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# Gas chromatographic-mass spectrometric analysis of urinary volatile organic metabolites: Optimization of the HS-SPME procedure and sample storage conditions

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ABSTRACT

Non-targeted metabolomics research of human volatile urinary metabolome can be used to identify potential biomarkers associated with the changes in metabolism related to various health disorders. To ensure reliable analysis of urinary volatile organic metabolites (VOMs) by gas chromatography-mass spectrometry (GC-MS), parameters affecting the headspace-solid phase microextraction (HS-SPME) procedure have been evaluated and optimized. The influence of incubation and extraction temperatures and times, coating fibre material and salt addition on SPME efficiency was investigated by multivariate optimization methods using reduced factorial and Doehlert matrix designs. The results showed optimum values for temperature to be 60 °C, extraction time 50 min, and incubation time 35 min. The proposed conditions were applied to investigate urine samples' stability regarding different storage conditions and freeze-thaw processes. The sum of peak areas of urine samples stored at 4 °C, −20 °C, and −80 °C up to six months showed a time dependent decrease over time although storage at −80 °C resulted in a slight non-significant reduction comparing to the fresh sample. However, due to the volatile nature of the analysed compounds, more than two cycles of freezing/thawing of the sample stored for six months at −80 °C should be avoided whenever possible.

## 1. Introduction

Metabolome is a complete set of metabolites (low molecular weight compounds) produced by cells and organisms, which varies according to the physiology, development or pathological state of the cell, tissue, organ or organism. Accordingly, metabolomics is the comprehensive analysis of metabolome of an isolated organism, cell system, tissue, or biological fluid [1]. Because metabolome is highly complex, it is hardly possible to analyse all metabolites in one single analysis.

Metabolomic techniques can be used as a powerful and reliable tool for the identification of biomarkers that are different between two samples under different biological conditions. Over the last few years non-targeted metabolomics research of human volatile urinary metabolome has attracted a great amount of scientific interest, particularly in oncological studies. Namely, cancer cells can produce some specific volatile metabolites that are not produced by healthy cells during normal physiological process and/or alter their concentration [2–4]. Several studies indicate that the profile of volatile organic metabolites (VOM) in urine may be useful to discriminate cancer patients from healthy subjects and the difference in VOM level can be potentially used for diagnostic and/or prognostic purposes. For example, VOMs in urine are considered potential cancer biomarkers in lung cancer [2], breast cancer [5], and renal cell carcinoma studies [6].

VOMs in urine are chemically very diverse. They include alcohols, aldehydes, furans, ketones, pyrroles, terpenes, and other heterocyclic compounds. These compounds generally contain up to twelve carbon atoms and are characterized by low molecular weight and boiling point less than 300 °C [7]. VOMs are concentrated in the kidneys before excretion so urine is a rich source of these compounds [8]. Another advantage of urine is its non-invasive sampling.

Analysis of VOMs in urine requires a procedure with good sensitivity and without sample losses and contamination. The development of solid-phase microextraction (SPME), which was introduced by Arthur and Pawliszyn [9], contributed to the simplification of sample preparation since it integrates sampling, extraction, concentration, and sample introduction in a single step. The headspace-SPME

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(HS-SPME) technique of solvent-free sample preparation coupled with gas chromatography mass spectrometry (GC-MS) proved to be most successful in VOM analysis [5,6]. SPME efficiency depends on several factors that can be optimized such as chemical nature of the compounds to be extracted (not changeable), the temperature of incubation and extraction, the coating material of the fibre, the incubation and extraction times, and the salting-out effect [10]. Optimization of the HS-SPME method for VOM analysis in urine generally involves examining the effect of one parameter at a time while all other variables are constant [5]. However, it is difficult and time consuming to study all these variables individually to obtain the best possible conditions. Multivariate optimization design methods offer a simultaneous study of several control variables and their interactions thus reducing the number of experiments in the SPME method optimization.

To the best of our knowledge, this is the first time that the combination of reduced factorial and Doehlert matrix designs was applied to optimize the SPME method for the extraction of the urinary volatile metabolome. Apart from a recently published study that used freeze-drying as its storage method [11], ours are the first data on VOM stability in urine with regard to sample handling and different storage conditions.

The aims of the current study were therefore to optimize the HS-SPME method using a multivariate design approach for urinary VOM analysis by GC-MS and to test whether different storage conditions [refrigerator (4 °C), freeze (−20 °C), or deep-freeze (−80 °C)] had a significant effect on the changes of urinary metabolites' concentration over time.

## 2. Material and methods

### 2.1. Reagents and materials

The following commercial chemicals were used: Suprapur nitric acid (65% $HNO_3$) from Merck (Darmstadt, Germany); hydrochloric acid (HCl) from VWR International (West Chester, PA, USA); sodium chloride (NaCl) from Kemika (Zagreb, Croatia) and alkane standard solution containing $C_8$-$C_{20}$ alkanes of 40 mg/L concentration in hexane from Fluka (Buchs, Switzerland). Clear glass 5 mL SPME vials (20.5 mm × 38 mm) with crimp caps were products of Macherey-Nagel GmbH & Co. (Düren, Germany) and grey butyl rubber septa with 20 mm diameter were product of Supelco (Bellefonte, USA). The thermoblock used for temperature control of HS-SPME extraction was supplied from Barnstead/Lab Line (Melrose Park, IL, USA). The commercial SPME holder for manual use and fibres coated with 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB), and 75 μm Carboxen/PDMS were purchased from Supelco (Bellefonte, USA). The fibres were conditioned by heating in the injection port of GC as recommended by the manufacturer and the blank level was checked before the first daily analysis.

### 2.2. Urine samples

Urine samples were obtained from three healthy male volunteers aged 26, 36, and 39 years who were on a mixed diet. All subjects were informed of the aim of the study and their written consent was obtained before inclusion in the study. The study was approved by the Institutional Ethics Committee and was performed in accordance with the Declaration of Helsinki.

Mid-stream urine samples were collected in the morning in plastic (phthalate free) vials filled completely to avoid any loss of volatile compounds. Nitric acid was used for adjusting the urine pH to 2–3.

A control sample was prepared according to Gika et al. [12] by pooling equal volumes from each of the three samples. This pool sample was analysed the same way as other samples. Pooled urine was used to provide a representative sample containing all the analytes that will be encountered during the analysis.

### 2.3. GC-MS analysis

The analyses of volatile compounds were carried out using a Trace 1300 gas chromatograph (Thermo Scientific, Milan, Italy) coupled to a ITQ 700 ion trap mass spectrometer (Thermo Scientific, Austin, TX, USA). Chromatographic separation was performed on a TG-5MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; Thermo Scientific, Runcorn, UK). The following chromatographic program was used: the oven temperature was held for 2 min at 40 °C, then increased to 150 °C at a rate of 5 °C/min (held for 2 min), then to 250 °C at a rate of 7 °C/min followed by an increase of 50 °C/min to 310 °C. The final oven temperature of 310 °C eluted all of the extracted compounds from the column, which was confirmed with the analysis of the blank sample after the urine sample. The carrier gas (He) flow was constant at 1 mL/min. The injection port was operated in the splitless mode at 280 °C. The operating conditions for the MS system were as follows: electron ionization mode at the energy of 70 eV; transfer line and ion source temperatures were 260 °C and 200 °C, respectively; full scan mode with a scan range of $m/z$ 30–400 was used for data acquisition.

To detect detector drift and to control the inertness of the analytical system [13], $C_8$-$C_{20}$ alkanes were used as reference standards. These were analysed after the last daily analysis in order to verify instrument conditions throughout the entire experiment.

### 2.4. Experimental procedure

The sum of peak areas of analytes with different volatile and polar characteristics selected across the entire GC chromatogram was considered for sample preparation optimization to accomplish the best SPME performance conditions during method development. The experimental conditions that achieved the highest sum of peak areas were selected. The peak area of the selected compounds was calculated by internally processing the setup method of the Xcalibur software (ver. 2.1, Thermo Scientific, San Jose, CA, USA).

The optimized HS-SPME GC-MS procedure was validated in terms of precision, and was applied for the evaluation of the storage conditions impact on VOM concentration in urine samples. Measurements were performed in duplicate.

#### 2.4.1. Optimization of HS-SPME conditions

The optimization procedure involved the selection of those experimental parameters that were important for the SPME extraction efficiency.

In the preliminary experiment, SPME fibres with three different coatings (100 μm-PDMS, 65 μm-PDMS/DVB, and 75 μm-Carboxen/PDMS) were compared in terms of their extraction efficiency. Extraction was performed by exposing the fibres to the headspace of 2 mL of acidified urine sample with 1 g of NaCl for 60 min at 60 °C after one hour incubation. In further experiments, Carboxen/PDMS fibre was used since it revealed the best VOM extraction efficiency.

To obtain the HS-SPME procedure with a maximum response area of the detected peak for VOM extraction from urine samples, the influence of NaCl amount (0.1, 0.5, and 1 g), agitation time prior to incubation (10, 65, and 120 s), incubation and extraction temperature (40, 60, and 80 °C), and incubation (10, 35, and 60 min) and extraction (10, 35, and 60 min) times were studied (Table 1). The minimum and maximum levels of each of the studied variables were arbitrarily selected to cover a wide range of experimental conditions, and the corresponding central points of factorial design were examined.

As an initial step, a $2^{5-1}$ reduced factorial design was performed to evaluate significant variables involved in HS-SPME. Optimization of sample preparation was evaluated on the total sum of 25 selected peak areas of VOMs covering the entire GC chromatogram (25 VOMs)