santa Clara, CA, USA). The chromatographic analysis was carried out using a capillary column DB-WAX with a 30 m length, 0.250 mm inner diameter, and 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA, USA). 1 mL/min He flow was used as a carrier. The injection temperature was investigated in a range from 200 to 250°C and 1 μL was injected in splitless mode. For the analysis of HMHA and 3MSH, the temperature program used was: 50°C (held for 2–10 min, to optimize the method) to 250°C at 20°C/min held for 3 min. The transfer-line temperature was 250°C, the mass selective detector (MSD) ion source temperature was 230°C and the MSD quadrupole 150°C. Electron ionization was performed at 70 eV. Measurements in full scan mode enabled the identification of peaks. Analytes quantification was instead performed in selected ion monitoring (SIM).

2.5 LOD, LOQ and linearity

The LOD for HMHA and 3MSH were determined as the sample concentrations resulting in a peak area with a S/N of at least 3:1. The LOQ was the lowest concentration that could be quantified with a S/N of at least 10:1 [39]. Laboratory limits of detection (L-LOD) and laboratory limits of quantification (L-LOQ) were defined as the mean concentrations of the procedural blanks plus three and ten times the SDs, respectively [35]. The linearity of the detector response was determined by analyzing a mixture of HMHA and 3MSH in 20 mL of ultrapure water containing analyte concentrations from 0.05 to 5 ng/mL.

3 Results and discussion

3.1 Optimization of thermal desorption by SPME–GC–MS

The chromatographic method was initially developed injecting 1 μL of a MTBE solution containing 5.0 μg/mL HMHA and 3MSH separately, and recording the mass spectrum of

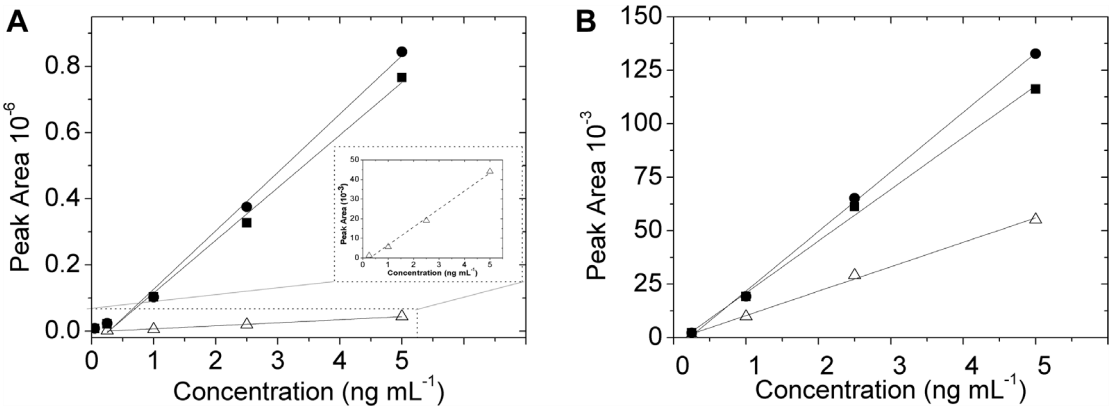

Figure 1. Peak area versus concentration of HMHA (A) and 3MSH (B) at different NaCl concentration.: saturated NaCl (●), 30% NaCl (■), and salt-free solution (△).

each compound. From the mass spectrum, target ion (I^T) and two control ions (I^C) were selected for HMHA (m/z 131 (I^T); m/z 103 and 87 (I^C)) and for 3MSH (m/z 114 (I^T); m/z 97 and 71 (I^C)). Their ratios were subsequently employed for checking the purity grade of the eluted compounds. Chromatograms obtained in SIM mode (chromatographic conditions are reported in Section 2) showed a good separation of the analytes that elute at retention time of 5.8 and 7.5 min for HMHA and 3MSH, respectively (Supporting Information Figs. S1).

SPME desorption conditions, such as the temperature of the injector (T_{inj}) and desorption time (t_{des}), were firstly optimized by spiking 100 ng of each compound studied in 20 mL of ultrapure water. Absorption conditions (120 min of extraction at room temperature) were kept fixed during these experiments. T_{inj} of 200°C and 250°C, and t_{des} of 2, 5, and 10 min were investigated, performing injections at each T_{inj}/t_{des} combination in triplicate ($n = 3$). Higher peak areas and more repeatable chromatograms (RSD = 6.2%) were recorded for T_{inj} of 250°C. A lower chromatographic signal was also observed for t_{des} of 2 min when compared with 5 and 10 min. However, desorption times of 5 and 10 min provided comparable responses (see statistical analysis in the Supporting Information), indicating that after 5 min all the analytes were completely desorbed. Thus, T_{inj} of 250°C and t_{des} of 5 min were used as desorption conditions in the following experiments.

3.2 Optimization of extraction by SPME

Absorption conditions, such as the ionic strength of the solution and absorption time (t_{abs}), were also investigated. Indeed, it was recognized that the ionic strength of the aqueous solution generally affects the yield of extraction [40, 41]. The increase in ionic strength of the solution was achieved by adding sodium chloride. The ionic strength conditions was optimized in 20 mL ultrapure water solutions spiked with different amounts of HMHA and 3MSH (5.0, 2.5, 1.0, 0.25, 0.05 ng/mL), where each solution contained either 30% NaCl ($NaCl_{30\%}$) or was saturated ($NaCl_{sat}$). For comparison, the same HMHA and 3MSH solutions were also prepared in salt-free solutions ($NaCl_{free}$). In this set of experiments, t_{abs} was kept at a constant value of 120 min. The peak area values obtained at different HMHA and 3MSH versus the concentration levels and NaCl contents are reported in Figure 1A and B, respectively. As expected, the peak area of both HMHA and 3MSH considerably increases in presence of NaCl. However, in NaCl-saturated solutions, a higher increase of the extraction yield was observed for HMHA (~18–22 times higher) rather than 3MSH (~1.9–2.4 times higher), respectively, suggesting that the partition coefficients of these two compounds changes depending on the ionic strength of the solution. This behavior is consistent with the higher hydrophilicity of HMHA. The use of NaCl-saturated solutions does not significantly improve the extraction yield, when compared with $NaCl_{30\%}$ (see statistical analysis in Supporting Information). Furthermore high NaCl concentration levels could shorten the lifetime of the fibers [42]. Thus, in this study, we considered that a NaCl concentration of 30% provides a useful balance between the increase of the extraction yield and the decrease in lifetime of the SPME fibers, and we therefore employed it in the following experiments.

The effect of t_{abs} was evaluated by keeping constant the ionic strength of the solution ($NaCl_{30\%}$), HMHA and 3MSH concentrations (100 ng spiked in 20 mL of water), while t_{abs} was varied between 10 and 180 min. As demonstrated elsewhere [43], the amount of analyte absorbed at the equilibrium (N^0) was a function of t_{abs}, according to the following equation:

$$\frac{N}{N^0} = 1 - e^{-at_{(abs)}} \quad (1)$$

where N is the amount of analyte absorbed at a time t and a is a parameter that determines the absorption equilibrium rate at the fiber surface.

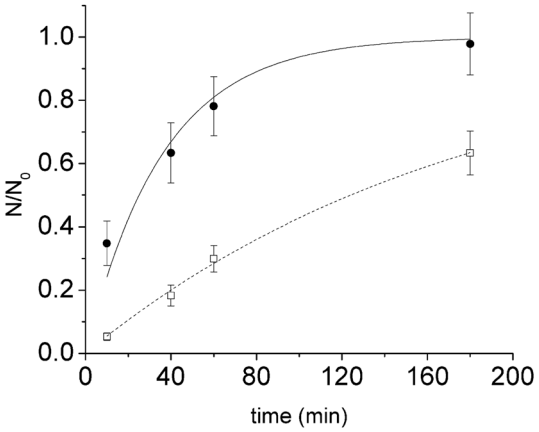

Figure 2. N/N_0 versus time at the SPME fiber of HMHA (●) and 3MSH (□) in solution containing 5 ng/mLof analytes and 30% NaCl. Fitted equation (1) for HMHA (—) and 3MSH (—).

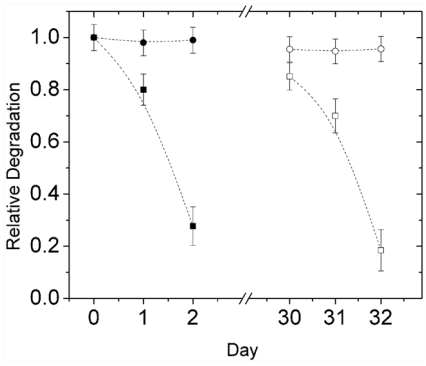

Figure 3. HMHA (●) and 3MSH (■)stability in water. HMHA (○) and 3MSH (□) stability in water after 30 days storage at –18°C.

In Fig. 2 the amount of absorbed analytes (N/N^0) versus t_{abs} is reported and fitted with equation (1) for both HMHA and 3MSH. The nonlinear fit curve for these values provided adjusted-r^2 of 0.935 and 0.997, for HMHA and 3MSH, respectively. The values obtained for the parameter *a* (0.028 for HMHA and 0.006 for 3MSH) indicate that the absorption equilibrium was reached faster for HMHA than for 3MSH. From the fitted equations, the times required for absorbing the 95% with respect to the equilibrium conditions were 107 and 499 min for HMHA and 3MSH, respectively. Although the absorption equilibrium of 3MSH was quite low, t_{abs} of 120 min was considered suitable for determining these two compounds, shortening the analysis time and obtaining nearly the total amount of extractable analyte for HMHA (96%) and 51% of 3MSH. The optimized absorption and desorption conditions of the method are: $t_{abs} = 120$ min; NaCl concentration = 30%, $t_{des} = 5$ min, and $T_{inj} = 250$°C. These conditions were used in the experiments reported in this paper.

The stability of the analytes was also investigated, considering 20 mL ultrapure water fortified with HMHA and 3MSH (5 ng/mL) kept at room temperature and analyzed daily for 3 days. In these conditions, whilst the HMHA signal did not vary significantly (RSD calculated between three daily measurements was ~5%), the peak area, corresponding to 3MSH, progressively decreased as shown in Fig. 3. After 24 h, the signal was ~80% of the initial value. The possibility of preserving 3MSH in aqueous environments was investigated by freezing 20 mL of spiked samples, stored at –18°C and analyzed after 30 days. As reported in Fig. 3, the analyses performed immediately after thawing the spiked water samples showed that the chromatographic signal of HMHA did not significantly vary (within 6%). Moreover, 3MSH signal appeared only slightly reduced (~15%) with respect to the signal initially obtained. However, 1 day after thawing, the same decreasing trend was observed. The behavior of 3MSH could be due to degradation phenomena in aqueous environment, as suggested by the oxidation of thiol compounds in aqueous solutions containing molecular oxygen [44, 45]. These oxidation reactions are likely slowed down when freezing the samples. In light of these results, we concluded that storing the aqueous samples at –18°C, until SPME analysis, ensures the reliability of the measurements.

The extraction efficiency of the method was evaluated considering the ratio between the peak area of the analytes extracted using DI-SPME in 20 mL of ultrapure water and the corresponding values obtained when injecting MTBE solutions in which the same amounts of HMHA and 3MSH were present. The major assumption is that the chromatographic signal is not affected by the presence of the solvent during the GC–MS analysis performed in splitless mode. In our view, this approach leads to a direct comparison between the amount of the analyte extracted and the amount of analyte initially present in the aqueous solutions. A set of experiments was carried out at two different HMHA and 3MSH concentration levels (0.25 and 5.0 ng/mL). As reported in Table 1, the extraction efficiency ranged between 10–12 and 2–3% for HMHA and 3MSH, respectively, depending on the concentration level. The extraction efficiency values obtained are comparable to similar methods that use DI-SPME extraction for the determination of other compounds, such as polycyclic aromatic hydrocarbons [46], phthalate esters [30, 31], and pesticides [47]. It must be noted that, in several studies [46–48], the extraction efficiency was considerably improved when using HS-SPME extraction instead of DI-SPME, especially when analyzing non-polar compounds. When extracting the headspace of a 50 mL vial in similar experimental conditions than those used in DI-SPME (40°C instead of room temperature), extraction efficiency values of ~2 and ~7% were obtained for HMHA and 3MSH, respectively, at 5.0 ng/mL. The increase of the extraction efficiency for 3MSH in HS-SPME, differently from HMHA, is consistent with the hydrophobicity of 3MSH, that preferentially distributes in the gas phase rather than aqueous. However, although the extraction efficiency of 3MSH increased, HS-SPME procedures proved scarcely reproducible for this latter compound (RSD>80%, $n = 6$). In this study, considering the potential applications of the method, we opt for higher precision more than higher extraction efficiency. Thus, in light

Table 1. Analytical performances of the method

Compound	Extraction efficiency %		Within-day precision RSD ($n = 6$)		Between-day precision RSD ($n = 6$)		Between-fibers precision RSD ($n = 6$)		Accuracy %		LOD ng/mL	LOQ ng/mL	Linearity r^2	
Concentration range (ng/mL)	0.25	5	0.25	5	0.25	5	0.25	5	0.25	5			0.05–5[a]	0.25–5[b]
HMHA	10.2	11.9	12.4	7.8	11.8	8.5	15.2	10.4	89.0	95.1	0.01	0.04	0.979	
3MSH	2.3	3.2	14.1	10.5	16.3	10.8	17.7	11.6	84.4	100.8	0.06	0.20	0.973	

[a] and [b] referred to HMHA and 3MSH, respectively.

of these considerations, DI-SPME results more suitable than HS-SPME for HMHA and 3MSH extraction in an aqueous matrix.

3.3 Analytical performance of the DI-SPME–GC–MS method

The within- and between-day precision was determined as RSD at high (5.0 ng/mL) and low (0.25 ng/mL) concentration levels of HMHA and 3MSH in 20 mL ultrapure water, repeating the DI-SPME–GC–MS procedure in six replicates for each concentration level and using the same SPME fiber. As shown in Table 1, the within-day precision for HMHA and 3MSH at higher concentration levels did not differ significantly (8 and 10%, respectively), indicating an acceptable repeatability of the method. At lower concentration levels, the within-day precision slightly increased (Table 1). The between-day precision showed similar results when using fresh spiked solutions (Table 1).

The reproducibility of the method was also investigated considering the variability between a batch of six different fibers at high (5.0 ng/mL) and low (0.25 ng/mL) HMHA and 3MSH concentration levels (Table 1). The concentration levels were chosen considering the LOQ values (see below). In particular, the lower concentration was set close to LOQ values, while the higher concentration was set at about one order of magnitude above the LOQ. At high concentrations, the variability between the fibers was 10% for HMHA and 12% for 3MSH, a result comparable to within-day and between-day precision values. At low-concentration levels, the variability was 15% for HMHA and 18% for 3MSH. However, it must be mentioned that the RSD values obtained at low concentration levels referred to five SPME fibers. Indeed, peak areas obtained with one SPME fiber were outliers, based on Dixon's Q test [49] and were therefore rejected.

Linearity, LOD and LOQ were determined as described in Section 2. Calibration curves were obtained in a concentration range 0.05–5.0 ng/mL for HMHA and 0.25–5.0 ng/mL for 3MSH. The concentrations compared to the chromatographic signal are linear when using up to 5 ng/mL for HMHA and 3MSH. LOD (LOQ) were: 0.01 ng/mL (0.04 ng/mL) and 0.06 ng/mL (0.20 ng/mL) for HMHA and 3MSH, respectively.

The contribution of the laboratory environment to the procedural blanks was evaluated by extracting in replicates ($n = 6$) 20 mL ultrapure water solutions containing 30% NaCl. In procedural blanks, for retention times corresponding to HMHA and 3MSH, the concentration of the analytes was less than three times the S/N ratio in all the replicates.

Since real samples are melted snow that potentially differs from pure water, we considered the possibility of matrix effect during analysis. An accepted method for evaluating matrix effect uses isotopically labeled internal standard calibration method [50–52]. Unfortunately, isotopically labeled HMHA and/or 3MSH are not available. Matrix effect was then evaluated considering the matrix (real melted snow,

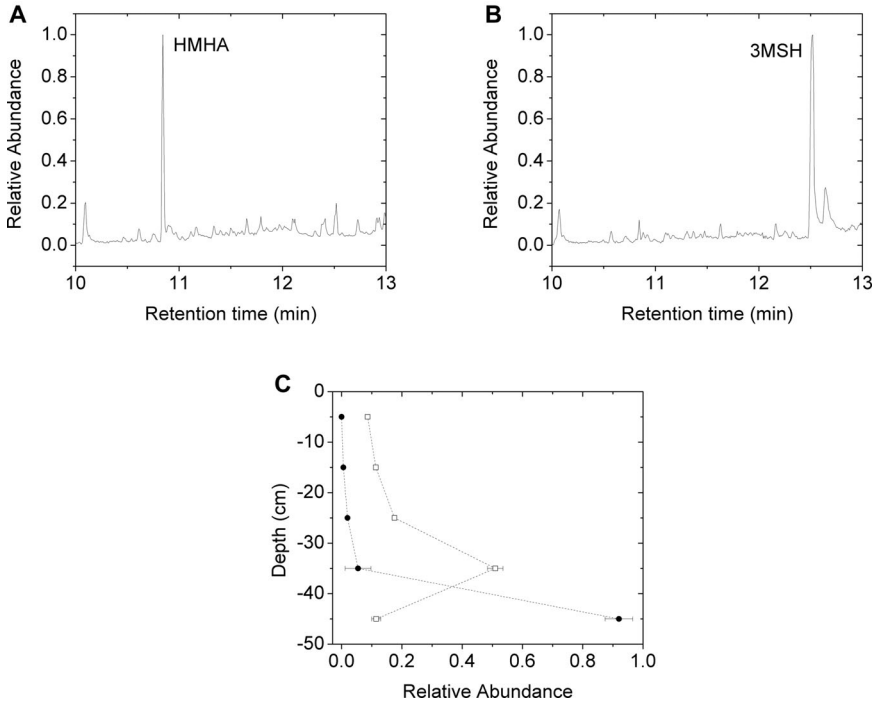

Figure 4. SIM Chromatogram obtained using DI-SPME in real samples of melted snow collected at 0–10 (A) and 10–20 cm (B) from the olfactory target. (C) HMHA (○) and 3MSH (□) vertical diffusion profile through 0.5 m snow column after 60 min.

20 mL) fortified with HMHA and 3MSH (0.25 and 5.0 ng/mL) in triplicate for each concentration level. Melted snow was previously analyzed (see field blanks in Section 3.3). As reported in Table 1, the recovery ranged from 89 to 95% for HMHA and from 84 to 101% for 3MSH, depending on the concentration level considered. These results indicate that, although for low concentrations (0.25 ng/mL) the recovery was slightly lower than 90%, for higher concentrations (5.0 ng/mL) the matrix does not really affect the recovery accuracy of the measurement. It must be noted, however, that snow is a matrix that could be considerably different from one sampling site to another. Thus, in our view, the evaluation of the matrix contribution should be performed in every specific sampling site.

3.4 Melted snow analysis

The possibility of performing diffusion studies of hydrophilic semi-volatile compounds through the snowpack was evaluated. The method was tested on aliquots of melted snow subsampled from a snow core collected at Passo Rolle (Italy) using a home-made corer (see Supporting Information) in correspondence with a target odorant, consisting of a cotton pad soaked with 500 ng of HMHA and 3MSH, placed at the bottom of the drilled core 1 h before coring. Field blanks ($n = 4$), consisting in surface snow collected nearby the coring spot, were firstly analyzed. In every sample, the chromatographic signals in correspondence of the retention times of HMHA and 3MSH were below the LOD.

Typical SIM chromatograms recorded in real samples of melted snow subsampled at 0–10 and 10–20 cm from the olfactory target are reported in Fig. 4A and B, respectively. As expected, the chromatogram in Fig. 4A shows an intense peak corresponding to HMHA, while the signal corresponding to 3MSH is weak. On the contrary, a more intense peak corresponding to 3MSH is observed in 10–20 cm snow section (Fig. 4B). Moreover, the matrix does not interfere with the analytes, as also suggested by the I^T/I^C ratios, which do not statistically differ from the values obtained in standard solutions.

The HMHA and 3MSH concentration profiles along the vertical section of the core are reported in Fig. 4C. As shown in the Fig. 4C, after 60 min, both compounds diffuse differently through the snow. Indeed, HMHA concentration profile fits a complementary error function(erfc)-like curve, as expected for a diffusive process when the flow rate of a flowing fluid is negligible or absent. On the contrary, 3MSH concentration profile showed a peak-shaped profile with its maximum at 10–20 cm from the target odorant. This latter profile could be due to a diffusive process in a flowing fluid medium, as it occurs in chromatography [46]. In this case, the flowing fluid could be the air or water vapor that flows toward the atmosphere, driven by a gradient of temperature from the interior of the snowpack that is warmer than the surface. However, although at this stage any interpretation of the different diffusion mechanism of the two compounds are merely speculative and beyond the scope of this paper, the different behavior of these compounds might be due to their differences in hydrophilic properties.

The results obtained in field demonstrated that the method proposed can be suitably applied to real melted snow samples and successfully used to investigate the diffusion mechanism of these specific biomarkers of human sweat through the snowpack.

4 Concluding remarks

In this paper, a novel DI-SPME–GC–MS method for extracting HMHA and 3MSH from aqueous systems was developed and optimized in terms of absorption and desorption conditions. The method does not need extensive sample treatment (extraction and clean-up procedures) and provided within- and between-day precision in the range of 8–16%. Although the extraction efficiency values ranged between 2 and 12%, depending on the analyte considered, the method is suitable for analyzing aqueous solution containing at least 0.04 ng/mL and 0.20 ng/mL of HMHA and 3MSH, respectively.

We also demonstrated the applicability of the method to melted snow samples fortified with HMHA and 3MSH. This method opens the possibility of studying and modeling the diffusion mechanism of these compounds through the snowpack toward the atmosphere.

This work was financially supported by Fondazione per l'Università e l'Alta Cultura in Provincia di Belluno. The synthesis of HMHA and 3MSH was carried out in the framework of the FP-7 funded project DOGGIES (Grant agreement no. 285446). We would also like to thank Daniela Almansi for the revision of the manuscript, Walter Di Mari, Michele Matteo Santoro and Valter Levis of Soccorso Alpino Guardia di Finanza (SAGF) Predazzo (TN), Dr.ssa Silvana Diverio of Laboratorio di Etologia e Benessere Animale (LEBA) – University of Perugia and Anselmo Cagnati, Renato Zasso and Mauro Valt of Associazione Interregionale Neve e Valanghe (AINEVA) (BL) for the field experiments and Andrea Spolaor, Valter Tomasi, Enrico Natin and Roberto Epis for the development and fabrication of the home-made snow corer. The authors gratefully acknowledge the help of ELGA LabWater in providing the PURELAB Pulse and PURELAB Flex that produced the ultrapure water used in these experiments.

The authors have declared no conflict of interest.

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