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Gas Chromatography

DETERMINATION OF VOLATILES IN MOUSE URINE BY HEADSPACE SOLID PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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The extraction and determination of volatile compounds in mice urine were performed using headspace solid-phase microextraction with by gas chromatography-mass spectrometry. In order to optimize the extraction conditions, experimental design was applied. A sample volume of 108 µl, a temperature of 148.6°C, and a time of 94 minutes were found to be the optimal conditions. Samples of male and female mouse urine were analyzed to determine volatile compound profiles. A total of 36 organic compounds including ketones, aldehydes, and terpenes were detected. The results revealed that compounds such as 2-isopropyl-4,5-dihydrothiazole, which is considered a male sexual pheromone, were only detected in male urine samples, whereas others like benzaldehyde were especially abundant in female mouse urine. A comparison of female samples corresponding to different stages of the estrous cycle was also performed.

Keywords: Experimental design; Gas chromatography-mass spectrometry (GC-MS); Headspace-solid-phase micro-extraction (HS-SPME); Mouse urine; Volatile organic compounds

INTRODUCTION

Urine is a complex biological fluid with enormous potential as a source of biochemical information. Therefore, the development of methods for the analysis of urine is an important objective. Urine contains diverse chemicals and plays an important role as a communication tool among animals. Of particular importance are volatile and semi-volatile substances functioning as pheromones. Examples of the extraction of volatile compounds in both rodent and human urine have been reported in literature using different techniques such as purge-and-trap (Prieto et al. 2000) and organic solvents (Achimaran et al. 2010; Zaporozhets, Tsyrulneva, and Ischenko 2012; Hardt and Angerer 2000; Zhang et al. 2008). On the other hand, solid-phase microextraction (SPME) has gained popularity as a simple, selective,

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and environmentally friendly tool for the sampling of a variety of volatile and semi-volatile compounds in different matrices. SPME presents several advantages for the extraction process such as small sample volume and reduced interferences of the matrix leading to cleaner extracts. Thus, SPME serves as a useful analytical tool prior to gas and liquid chromatography analyses (Biniecka and Caroli 2011; Vas and Vékey 2004). Examples of the extraction of organic volatile compounds in diverse matrices such as environmental samples (Krutz, Senseman, and Sciumbato 2003), cosmetics (Alvarez-Rivera et al. 2013), food samples (Lee et al. 2013; Bryant and McClung 2011; Palma-Harris, McFeeters, and Fleming 2001; Kumari et al. 2013; Pozo-Bayón et al. 2001), and biological matrices (Aresta et al. 2008; Mills and Walker, 2001) have been published in literature.

The purpose of this study was to determine small volatile compounds present in mouse urine that may act as rodent pheromones and influence rodent behavior. HS-SPME is well suited to identify these compounds, as it has already been applied to identify pheromones in the glands of Lepidoptera (Frérot, Malosse, and Cain 1997), elephant urine (Kayali-Sayadi et al. 2003), and rodent urine (Dehnhard et al. 2003; Osada et al. 2008; Gutiérrez-García et al. 2007). From the experimental point of view, SPME techniques may be influenced by a relatively large number of factors. To address this issue, experimental design allows rational and systematic optimization of conditions leading to better and more reproducible results (Salafranca et al. 2003). The advantages of using experimental design applied to SPME extraction have been demonstrated for the determination of drugs in biological matrices in humans (Reubsaet et al. 1998) or organic compounds in water (Mousavi et al. 2007; De Lima Gomes et al. 2011). The goal of this study was to optimize the SPME conditions using experimental design to perform the analyses and to establish the best extraction conditions for volatile compounds in mouse urine samples.

EXPERIMENTAL

Urine Samples

Mouse urine samples were provided by Faculty of Medicine, University of Valladolid (Spain). All samples were stored at −20°C following collection until analysis.

Solid Phase Micro-Extraction

85 μm StableFlex carboxen/polydimethylsiloxane (CAR/PDMS) fibers, a solid phase micro-extraction holder for manual use, and an SPME inlet guide were purchased from Supelco (Bellefonte, PA, USA). Head-space solid phase micro-extraction was performed in septum sealed vials. The urine was placed in sealed vials and diluted to a total volume of 1 ml with a NaCl solution. Fibers were inserted through the septum and maintained 1 cm above of the samples which were heated and stirred during all the extraction process. After extraction, the fibers were desorbed for 15 minutes in the injector of the gas chromatograph system using manual injection, the SPME manual holder, and the inlet guide. Fibers were conditioned before use as recommended by the manufacturer and cleaned at 275°C

for one hour in the injection port of the gas chromatograph. Blank extractions and analyses were performed in order to ensure correct identification.

Chromatographic Conditions

Analysis were performed using a 6890N Gas Chromatograph system coupled with a Mass Selective Detector 5975 supplied by Agilent Technologies Network. The injection port was equipped with a 0.75-mm i.d. inlet liner (Supelco, Bellefonte, PA, USA) to optimize fiber desorption and sample delivery on the column (HP-1MS capillary column; $30\,m \times 250\,\mu m \times 0.25\,\mu m$). Helium, at a flow rate of 1 ml/min, was used as the carrier gas. The oven temperature program started at 35°C. After 2 minutes, the temperature was increased to 80°C at $10°C\,min^{-1}$, to 250°C at $5°C\,min^{-1}$, and finally to 300°C at $20°C\,min^{-1}$. This temperature was held for 10 minutes. The interface was maintained at 300°C, the ion source at 230°C, and the quadrupole analyzer at 150°C. Electron impact (EI) spectra were obtained with an electron energy of 70 eV. Mass spectral acquisition was performed from 35 to 800 amu. The identity of the compounds was assigned according to the NIST (National Institute of Standards and Technology, Maryland, USA) database.

RESULTS AND DISCUSSION

Experimental Design

The optimal extraction conditions for the volatile compounds were established using experimental design implemented with the statistical program Statgraphics Centurion XVI.I. An orthogonal central composite design was selected involving three experimental factors (volume of sample, temperature, and time of extraction) and two response variables (the total peak area of the analytes of interest and the number of compounds detected). In order to obtain input data for the calculations, 16 experiments using male mouse urine samples were performed. The resulting Pareto charts are shown in Figure 1.

With respect to the total area response (Figure 1a), there are three effects with p-values less than 0.05 (volume, temperature, and the quadratic effect of time), indicating that they are significantly different from zero at the 95.0% confidence level. In the case of the number of peaks (Figure 1b), there is only one significant effect with *p*-value less than 0.05, the temperature.

Surface response graphs were generated as a function of total peak area and number of compounds (Figure 2). Improved responses were obtained by increasing both the volume of sample and temperature of extraction.

Response surface modeling (RSM) was used in order to find the optimal conditions for the HS-SPME extractions (Figure 3). The desirability was found to increase with temperature and time whereas volume of sample should be above 60 μL to optimize the extraction conditions. The optimal conditions were found to be a volume of 108 μl, 148.6°C, and 94 minutes.

Determination of Volatile Compounds in Mouse Urine

Male and female mouse urine samples were analyzed under the optimized HS-SPME conditions. Moreover, female urine samples obtained at different stages

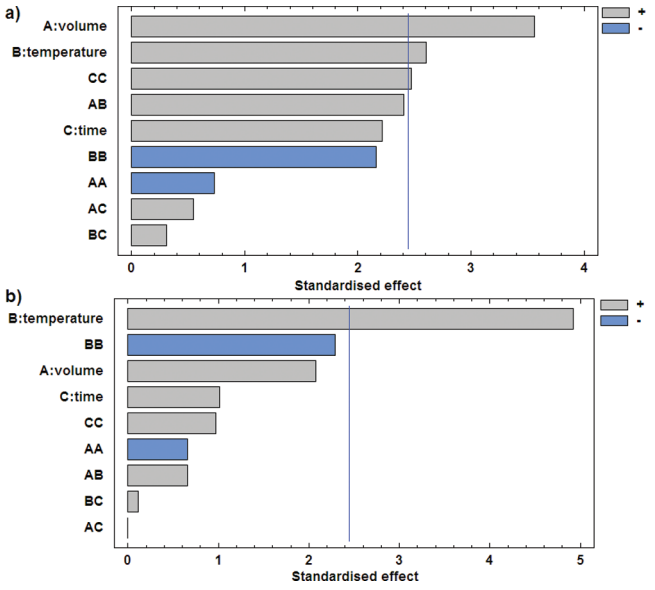

Figure 1. Pareto charts for the response variables: (a) total area and (b) number of compounds.

of the estrous cycle were included in the study. Characteristic chromatograms corresponding to male and proestrus female mice urine samples are shown in Figure 4.

The results obtained in neutral extraction conditions, diluting urine samples with NaCl solution in order to increase the ionic force, are summarized in Table 1.

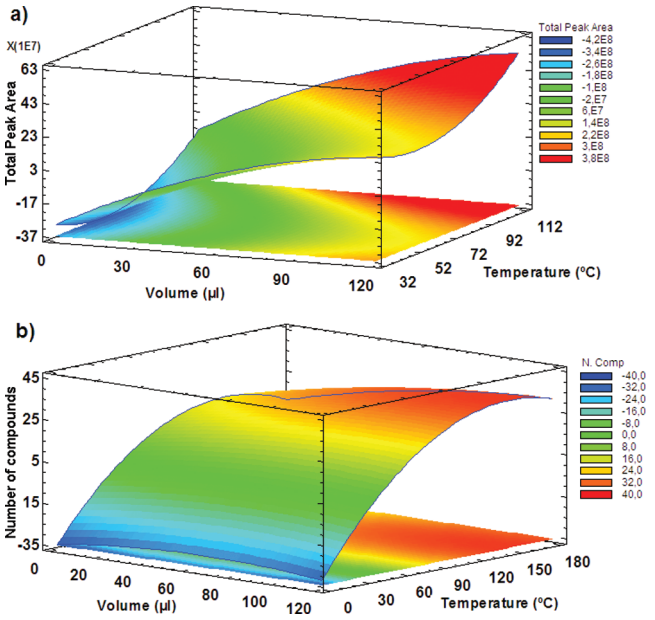

Figure 2. Surface response graphs: (a) total peak area and (b) number of compounds.

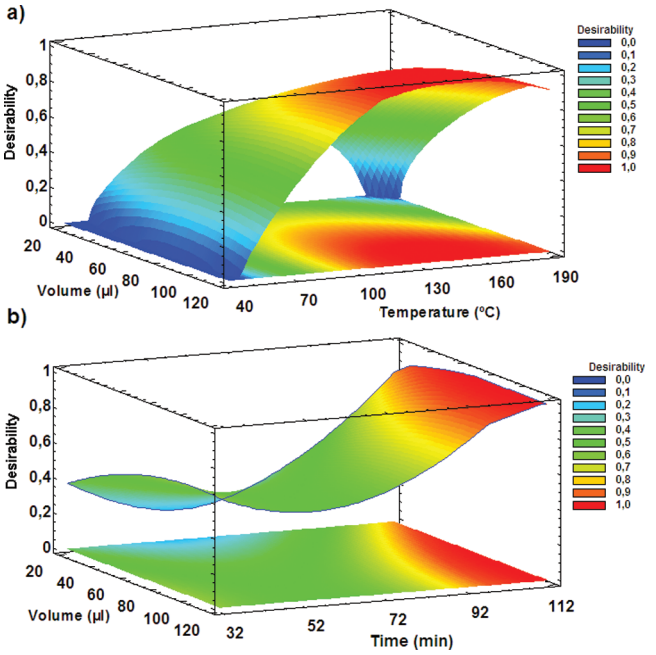

Figure 3. Response surface applied for the optimization of HS-SPME extractions.

The composition of the urine samples is expressed as percentages of the different compounds. The relative abundance of each compound was given as the ratio between the area of the target ion and the total area of all compounds.

A total of 36 organic compounds were identified in the urine samples. Twelve terpenes were identified. Seven compounds (β-myrcene, linalool, geraniol, citral, β-farnesene, α-farnesene, and farnesol) were satisfactorily identified, with farnesol most abundant. The profiles of the male and female samples, both male and female, are similar, although some significant differences were observed. The amount of benzaldehyde detected was much higher in female compared to male

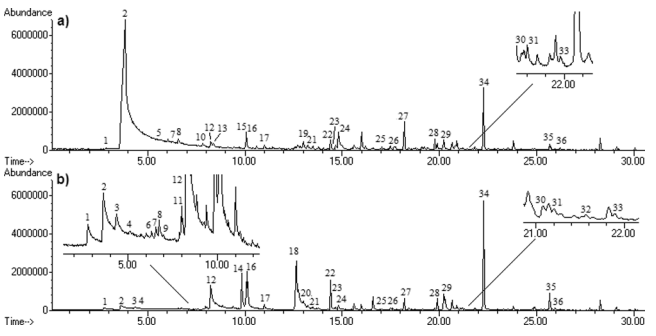

Figure 4. Representative total ion chromatograms of SPME extracts: (a) female mouse urine and (b) male mouse urine.

Table 1. Relative abundances of identified substances in mouse urine

			Male		Female	
				Proestrus	Diestrus	Estrus
N°	Compound name	Diagnostic ions m/z (A)	%	%	%	%
1	3-hepten-2-one	55 (100), 97 (86), 43 (70), 112 (33)	2.10	0.34	0.57	1.48
2	Benzaldehyde	106 (100), 77 (95), 105 (90), 51 (48.9)	9.81	84.34	73.10	66.34
3	Dimethyl sulphide	126 (100), 45 (60), 79 (50.6), 111 (15)	1.74	–	–	–
4	6-methyl-3-heptanone	57 (100), 43 (80), 72.05 (60), 81 (54), 128 (16)	0.09	–	–	–
5	4-octen-3-one	55 (100), 70 (93), 43 (18)	–	0.07	0.28	0.37
6	5-methyl-5-hepten-3-one	57 (100), 41 (46), 69 (39), 97 (22)	0.36	–	0.14	0.21
7	2-pentyl furane	81 (100), 53 (14), 138 (21)	0.73	0.17	1.39	1.61
8	Terpene 1 (-myrcene)	93 (100), 41 (66), 69 (66), 79 (15), 121 (6)	0.65	0.11	0.28	0.31
9	2-isopropyl-4,5-dihydrothiazole	60 (100), 129(40), 114 (15), 59.05 (25)	0.08	–	–	–
10	3-octen-2-one	55 (100), 43 (90), 111 (80), 126 (15)	–	0.46	0.50	0.78
11	3-ethyl-2-methyl-1,3-hexadiene	95 (100), 67 (91), 57 (79), 41 (56)	1.40	–	–	–
12	1-phenyl-ethanone	105 (100), 77 (68), 120 (32), 95 (20), 51 (19)	33.93	1.35	2.99	3.22
13	Octanal	43 (100), 56 (75), 84 (60), 69 (25), 100 (10)	–	0.18	0.23	0.32
14	2-sec-butyl-4,5-dihydrothiazole	115 (100), 60 (95), 59 (25), 128.05 (19)	13.32	–	–	0.04
15	Nonanal	57 (100), 44 (80), 98 (35), 70 (25)	–	0.11	0.19	0.23
16	Terpene 2 (linalool)	71 (100), 93 (90), 80 (34), 115 (32), 121 (29), 136 (10)	2.20	0.61	1.58	2.07
17	3-nonen-2-one	55 (100), 43 (55), 71.10 (23), 97 (24), 125 (41)	1.41	0.44	0.63	0.66
18	3-methyl pyridine	93(100), 65 (33), 79 (11), 43 (7),	0.75	–	–	–
19	4-pentyl-phenol	107 (100), 108 (88.2), 77 (22.2), 27 (5.1)	–	1.12	2.07	2.67
20	N-phenyl-formamide	43 (65), 93 (100), 87 (39), 121 (83)	0.32	–	–	–
21	2,3-dihydro-benzofurane	120 (100), 91 (80), 107 (30)	2.00	0.11	1.35	1.73
22	Terpene 3 (geraniol)	69 (100), 41 (57), 93 (24), 123 (13), 111 (8)	3.26	0.72	1.46	2.07
23	Terpene 4 (citral)	69 (100), 41 (75), 84 (24), 121 (13), 93 (22)	0.44	0.07	0.12	0.14
24	1H-indole	117 (100), 89 (30), 90 (30), 91 (10), 43 (10)	3.93	5.01	3.89	4.46
25	Ester of propanoic acid	71 (100), 113 (83), 43 (64), 56 (53), 98 (24)	0.08	0.07	0.09	2.07
26	N-ethyl-benzenamine	106 (100), 153 (30), 77 (14), 136 (5)	0.52	0.04	0.09	0.14
27	2,6-bis(1,1-dimethyl-ethyl)-phenol	191 (100), 149 (36), 206 (24)	2.55	1.88	2.21	1.52

(Continued)

Table 1. Continued

28	Terpene 5 (-farnesene)	69 (100), 41 (53), 93 (64), 133 (35), 81 (22)	1.80	0.28	0.68	0.75
29	Unassigned (cyclohexanol derivative)	57 (100), 123 (41), 109(21), 85(20)	3.07	0.63	1.44	1.63
30	Terpene 6 (α-farnesene)	93 (100), 41 (85), 69 (80), 55 (57), 107 (52), 119 (48)	0.22	0.03	0.08	0.09
31	Terpene 7	69 (100), 41 (62), 93 (93), 119 (30), 161 (34), 204 (28)	0.22	0.03	0.07	0.10
32	Terpene 8	69 (100), 93.05 (60), 107 (22), 123 (14), 136 (15),	0.06	–	–	–
33	Terpene 9	93 (100), 119 (38), 109 (28), 41 (29)	0.11	0.02	0.04	0.06
34	Terpene 10 (farnesol)	69 (100), 41 (57), 93 (76), 107 (46), 136(26), 204(3)	11.00	1.52	3.73	4.02
35	Terpene 11	69 (100), 41 (59), 93 (39), 79 (17), 107 (16) 121 (11))	1.65	0.25	0.74	0.83
36	Terpene 12	69 (100), 84 (48), 123 (12), 41 (44), 57 (29)	0.20	0.03	0.08	0.08

Analytes not detected in the samples or low than 0.01%.

mice. Benzaldehyde was the main compound detected in all the stages of the female estrous cycle (84.34% in proestrus, 73.10% in diestrus, and 66.34% in estrus), whereas in male urine samples, this percentage dropped to approximately 10%. Percentages of phenyl ethanone in male urine samples (close to 34%) were higher than those detected in females (1–3% depending on their estrous cycle). All terpenes were detected in male and female mice, except that terpene 8 was found exclusively in males. The detected amount of terpenes was in general higher in males than in females. For instance, the farnesol percentage in males was found to be 11%, whereas it decreased to 4%–1.5% in females. The same trend was observed for other terpenes such as β-farnesene (1.8% in males, 0.28–0.75% in females), geraniol (3.26% in males, 0.72–2.07% in females), and linalool (2.20% in males, 0.61–2.07% in females).

Some compounds were detected exclusively in urine from one gender. Thus, dimethyl sulfide, 6-methyl-3-heptanone, 2-isopropyl-4,5-dihydrothiazole, 3-ethyl-2-methyl-1,3-hexadiene, 3-methylpyridine, and N-phenylformamide were found only in males. On the other hand, compounds like 4-octen-2-one, 3-octen-2-one, octanal, nonanal, and 4-pentylphenol were exclusively observed in females. Compounds considered as sexual pheromones in mice such as 2-isopropyl-4,5-dihydrothiazole and 2-sec-butyl-4,5-dihydrothiazol were also detected.

Urine samples collected at different stages of the female mice estrous cycle also showed similar profiles. However, a slightly increase in the percentage of ketones during the estrus stage was observed. This observation is in agreement with the experiments reported by Schwende, Wiesler, and Novotny (1984) in female rats during this stage. The sexual pheromone 2-sec-butyl-4,5-dihydrothiazole was also identified in samples of urine obtained during the estrus stages of female mice.

CONCLUSIONS

Experimental design was implemented using response surface methodology and applied to the analysis of mouse urine in order to determine the best conditions for the extraction of volatile and semi-volatile compounds using HS-SPME with GC-MS. The use of the optimized extraction conditions (108 µl, 148.6°C, and 94 minutes) resulted in robust, reproducible results. 36 organic compounds were detected in those samples. Several differences were observed when comparing male and female mouse urine samples. Benzaldehyde was found to be the main compound detected in the female urine samples. Depending on the gender, specific compounds were detected. Thus, compounds such as 2-isopropyl-4,5-dihydrothiazole were exclusively detected in males whereas ketones like 4-octen-2-one, 3-octen-2-one were detected in females. This method may have future application for the determination of volatile and semi-volatile compounds in urine.

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