Headspace solid phase microextration *vs.* dynamic headspace extraction to explore breast milk volatile fraction

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Abstract

Biological matrices of mammals contain many volatile compounds which can act as specific chemical cues for congeners modifying their behavior or their physiological parameters. Gas-chromatography (GC) can be used to assess such matrices, however, this is a challenging technique because of low concentrations of highly volatile analytes. Thus, an extraction technique that (i) preserves the original profile of the volatile compounds, and (ii) concentrates the analytes is required. Headspace extraction methods, such as headspace solid phase micro-extraction (HS-SPME) or dynamic headspace (DHS), show many advantages. Therefore, they are promising methods for the analyses of biological matrices.

In this study, DHS was compared by using two sorbent cartridges, and HS-SPME was performed for the exploration of human milk composition. Volatile compounds of pooled breast milk samples were extracted by HS-SPME using a Car/PDMS fiber, by DHS associated to Tenax® or Bio-monitoring sorbent tubes. Extracts were analyzed by GC coupled to a mass spectrometer and a flame ionization detector. Extraction yields were compared on the basis of qualitative and semi-quantitative chromatograms.

As a result, HS-SPME with Car/PDMS fiber extractions enabled the recovery of a large diversity of compounds from breast milk and displayed a great reproducibility, whereas DHS enabled to recover a larger number of compounds with an about 10-fold higher yield. The DHS method allowed four compounds to be newly identified in breast milk: cyclohexanone, 6-methyl-5-hepten-2-one, pyridine and phenol. Thus, despite a challenging implementation, DHS is a better option than HS-SPME with Car/PDMS fiber for the investigation of volatile fractions of small-scale or low concentrated samples like breast milk. Since the two sorbents used in DHS, Tenax® and Bio-monitoring, demonstrated equivalent extraction capabilities, Tenax®, which is widely used, is the preferred option for a better comparison of the results with the current literature.

Introduction

Biological matrices of mammals contain many volatile compounds which can act as specific chemical cues for congeners modifying their behavior or their physiological parameters[1]. Extraction of volatile compounds and their subsequent analysis by means of gas-chromatography (GC) could enable key components from biological matrices to be identified. However, since volatile compounds of biological matrices were present in very-small amounts[2] their exploration is particularly challenging. Indeed, analytical methods require the extraction technique to have a strong concentration capability without distorting the original volatile profile. Regarding this issue, the headspace extraction techniques could be of interest since they involve solvent-free procedures and respect matrix integrity[3]. Among them, headspace solid phase micro-extraction (HS-SPME) is widely employed because it is relatively easy to apply and low in costs. However, the low amount of sorbent on fibers could limit its application for samples with low-concentrated headspace. Dynamic headspace (DHS) methods demonstrate enriched

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capabilities through the constant displacement of the headspace equilibrium and the large quantity of sorbent involved. However, the implementation of this technique can be complex, notably due to leaking during desorption of the target compounds.

The objective of this study was to evaluate the capability of DHS associated with the commonly used sorbent, Tenax® or a new combination of sorbent Bio-monitoring and HS-SPME for the exploration of a low-concentrated biological fluid: the breast milk.

Experimental

Samples

Four samples of human milk (50mL) were collected into a pre-cleaned 120mL wide-mouth bottle. The bottle was capped and the sample stored at -20°C until all samples were collected. Once the four samples were collected, they were defrosted and pooled together. The pooled sample was divided into 5mL aliquots, placed into 22,5 mL amber screw cap *vials, sealed with a PTFE septum and stored at -80°C prior to analysis. Before extraction*, samples were defrosted during 10 minutes at ambient temperature.

Extraction methods

DHS extraction. A nitrogen flow was bubbled into 5mL sample of human milk with a purge flow of 20mL.min^{-1} during 2 hours adapted from conditions previously published[4]. The compounds were trapped either in a Tenax® (Markes international Ltd, Llantrisant, UK) or a Bio-monitoring cartridge (composed of Tenax® TA and Carbograph 5 TD, Markes).

HS-SPME extraction. Volatile compounds from a 5mL sample of human milk were extracted by HS-SPME with a Car/PDMS fiber (10mm length, 85µm film thickness) placed in the headspace of the vial for 2 hours at 34°C. SPME extraction time was set in order to have comparable conditions with DHS.

Desorption and chromatographic conditions

Analyses were carried out using a GC (7890A System Agilent, Wilmington, DE, USA) equipped with a mass spectrometer (5975 inert MSD with Triple Axis Detector MS, Agilent) and a Flame Ionization Detector (FID, Agilent). Triplicates of HS-SPME and DHS extracts were analyzed in a random sequence. Compounds on the SPME fiber were desorbed into the injection port of the chromatograph (T=260°C, splitless) whereas DHS cartridges were desorbed on a thermal desorption system (Unity 2 thermal desorption, Markes) for 20 minutes at 240°C. The thermal desorption system uses a two-stage procedure, where the first stage is a desorption of the cartridge followed by a re-trapping on a Peltier-cooled (10°C) trap. Compounds were then transferred to the GC column during the rapid heating of the Peltier trap from 10°C to 320°C with a rate of 100°C.s^{-1}. After desorption, volatile compounds were separated on a DB-WAX column (30m x 0.25mm x 0.5µm film thickness, Agilent). Hydrogen was used as a carrier gas at constant flow (1mL.min^{-1}). The oven temperature was programmed from 50°C (0min) to 80°C at 5°C.min^{-1}, then from 80°C to 200°C (0min) at 10°C.min^{-1}, and finally from 200°C to 240°C (4min) at 20°C.min^{-1}. Peak areas were integrated using the MSD Chemstation software (Agilent). Mass spectra were recorded in electron impact mode (70eV) between a mass range of 33 and 300 m/z at a scan rate of 2.7 scan.s^{-1}. Compounds were identified by comparing their mass spectra and their linear retention indices (LRI) with those of reference databases (Nist and internal database) and with relevant literature.

Data processing and statistical analyses.

One-way analysis of variance (ANOVA) and Least Significant Difference (LSD) tests were performed on FID peak areas obtained from the analysis of HS-SPME or DHS (Tenax® or Bio-monitoring cartridges) milk extracts with a 95 % confidence level. A normalized principal component analysis (PCA) and Ascendant Hierarchical Classification (AHC) were conducted on the data. The Xlstat software (Addinsoft) was used to perform statistical analyses.

Results and discussion

Chromatograms obtained from the three extraction methods are presented in Figure 1.

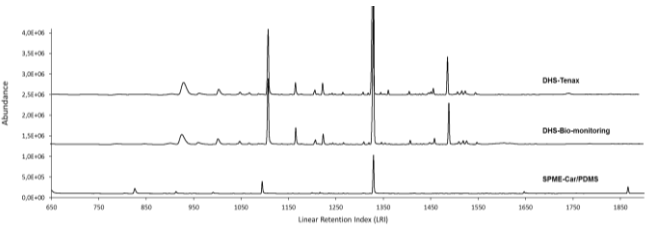

Figure 1: FID chromatograms of breast milk extracts obtained with DHS with Tenax® or Bio-monitoring cartridges and HS-SPME with Car/PDMS fiber

Fifty-five peaks were detected in at least one of the three extracts, mostly carbonyl compounds, alcohols, terpenes and carboxylic acids (Fig.2).

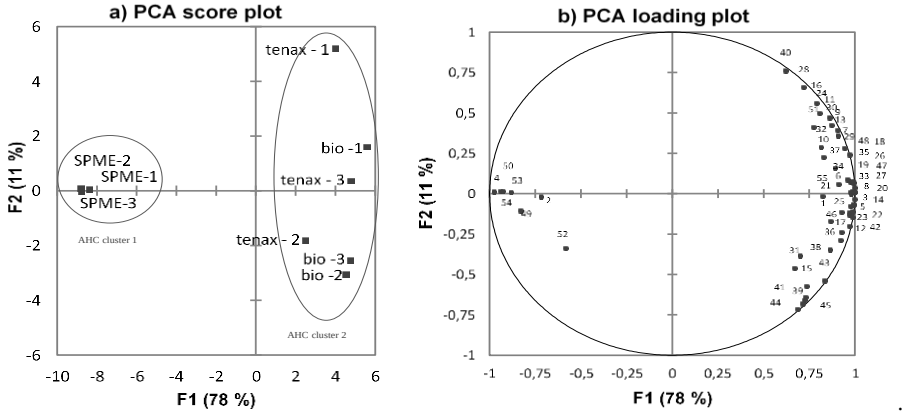

Figure 2: Principal component analysis performed on the peak areas of the volatile compounds detected in human milk extracted by HS-SPME, DHS (Tenax®) and DHS (Bio-monitoring) and projection of clusters (AHC). a- Score plot from human milk different extracts. b- Loading plot for volatile compounds 1: Hexane; 2:Unidentified-1; 3: Octane; 4 :2-propanone; 5: Butanal; 6: 2-Butanone; 7: Heptane, 2,2,4,6,6-pentamethyl-; 8: 2-Pentanone; 9: α-Pinene; 10: Toluene; 11: Camphene; 12: Hexanal; 13: β-Pinène; 14: Ethylbenzene; 15: 1-Butanol; 16: 3-Carene; 17: Unidentified-2; 18: 2-Heptanone + Heptanal; 19: Pyridine; 20: dl-Limonène; 21: (E)-2-Hexenal; 22: 2-Pentylfuran; 23: 1-Pentanol + γ-Terpinene; 25: p-Cymene; 26: Octanal; 27: 1-Hexanol; 28: Cyclohexanone; 29: (E)-2-Heptenal; 30: 6-Methyl-5-hepten-2-one; 31: 1-Hexanol; 32: Unidentified-3; 33: 2-Nonanone; 34: Nonanal; 35: Unidentified-4; 36: Unidentified-5; 37: (E)-2-Octenal; 38: Acetic acid; 39: Unidentified-6; 40: Unidentified-7; 41: 2-Ethyl-1-hexanol; 42: Unidentified-8; 43: Unidentified-9; 44: Unidentified-10; 45: Unidentified-11; 46: 3,5-Octadien-2-one; 47: (E)-2-Nonenal + Benzaldehyde; 48: Camphor; 49: Butanoic acid; 50: Unidentified-12; 51: Acetophenone; 52: Pentanoic acid; 53: Hexanoic acid; 54: Heptanoic acid; 55: Phenol

Among detected compounds, 43 were associated with at least one identified compound. All of them have already been identified in human milk except cyclohexanone, 6-methyl-5-hepten-2-one, pyridine and phenol which were tentatively identified. These compounds have already been found in other human materials[5]. The analysis of chromatographic profiles of breast milk samples showed that respectively 38 and 53 compounds were identified in HS-SPME and DHS extracts. The following 20

compounds were only detected in the DHS extracts: butanal, (E)-2-hexenal, 3,5-octadien-2-one, acetophenone, camphene, 3-carene, camphor, 1-hexanol, 2-ethyl-1-hexanol, octane, heptane,2,2,4,6,6-pentamethyl, ethylbenzene, styrene and 7 unidentified compounds (LRIs: 1372, 1418, 1441, 1456, 1493, 1503, 1510) while four compounds: 2-propanone, an unidentified compound (LRI: 1651), hexanoic acid and heptanoic acid where only detected in the HS-SPME extracts. The ANOVA and LSD tests performed on individual peak areas (data not shown) demonstrated that among the 34 compounds common to HS-SPME and DHS extracts, all of them, except butanoic acid, were found at a larger extent in the DHS extracts with a 10-fold higher yield. However, no specific trends were observed for any chemical family. The ratio of five compounds, two mono-terpenes (α-pinene and p-cymene) and three other components (1-butanol, pyridine and 6-methyl-5-hepten-2-one) even exceeded a ratio of 20. Some of these compounds, could have originated from the mother's diet, like terpenes (vegetables) or pyridins (roasted food), and could be flavor cues present in the mother's milk influencing the early and future newborn's feeding behavior[6]. Moreover, among the 53 compounds detected in DHS extracts, none was statistically different between the Tenax® and Bio-monitoring cartridges, except hexane and acetic acid, which were significantly more abundant when using the Bio-monitoring cartridge, although these could also have been artifacts of sorbents.

A PCA performed on the peak areas of each compound (Fig. 1) allowed visualization of these previous observations. Indeed, 89% of the variance was recovered in the PCA map. In the PCA score plot, DHS extracts were positively correlated to the first axis, which represents 78% of the variance, while the HS-SPME extract was negatively correlated to this axis. HS-SPME extraction exhibited a greater repeatability than DHS. However, the loading plot showed that the majority of peaks (48) was positively correlated to DHS extracts, whereas only 7 peaks were correlated to the HS-SPME extract. The AHC shows that HS-SPME and DHS extracts were discriminated, however Tenax® and Bio-monitoring cartridges were not.

In conclusion, even if HS-SPME with the Car/PDMS fiber extraction enables the recovery of a large diversity of compounds from milk and displays a great reproducibility, DHS enables the recovery of a larger number of compounds and in a greater extent than HS-SPME. Thus, despite a challenging implementation, DHS is a better option than HS-SPME to investigate volatile fraction of small-scale or little concentrated samples like breast milk. Tenax® and Bio-monitoring sorbents, tested in DHS, exhibited equivalent extraction capabilities. Thus, Tenax®, which is widely used, should be preferred to Bio-monitoring sorbent to enhance comparison of results with the current literature.

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