Quantification of Volatiles in Mammalian Urine by Stir Bar Sorptive Extraction (SBSE) Techniques and Gas Chromatography

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Summary
Stir Bar Sorptive Extraction (SBSE) has been found as a successful extraction method for profiling volatile and semivolatile putative chemosignaling compounds in hamster and mouse urine. When combined with an automated thermal desorption system (TDSA) for the gas chromatographic analyses, SBSE has proven to be highly reproducible in quantitative comparisons of the urinary profiles. Relative standard deviations (RSD) of 1-5% were achieved for a wide range of compounds in undiluted male hamster urine without using an internal standard. Hydrophilicity/hydrophobicity did not affect reproducibility of extraction. Quantitative estimation with selected standard compounds was also made. The effect of sample dilution prior to extraction was demonstrated with sulfur compounds extracted from male mouse urine.

1 Introduction
Mammalian chemical signaling is at least partially mediated by relatively small volatile and semivolatile compounds found in urine and glandular secretions [1]. Some of these compounds possess pheromone activity influencing social behavior and reproductive functions of the recipient animals [2,3]. Quantitative analysis of the chemosignaling compounds in urine and other biological specimens is challenging due to the complex sample matrices, low concentrations and a wide variation in chemical and physical properties. One isolation approach may be optimal for one class of compounds, but it may be discriminating toward other classes of compounds. Solventless trapping of the volatiles into sorption materials, carried by a helium stream (dynamic headspace), has been used for a long time as a successful approach for biological samples like urine prior to their GC-MS analysis [2,4]. One of the disadvantages of this technique is a low reproducibility due to differences in the trapping material quantity and composition. Therefore, an appropriate internal standard must be used for compensating the problem. The set-up of the system also requires specialized glassware and additional care to clean large surface areas. A sample throughput using the headspace sampling becomes a limiting step for the analysis of a large number of samples which are often required in biological studies.
A new approach of Stir Bar Sorptive Extraction (SBSE) technique consists of a polymer-coated magnetic Twister™ bar for extraction of a wide range of volatile and semivolatile compounds in aqueous samples [5]. The volume of polydimethylsiloxane (PDMS) coating on stir bars can be precisely controlled, increasing the reliability of extraction. As the Twister™ technique utilizes sorptive extraction principles, the extraction process can reach equilibrium within a reasonable time. This facilitates parallel sample preparation and increases sample throughput. To this date, the PDMS sorption techniques have been applied successfully in environmental [6] and food flavor analytical applications [7]. In this communication, we demonstrate the use of SBSE for qualitative profiling and quantitative measurements of volatile and semivolatile compounds in hamster (*Phodopus campbelli*) and mouse (*Mus domesticus*) urine. The effects of sample dilution on quantification are demonstrated.

2 Materials and Methods
2.1 SBSE Extraction
All used glassware was washed with distilled water and acetone, and dried at 80 °C. Volatile and semivolatile compounds were extracted from 1.0 ml of undiluted or diluted urine by sorptive extraction with a Twister™ PDMS polymer coated stirbar (10 mm, 0.5 mm film thickness, 24 µl PDMS volume, Gerstel GmbH, Mülheim an der Ruhr, Germany) for 60 minutes. Stirring speed was 800+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnic, Oberschleissheim, Germany). After extraction, stirbars were rinsed with a small amount of distilled water, dried gently on the paper tissue and placed in the glass injector liner for mass-spectrometric (MS) identification, or in the TDSA autosampler tube for the gas chromatographic (GC) quantitative analysis. Standard compounds were obtained from Aldrich Chemical Company (Milwaukee, WI).

2.2 Gas Chromatography
Gas chromatographic equipment for the quantitative analysis consisted of an Agilent GC Model 6890 with an Atomic Emission Detector (AED) Model G2350A (Agilent Technologies Inc., Wilmington, DE) and a Thermal Desorption Autosampler (TDSA, Gerstel GmbH, Mülheim an der Ruhr, Germany). The separation capillary was DB-5 (30 m x 0.25 mm, i.d., from J&W Scientific, Folsom, CA). Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection system CIS-4. TDSA operated in a splitless mode. Temperature program for desorption was 20 °C (0.5 min), then 60 °C/min to 280 °C (10 min). Temperature of the transfer line was set at 280 °C. CIS was cooled with liquid nitrogen to -60 °C. After desorption and cryotrapping, CIS was heated at 12 °C/s to 280 °C with the hold time of 10 min. Temperature program in the GC was 40 °C for 5 minutes, then increasing to 200 °C at the rate of 2 °C/min. The final temperature was held for 10 minutes. Carrier gas head pressure was 14 psi (flow rate, 3.9 ml/min). The GC unit was operated in the constant flow mode. The emission
lines for carbon (193 nm), sulfur (181 nm) and nitrogen (174 nm) were monitored in the atomic plasma emission detection.

2.3 Capillary Gas Chromatography-Mass Spectrometry
Finnigan MAT Magnum ion trap GC-MS system was used for the compound identification (Finnigan MAT, San Jose, CA). The system was provided with a DB-5 capillary column (30 m x 0.25 mm, i.d., J&W Scientific, Folsom, CA). Helium carrier gas head pressure was 12 psi. At the beginning of the column, a loop of uncoated deactivated silica tubing (30 cm x 0.25 mm, i.d.) was attached using an universal Press-Tight Connector (Restek Corporation, Bellefonte, PA) as described earlier [4]. The loop was cooled at liquid nitrogen while the Twister™ stirbar was held in the injector liner for 15 minutes at 250 °C for the thermal desorption of the analytes. Subsequently, the desorbed compounds were cryotrapped into the liquid nitrogen cooled loop. After removing liquid nitrogen cooling, the GC temperature was held at 40 °C for 5 minutes and increased to 200 °C at the rate of 2 °C/min. The final temperature was held for 10 minutes. The manifold and transfer line temperatures were 220°C and 300 °C, respectively. The ion trap was operating in the positive electron ionization mode. Spectra were scanned from 40 to 350 msu (1 scan/s).

3 Results
3.1 Chromatographic Profiles
Gas-chromatographic profiles of the extracted compounds were compared with the GC-MS profiles while the peak identities were established based on retention times, mass spectra and comparisons with the known standard compounds. An urinary volatile compound profile from a pooled male Campbelli hamster urine is shown in Figure 1. The numbers indicate a compound identity (see Table I). Male hamster urine characteristically contained a number of partially identified pyrazines, indicated as “p”. Their strong endocrine dependency (data not shown) suggested that alkylated pyrazines are putative chemosignals for this hamster species. Work is underway for a complete characterization of the individual pyrazine compounds. The peaks marked as “s” were verified as sulfur compounds with a GC-AED. Their levels were too low for mass-spectral characterization.

3.2. Reproducibility and Quantitative Analysis
Partition into the PDMS phase can be estimated through water-octanol partition coefficients (EpiSuite™). Using the phase ratios of 24 µl PDMS/10 ml water, the theoretical recoveries could be calculated. Relative standard deviations of the peak areas for seven compounds differing in their theoretical extraction efficiencies into the PDMS phase ranged from 3 to 88 %, as shown in Table I. Relative standard deviations varied between 1-5 % (n=4). Hydrophobic compounds (eg., geraniol) with high theoretical recoveries did not show improved extraction reproducibility compared with
more hydrophilic compounds (e.g., 2,5-dimethylpyrazine). In conclusion, quantitative reproducibility of the SBSE extraction of volatiles from hamster urine was relatively constant over a respectable range of hydrophilic and hydrophobic compounds.

![Chromatogram of the SBSE extracted and thermally desorbed compounds from male hamster urine (GC-AED, carbon emission, 193 nm). The numbers indicate quantitatively measured compounds. “s” = sulfur compounds verified on sulfur emission (181 nm); “p”= partially identified pyrazines by GC-MS; Sil= siloxanes from a stir bar; IS = an internal standard, 7-tridecanone.](image)

**Figure 1.** Chromatogram of the SBSE extracted and thermally desorbed compounds from male hamster urine (GC-AED, carbon emission, 193 nm). The numbers indicate quantitatively measured compounds. “s” = sulfur compounds verified on sulfur emission (181 nm); “p”= partially identified pyrazines by GC-MS; Sil= siloxanes from a stir bar; IS = an internal standard, 7-tridecanone.

**Table I** Reproducibility of SBSE extraction for selected compounds in a male hamster (*Phodopus campbelli*) pooled urine samples.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Compound</th>
<th>*log K&lt;sub&gt;ow&lt;/sub&gt;</th>
<th>Peak area (average, n=4)</th>
<th>SD</th>
<th>RSD %</th>
<th>Theoretical recovery %</th>
<th>Quantitatively detected ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-heptanone</td>
<td>1.73</td>
<td>3399</td>
<td>116</td>
<td>3.41</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>2-heptanone</td>
<td>1.73</td>
<td>1221</td>
<td>28</td>
<td>2.30</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>2,5-dimethylpyrazine</td>
<td>1.03</td>
<td>3652</td>
<td>86</td>
<td>2.35</td>
<td>3</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>4-nonanone</td>
<td>2.71</td>
<td>3492</td>
<td>25</td>
<td>0.72</td>
<td>57</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>2-nonanol</td>
<td>3.22</td>
<td>3774</td>
<td>111</td>
<td>2.95</td>
<td>80</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>geraniol</td>
<td>3.47</td>
<td>1682</td>
<td>81</td>
<td>4.80</td>
<td>88</td>
<td>69</td>
</tr>
<tr>
<td>7</td>
<td>Vitamin K&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.21</td>
<td>9522</td>
<td>526</td>
<td>5.52</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>

*EPISuite™ v. 3.10 from 2000 U.S. Environmental Protection Agency, K<sub>ow</sub> is a partition coefficient between*
water and octanol.

3.3. Effect of Dilution in Mouse Urine

Mouse urine contains relatively high concentration (1-5 mg/ml) of major urinary proteins, MUPs [8]. Several mouse pheromones are known to bind strongly inside the protein hydrophobic cavity [9]. We have investigated whether a dilution of mouse urine with water would change extractability of the mouse urinary constituents. Figure 2 shows a chromatogram of the extracted sulfur compounds from a pooled male mouse urine after a 50:50 dilution with water. Dilutions of 500 µl, 250 µl and 100 µl of urine to 1 ml total volume with water decreased almost linearly the peak areas of selected sulfur compounds (data not shown). Detectability of about 0.2 ng of dimethyldisulfide and bis(methylthio)methane was achieved with the SBSE method using 100 µl of mouse urine.

![Figure 2. Chromatogram of the SBSE-extracted and thermally desorbed compounds from male mouse urine (GC-AED, sulfur emission, 181 nm). 1 = dimethyldisulfide; 2 = bis(methylthio)methane; 3 = 2-sec-butyl-4,5-dihydrothiazole; 4 = benzothiazole; X =sulfur compounds from blank.](image-url)
References


