

Headspace Solid-Phase Microextraction Coupled with Gas Chromatography–Mass Spectrometry for the Determination of Volatile Organic Compounds in Urine

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Abstract—Determination of volatile organic compounds (VOCs), the metabolites produced in human body, contributes to better understanding of the mechanisms responsible for the progression of diseases and facilitates monitoring of the responses to treatment. Urine samples are a rich source of VOCs and they constitute a non-invasive tool for diagnosis of various metabolic and pathological states. The aim of this study was to optimize the headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME–GC/MS) method for analysis of urine samples on the example of urine collected from children with celiac disease. Pooled urine samples from three children with celiac disease were used to select the optimal fibers, time and temperature of extraction, salt dose, and pH to achieve the highest SPME efficiency. Analysis performed with the use of divinylbenzene/carboxen/polydimethylsiloxane fiber in acidic pH with the addition of 1.5 g of sodium chloride supported the extraction of the highest number of VOCs with the largest total peak area. The extraction conducted at 60°C for 45 min led to an optimal improvement in efficiency with a reduced number of siloxane derivatives, indicative of degradation of septa, fiber, and/or GC column stationary phase. The optimized HS-SPME–GC/MS method can be successfully applied to the determination of VOCs in urine samples.

Keywords: volatile organic compounds, solid phase microextraction, mass spectrometry, urine, volatolomics

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Diseases are often diagnosed too late and treatments are not always sufficient. Effective methods are required for diagnosis of diseases at an early stage, facilitating personalized therapy and evaluating short-term results to improve therapy. For this reason, efforts should be made to develop low-cost and non-invasive diagnostic methods. Volatolomics is a new branch of chemistry that can offer effective solutions to the above problems. Volatolomics describes the profiles of volatile and semi-volatile organic compounds produced by biological systems, including the human body [1]. Volatile organic compounds are synthesized in metabolic pathways and are found in cells and their microenvironment, thus can be detected in all biological fluids, including breath condensate, skin emanations, urine, feces, saliva and blood [2]. Volatolomics is a particularly interesting diagnostic tool because each disease induces specific changes in the metabolic pathway, which influences the formation of VOCs and changes their profile.

In recent decades, considerable research efforts have been made to determine VOCs in biological samples. Selected VOCs were found to be correlated with

the prevalence of certain diseases, including gastrointestinal and metabolic disorders [2, 3]. Determination of VOCs can be an innovative tool for diagnosing non-invasive diseases and monitoring body responses to treatment. This diagnostic method is relatively simple, quick and patient-friendly.

Solid-phase microextraction (SPME) is one of the most popular methods for VOCs extraction [4]. The SPME method has been developed through a combined effort in the fields of pharmacology, forensic sciences, clinical diagnostics, environmental research, and occupational health analyses [5]. Effective diagnostic techniques support detailed identification. Aranda-Rodriguez et al. [6] monitored a group of patients exposed to environmental sources of VOCs. The cited authors relied on the SPME method coupled with gas chromatography and tandem mass spectrometry (MS/MS) to quantify compounds in an isotope dilution analysis.

The blood concentrations of many metabolites are too low to be detected by the available analytical methods. Urine that has been filtered and concentrated by the kidneys contains higher and detectable levels of VOCs. For

this reason, urine can be effectively used in diagnostics. Urine sampling is also a much more patient-friendly method. Therefore, the aim of this study was to optimize the HS-SPME-GC/MS for quantifying VOCs in urine samples. The variable parameters affecting VOC extraction were optimized. Six types of SPME fibers, including divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), polydimethylsiloxane (PDMS), divinylbenzene/polydimethylsiloxane (DVB/PDMS), carboxen/polydimethylsiloxane (CAR/PDMS), polyacrylate (PA), and carbowax-polyethylene glycol (PEG), were studied to optimize the extraction conditions. To our knowledge, this study makes the first attempt to apply PEG fiber coating in VOCs determination in urine samples. The applied method was modified to investigate such factors as ionic strength, extraction temperature, and extraction time for optimal analytical performance. To our knowledge, there is a general scarcity of research concerning the VOC concentrations in urine of celiac disease patients. Therefore, we performed the presented study with the use of pooled urine samples collected from celiac disease children. Untreated celiac disease can lead to chronic inflammation and malabsorption with serious health consequences, which is why effective methods for diagnosing the disease are needed. And still, even when approved diagnostic tools are used, the high percentage of patients remain undiagnosed or are diagnosed incorrectly [7].

EXPERIMENTAL

Chemicals. Sodium chloride (NaCl) was purchased from Sigma (Sigma-Aldrich, USA). Sodium hydroxide (NaOH, 99%) and hydrochloric acid (HCl, 37%) were purchased from Fluka (Sigma-Aldrich, USA). Water was purified with the Mili-Q system (Milipore, Bedford, USA) and was used to prepare HCl and NaOH solutions.

Urine samples. Urine samples were collected from three children (aged 4–5 years) with celiac disease to develop and optimize the HS-SPME-GC/MS method. The collected urine was pooled, portioned into an appropriate number of vials calculated for both optimization and validation of the method, capped with a PTFE septum and a screw cap (Supelco, Bellefonte, PA, USA), and refrigerated (at 4°C) until analysis. The sample collection procedure was consistent with the provisions of the Declaration of Helsinki and was approved by the Bioethics Committee of the Faculty of Medical Sciences of the University of Warmia and Mazury in Olsztyn (agreement no. 23/2015 of 16 June 2015).

Solid-phase microextraction optimization parameters. The development of SPME method involved the selection and optimization of the parameters affecting extraction efficiency. Fiber type, pH, ionic strength, temperature, and time of extraction were carefully optimized. All fibers were exposed to 4 mL of urine samples in the headspace mode under identical condi-

tions. The analyses were performed using a manual SPME fiber holder. The time and temperature of incubation, extraction and desorption, and salt dose were determined based on the conditions specified in the literature [8]. The extraction efficiency of selected parameters was determined by plotting a total ion chromatogram and identifying the total number of extracted compounds.

Selection of fiber type. Six fiber coatings with different stationary phases and film thicknesses were purchased from Supelco (Bellefonte, PA): 50/30 μm DVB/CAR/PDMS (stable flex), 65 μm DVB/PDMS (stable flex), 100 μm PDMS, 75 μm CAR/PDMS (stable flex), 85 μm PA (stable flex) and 60 μm PEG. Before analysis, fibers were conditioned according to the producer recommendations to remove contaminants and stabilize the solid-phase.

Before extraction, 0.8 g of NaCl was added to each sample and the mixture was stirred at 650 rpm for 60 min at 60°C before incubation. Then, the fiber was exposed to headspace at 50°C for 60 min (extraction step). Next, the fiber was inserted into a GC injector port for thermal desorption of the analytes for 6 min at 250°C. The most effective fiber was used to optimize the remaining parameters.

Evaluation of the influence of ionic strength. The influence of ionic strength was determined by comparing solutions with different sodium chloride doses: none, 0.8 and 1.5 g. Analyses were performed at the physiological pH of urine (pH 7) using the most effective fiber.

Evaluation of the influence of pH. Urine samples are complex mixtures of compounds with different structural and chemical properties. In general, fresh urine samples contain a limited number of VOCs [4]. For this reason, the concentration of VOCs in headspace was enhanced by modifying the pH of the samples. To produce an acidic environment (pH 1–2), 4 mL of urine was added to a 20 mL glass vial (Supelco, Bellefonte, PA, USA) and mixed with 100 μL of 6 M HCl solution. To produce an alkaline environment (pH 12–14), 4 mL of urine sample was combined with 100 μL of 6 M sodium hydroxide solution.

Selection of extraction temperature and time. The optimal pH and dose of sodium chloride selected in the previous steps were applied in this step. Extraction temperatures of 50, 60 and 70°C were tested over the extraction time of 60 min. The optimal temperature was used to compare extraction times of 45, 60 and 75 min.

Parameters of gas chromatography-mass spectrometry detection. Volatile organic compounds were analyzed using the Hewlett-Packard 5972 mass selective detector coupled with the HP 5890 gas chromatograph (Agilent Technologies, Santa Clara, United States) and a data station containing the NIST/EPA/NIH mass spectral library (Version 2). The compounds were separated in the 30 m DB-WAX capillary column

(0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, United States). The injection port was lined with the 0.75 mm ID splitless glass liner (Supelco, Bellefonte, PA). Front inlet temperature was set at 250°C. The oven temperature program was 35°C for 5 min, it was increased at intervals of 2.5 grad/min to 220°C and maintained for 5 min. The injection port was set in splitless mode. The carrier gas was helium at a constant flow rate of 1 mL/min. Mass spectra were obtained by electron ionization in the range of 35–550 m/e. Ion source temperature was 230°C, and electronic impact energy was 70 eV. Total ion chromatograms were analyzed with the MSD ChemStation E.02.02.1431 software (Agilent Technologies, Santa Clara, United States) with an initial threshold of 13.0.

The compounds were identified by comparing spectra in the mass spectral library of the National Institute of Standards and Technology (2005), comparing retention times and calculating linear retention indices relative to a series of alkanes (C₆–C₃₀).

Precision. The practical applicability of the optimized HS-SPME method is determined by evaluating its precision in terms of repeatability and intermediate precision. The repeatability and intermediate precision of the optimized method were evaluated in 10 aliquots of the pooled urine samples. Five urine samples were extracted in line with the described protocol under optimal conditions, and the remaining 5 samples were extracted and analyzed in the same manner on the following day. The total peak areas of selected compounds and the total number of extracted compounds were used to calculate relative standard deviation (RSD).

Statistical analysis. All experiments and analytical measurements were performed in triplicate. Solid-phase microextraction variables were optimized and analyzed using Statistica v. 12 software (StatSoft, USA).

RESULTS AND DISCUSSION

Selected extraction parameters were optimized by choosing the most effective conditions in terms of total peak area, number of compounds, and reproducibility. The experiments were performed using the one-factor-at-a-time (OFAT) method [9] where factors were tested individually rather than simultaneously.

Selection of fiber type. This study made the first attempt at investigating six fiber coatings with different polarity, including a PEG fiber, to determine the most effective method for extracting volatile metabolites from urine samples. The total peak areas of urine metabolites are presented in Fig. 1a. Based on the number of extracted compounds, DVB/CAR/PDMS was selected as the most effective coating for extracting metabolites from urine samples. The total peak areas of DVB/CAR/PDMS, PEG, and PDMS fibers were

similar, however, the repeatability of the results based on standard deviation values was the lowest for DVB/CAR/PDMS coating. The highest number of separated compounds was also achieved with the DVB/CAR/PDMS fiber. The CAR/PDMS fiber was characterized by the lowest efficiency. DVB/CAR/PDMS coating had a complex structure which supported the extraction of volatile compounds within a wide range of polarities. Mixed phase coatings facilitate the extraction of a higher number of compounds with different chemical properties within a wide range of pH values, which makes them suitable for metabolite profiling [10].

Evaluation of ionic strength effect. The addition of a soluble salt to a sample can change the ionic strength of the matrix and influence extraction efficiency. The effect of ionic strength on metabolite extractability was investigated at different concentrations of sodium chloride. The results of this procedure are presented in Fig. 1b. The total response values indicate that sodium chloride significantly enhanced the extraction of urine metabolites in a dose-dependent manner. Therefore, the highest sodium chloride dose (1.5 g) was used in further studies.

Extraction efficiency can be improved by supersaturating the samples which facilitates analyte extraction onto the fiber due to the salting-out effect and easy passage of molecules from the sample matrix to headspace [11]. However, it should be noted that salt addition is safe only in the HS mode, whereas higher salt doses in the direct immersion mode can damage the coating [10]. According to our preliminary studies (data not shown) sodium chloride dose above 1.5 g does not increase efficiency of VOCs extraction. Our observations are in agreement with Monteneiro et al. [12] who optimized sodium chloride dose at 0.59 g for 2 mL urine sample.

Evaluation of the effect of pH conditions. Acidic, neutral, and alkaline conditions were compared to increase the number of extracted compounds. The effectiveness of the tested conditions is presented in Fig. 2. The number of produced compounds was significantly higher under exposure to hydrogen chloride than under the remaining pH conditions, which can be attributed to increased compound volatility and easier transition to the headspace [13].

Acidic pH supported the recovery of a higher number of compounds, however, the limitation of the application of acidic pH is the fact that some of the detected compounds could be formed under exposure to extreme pH and high temperature of SPME extraction [4], therefore it should be considered during the data analysis.

Selection of optimal extraction temperature and time. The time of extraction is a crucial step in the SPME procedure. The influence of temperature on the extraction efficiency is presented in Fig. 3a. Our study showed that longer extraction time had no sig-

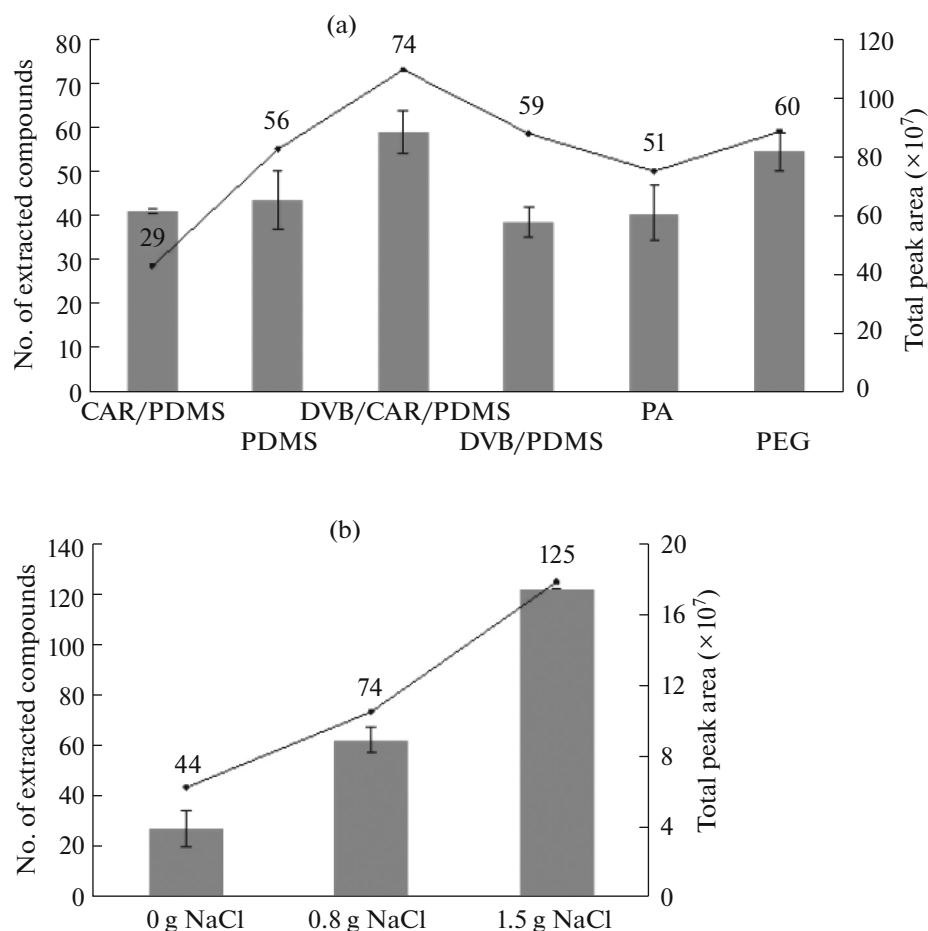


Fig. 1. The results produced by the optimized method in terms of fiber type (a) and ionic strength (b) (plot bar is related to results of total peak area whereas no. of extracted compounds is presented by linear plot).

nificant influence on extraction efficiency. The shortest extraction time of 45 min produced satisfactory extractability and prevented fiber degradation to polysiloxane compounds (data not shown), and therefore was selected as the most effective.

The data in Fig. 3b indicate that temperature had a significant impact on the extraction efficiency. An increase in temperature from 50 to 60°C significantly improved extraction efficiency in terms of the total peak area and the number of extracted compounds. Higher extraction temperatures increased the extraction rate and decreased the distribution constant (Kataoko et al. [11]). At higher temperature, the equilibrium state was achieved in a shorter period of time, which decreased the duration of the SMPE analysis [10]. A further increase in temperature to 70°C increased the number of extracted metabolites, but reproducibility was very low. Higher temperature can degrade the sample and less thermally stable compounds, which may also affect the total number of compounds [14]. Secondly, high temperature can influence fiber coating, which leads to premature desorption of analytes and formation of coating arte-

facts [8]. In our study, the highest number of peaks representing siloxane derivatives was determined in the samples extracted at 70°C. In this case, siloxane derivatives are identified as degradation products of the fiber coating. Therefore, 60°C was chosen as the optimal temperature that contributed to the highest extraction yield without the risk of fiber destruction or metabolite degradation. Furthermore, all extractions were performed with continuous stirring at about 650 rpm, which might significantly influence equilibrium establishment.

Precision. According to the literature, non-target analyses are difficult to validate [15]. In this study, attempts were made to compare parameters that could be easily checked even in non-target profiling. Therefore, repeatability and intermediate precision were monitored and maintained in the same set of samples that were divided into 10 portions and analyzed under optimized conditions. Repeatability can be measured by examining the variation in peak areas on the day of analysis, whereas intermediate precision can be evaluated by comparing the results obtained on different days. In our study, two parameters of non-target anal-

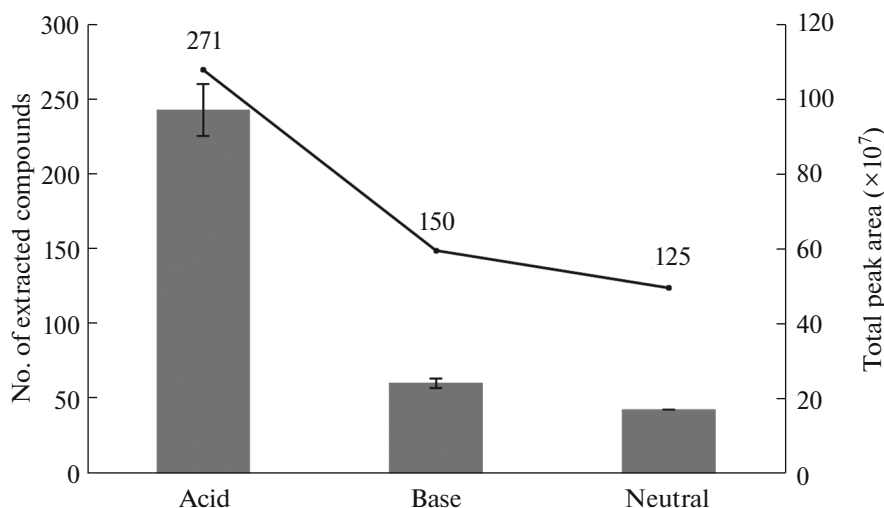


Fig. 2. The effectiveness of extraction in acidic (pH 1–2), alkaline (pH 12–14), and neutral (pH 7) conditions (plot bar is related to results of total peak area whereas no. of extracted compounds is presented by linear plot).

ysis were compared: total peak area and total number of extracted compounds. The RSDs of the total peak area and total number of compounds were 5.5 and 3.7%, respectively. A comparison of the values obtained on the first and second days revealed similar repeatability on both days. The RSDs of intermediate precision were 6.4% for the total peak area and 3.5% for the total number of compounds. This result is satisfactory, especially for the manual injections, and it indicates that the proposed procedure is repeatable. The results of repeatability and intermediate precision are summarized in Table 1.

Compound identification. The identified VOCs included sulphur-containing compounds, carboxylic acids, aldehydes, alcohols, ketones, aromatic compounds, single esters, and amines. The list of the metabolites tentatively identified based on the NIST database and the available literature is presented in Table 2.

Also, the presence of 4-methylphenol, 4-heptanone, phenol, dimethyl disulphide, dimethyl trisulphide was confirmed by comparing to the commercial standards. The highest responses were observed for 4-methylphenol, 4-heptanone, 2,6-dibromoaniline, and 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one (**β -damascenone**). Then, lower peak areas were recognized for phenol, dehydro- β -ionone, octanoic, and nonanoic acids. The sums of peak areas of ketones and then aldehydes and alcohols were noted as the highest. Some of selected volatile compounds had been previously identified in the human urine metabolome by HS-SPME-GC/MS [16], including dimethyl disulphide, dimethyl trisulfide, α -methyl- α -(4-methyl-3-pentenyl)oxirane methanol (**1,2-oxolin-alool**), phenol, 4-methylphenol, 4-heptanone, β -damascenone, 4-(2,2,6-trimethylcyclohexa-1,3-die-

nyl)but-3-en-2-one (**dehydro- β -ionone**), 2,6,10,10-tetramethyl-1-oxa-spiro-dec-6-ene, methyl salicylate, and α -ionone. The chromatogram obtained with the use of the optimized extraction method in acidic conditions is shown in Fig. 4.

Sulphur-containing compounds. Acidic compounds, such as organic acids and sulphur-containing compounds, were extracted at low pH (1–2) [17]. It has been reported that acidic pH supports the extraction of sulphur-containing metabolites. Our findings are consistent with the results of Mills et al. [17] who reported that sulphur-containing molecules were more effectively extracted in acidic pH. Sulphur-containing compounds are products of detoxification mechanisms related to neutralization of hydrogen sulphide which is generated from sulphur-containing substances in the diet due to the presence of sulphate reducing bacteria [18]. Methanethiol has the aroma of rotten cabbage and it occurs naturally in biological fluids. It is present in various biological fluids, including saliva, feces, and urine [14, 19, 20]. Methanethiol originates from methylation of hydrogen sulphide by mucosal S-thiolmethyltransferase as a detoxification mechanism to avoid damages to intestinal epithelium [18]. It was previously reported that the variations in methanethiol levels in feces and blood could be used to diagnose liver and intestinal diseases [18, 20].

Table 1. Repeatability and intermediate precision expressed by the relative standard deviation (%)

Precision	Total peak area	Total number of compounds
Repeatability	5.5	3.7
Intermediate precision	6.4	3.5

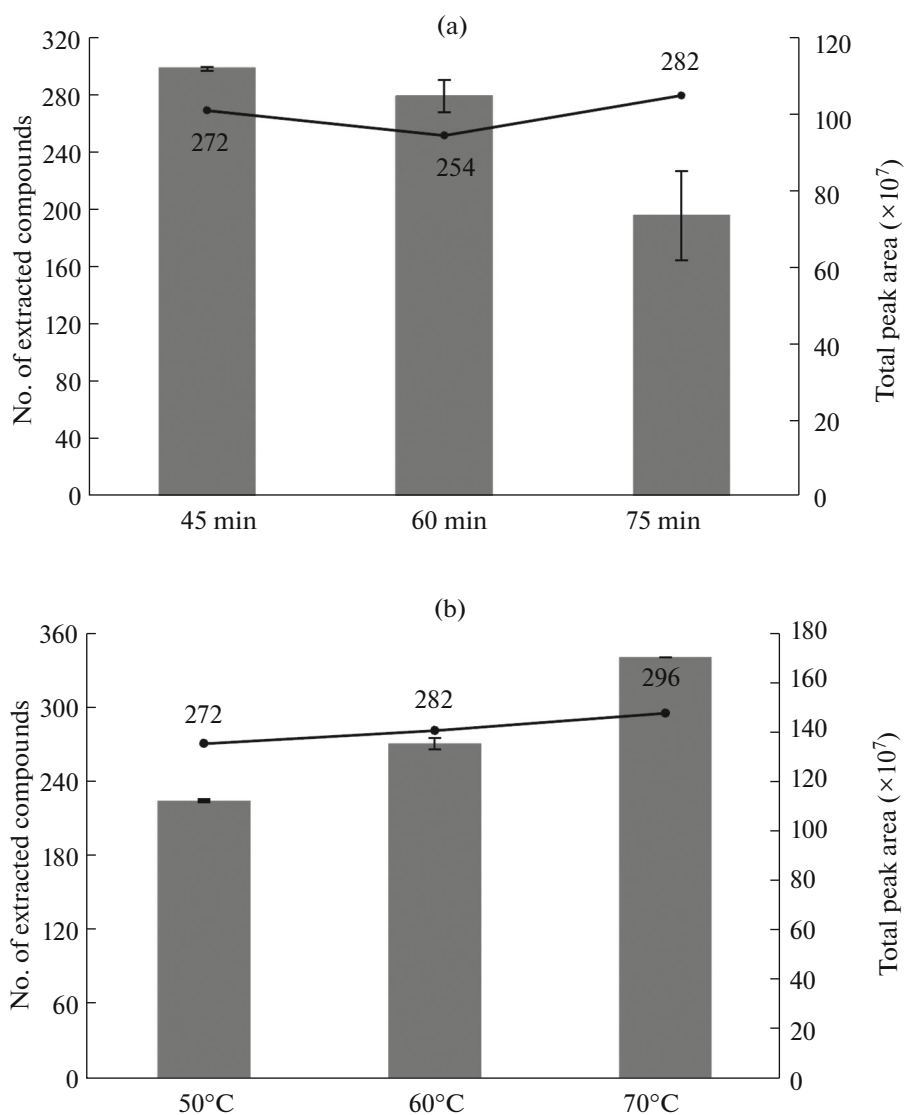


Fig. 3. Optimization of extraction time (a) and temperature (b) (plot bar is related to results of total peak area whereas no. of extracted compounds is presented by linear plot).

Another important sulphur containing metabolite is dimethyl disulphide, a protein fermentation product which is normally found in the metabolome of healthy individuals. Lower levels of dimethyl disulphide were noted in patients with Crohn's disease and ulcerative colitis [21]. Lower levels of dimethyl disulfide are also considered as an indication of cancer [14]. Dimethyl trisulfide was also identified in the urine of healthy volunteers [22]. Both compounds, dimethyl trisulfide and dimethyl disulfide have been reported at higher levels in the urine samples of the children with celiac disease as compared to the healthy ones [23]. Sulphur-containing compounds, such as dimethyl disulphide, methanethiol, and 2-methyl-5-(methylthio)furan, were characterized by high concentrations in patients with homocystinuria [17]. Furan derivatives, 2-methyl-5-(methylthio)furan and tetra-

hydro-2,2-dimethyl-5-(-1-methyl-1-propenyl)furan, are the compounds produced during digestion of food.

Carboxylic acids. The major saturated fatty acids, octanoic and nonanoic acids, were effectively extracted. Fatty acids are the end products of dietary fiber fermentation by anaerobic intestinal microbiota and they are beneficial for human health [24]. Our findings are consistent with the results of Weijers and van de Kamer [25] who reported higher levels of saturated than unsaturated fatty acids in the feces of patients with celiac disease. Their findings can be attributed to the gluten-free diet of celiac patients where wheat flour is replaced with rice flour. Tjellström et al. [26] reported significantly higher levels of short-chain fatty acids in feces of children with celiac disease. Further research is required to determine the presence of fatty acids with more than 7 carbon atoms

Table 2. Compounds identified in urine by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry using divinylbenzene/carboxen/polydimethylsiloxane fiber

Compound	CAS-number	Calculated retention index	Literature retention index	Peak area ^a × 10 ⁻⁵	RSD, %
Sulphur-containing compounds					
Methanethiol	74-93-1	709	699 ^b	5.1	20.1
Dimethyl disulfide	624-92-0	886	844 ^c	51.5	4.0
Dimethyl trisulfide	3658-80-8	1459	1412 ^b	38.7	1.4
2-Methyl-5-(methylthio)furan	13678-59-6	1468	938 ^c	65.4	9.0
				Σ 160.7	
Carboxylic acids					
Octanoic acid	124-07-2	2026	2098 ^c	117.4	4.8
Nonanoic acid	112-05-0	2136	2202 ^c	81.1	10.1
				Σ 198.5	
Aldehydes and alcohols					
α-Methyl-α-(4-methyl-3-pentenyl)oxirane methanol	— ^d	1678	1570 ^b	37.7	5.6
Phenol	108-95-2	2030	—	188.9	1.7
4-Methylphenol	106-44-5	2043	2100 ^c	798.6	3.2
				Σ 1025.2	
Ketones					
4-Heptanone	123-19-3	973	1118 ^b	548.5	4.3
5-Ethenyldihydro-5-methyl-2(3H)-furanone	1073-11-6	1692	1669 ^b	20.3	5.6
1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	23726-93-4	1838	1836 ^c	265.0	1.5
4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	1203-08-3	1822	1854 ^b	151.3	0.6
4-(2,3,6-Trimethylphenyl)-2-butanone	58720-40-4	2294	—	46.1	7.7
				Σ 1031.2	
Esters					
Methyl salicylate	119-36-8	1748	1782 ^c	23.9	1.1
Aromatic compounds					
Tetrahydro-2,2-dimethyl-5-(1-methyl-1-propenyl)-furan	7416-35-5	1185	1198 ^b	23.9	2.3
1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene	475-03-6	1895	1889 ^b	33.0	9.8
2,6,10,10-Tetramethyl-1-oxaspiro-dec-6-ene	36431-72-8	1543	1533 ^b	45.4	1.8
2-Chloro-1-methyl-4-(1-methylethyl)-benzene (2-chloro- <i>p</i> -cymene)	4395-79-3	1293	1282 ^c	58.1	1.4
1,6-Dimethyl-4-(1-methylethyl)naphthalene	483-78-3	2292	2256 ^b	58.8	0.2
				Σ 219.2	
Amine					
2,6-Dibromo-4-chloroaniline	874-17-9	2309	—	360.0	6.0

^aPeak area as a mean, ^bNIST database for polar column, ^cpherobase.org for DB-WAX column, ^d“—” — no data available.

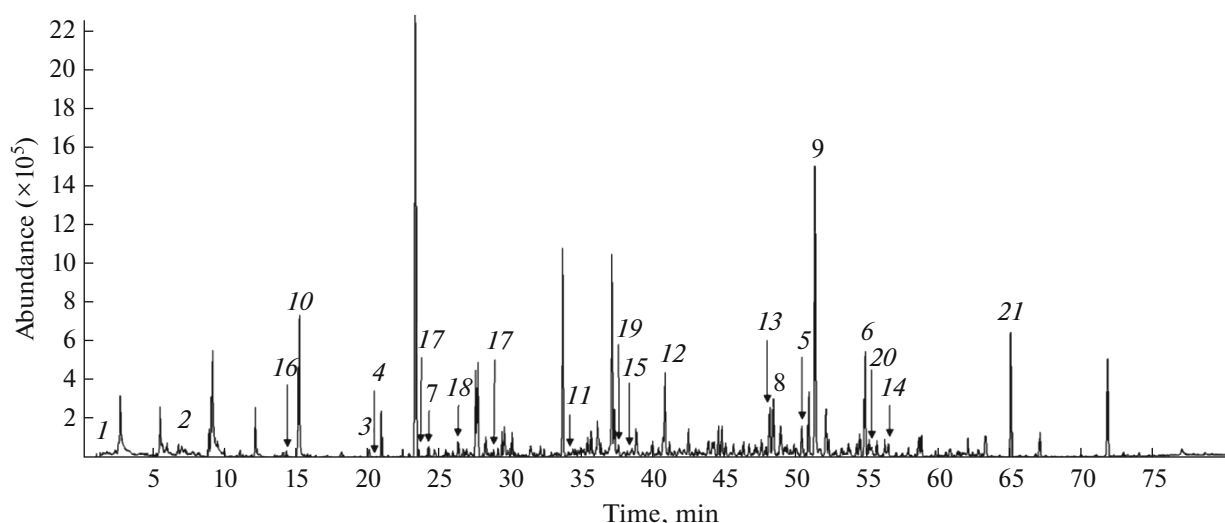


Fig. 4. Representative total ion chromatogram of volatile organic compounds identified in urine under optimal extraction conditions: (1) methanethiol, (2) dimethyl disulfide, (3) dimethyl trisulfide, (4) 2-methyl-5-(methylthio)furan, (5) octanoic acid, (6) nonanoic acid, (7) 1,2-oxalinalool, (8) phenol, (9) 4-methylphenol, (10) 4-heptanone, (11) lavender lactone, (12) β -damascenone, (13) dehydro- β -ionone, (14) 4-(2,3,6-trimethylphenyl)-2-butanone, (15) methyl salicylate, (16) tetrahydro-2,2-dimethyl-5-(-1-methyl-1-propenyl)furan, (17) α -ionene, (18) 2,6,10,10-tetramethyl-1-oxa-spiro-dec-6-ene, (19) 2-chloro-*p*-cymene, (20) 1,6-dimethyl-4-(1-methylethyl)naphthalene, (21) 2,6-dibromo-4-chloroaniline.

and to identify any correlations in their formation. According to Tanaka et al. [27], octanoic and nonanoic acids are present in urine of healthy subjects. Decreased levels of nonanoic acid have been reported in patients with schizophrenia [28]. Changes in the levels of this metabolite explain abnormalities of the homocysteine metabolism, creatine kinase-emia, and oxidative stress, that is hypothesized as a reason in disease development. In vivo studies also demonstrated that octanoic acid from food is an effective antioxidant [29].

Aldehydes and alcohols. Phenol and 4-methylphenol (*p*-cresol) were the most prevalent alcohols in this study. High phenol concentrations were previously determined in patients with gastrointestinal disorders [30]. The cited authors also suggested that higher phenol content could be associated with bacterial degradation of unabsorbed tyrosine to volatile phenols. Silva et al. [14] demonstrated that phenol concentrations were elevated in patients with different types of cancer. *p*-Cresol can be produced by bacterial protein fermentation in the human large intestine [31]. Interesting results were reported by Gabriele et al. [32] who observed significant changes in urinary *p*-cresol content only in children with an autism spectrum disorder (ASD) who were younger than 8 years. Their study confirmed that metabolic disorders could have a wide range of side effects, including mental. Total *p*-cresol levels were estimated at 98 $\mu\text{g}/\text{mL}$ in children with ASD and at 52 $\mu\text{g}/\text{mL}$ in healthy controls. Selected terpene alcohols in urine samples, such as 1,2-oxalinalool, can also originate directly from dietary sources. Linalool is produced mainly by herbs and spice plants,

such as mint, laurel, and cinnamon, and it is responsible for the characteristic sweet and floral aroma of plants [33]. Another aromatic compound, 2,6,10,10-tetramethyl-1-oxa-spiro-dec-6-ene, is also a flavor ingredient that occurs naturally in raspberries, tea, yellow passion fruit, or black chokeberry [34].

Ketones. In our study, selected ketones were extracted from urine. Urine contains many ketones due to the enzymatic function of the liver [35]. Ketones can be derived from dietary sources, but they are also useful in diagnoses of metabolic disorders. Elevated ketone levels were found in patients with diabetes mellitus [17]. Increased concentration of 4-heptanone in urine can be a symptom of diabetes [36]. 5-Ethenyl-5-methyl-2(3H)-furanone (**lavender lactone**) with a fruity and minty aroma is a natural food flavoring. β -Damascenone is an important compound with a strong fruity and floral aroma that resembles cooked apples [33]. β -Damascenone is widely synthesized in the plant kingdom and it is found in grapes, apples, lychees, starfruits, and mangoes [37]. β -Damascenone is the product of carotenoid degradation in plants [38].

Aromatic compounds. 4-(2,2,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one, 1,1,6-trimethyl-1,2,3,4-tetrahydronaphthalene and 1,2,3,4-tetrahydro-1,1,6-trimethylnaphthalene (**α -ionene**) have the same origin as β -damascenone in addition to food. 1,6-Dimethyl-4-(1-methylethyl)naphthalene occurs naturally in essential oils.

Other volatiles. In our study, only one ester and one amine were identified. Methyl salicylate occurs in

dietary sources. It is present in high concentrations in fresh green tea leaves [39].

CONCLUSIONS

An optimized HS-SPME-GC/MS procedure for non-target analysis of urine was proposed in this study. Selected extraction parameters, including fiber type, ionic strength, extraction time, and temperature, were compared to enhance analytical performance in terms of extraction efficiency and sensitivity. DVB/CAR/PDMS coating was the most effective fiber for VOCs determination in comparison to the remaining stationary phases. The conditions for non-targeted analysis of the urinary metabolome were optimized based on the obtained results. In the analysis of urine samples of 4 mL, the response values and reproducibility were optimized by adding 1.5 g of NaCl and extracting the samples at 60°C for 45 min in acidic (pH 1–2) conditions. A higher number of volatile metabolites with different physicochemical properties were extracted from urine solutions with an acidic rather than a neutral pH. The obtained results were characterized by high repeatability and intermediate precision. In the group of around 280 extracted compounds, a total of 21 volatile metabolites belonging to various chemical classes were tentatively identified based on the NIST database and the literature and confirmed by external standards. The most prevalent VOCs in the analyzed urine samples were sulphur-containing heterocyclic compounds, ketones and aromatic compounds. The described method can be a useful tool for identifying VOCs in biological fluids. As the VOC profile of urine samples provides valuable information about the metabolic status of patients, the proposed procedure could be used in biomedical research, and in future, it might be applied as a diagnostic method.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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