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Abstract: Occupational Exposure to Aromatic Hydrocarbons at a Coke Plant: Part I. Identification of Hydrocarbons in Air and their Metabolites in Urine by a Gas Chromatography-Mass Spectrometry Method: Grażyna Bieniek, et al. Department of Molecular Biology, Biochemistry and Biopharmacy, Faculty of Pharmacy, Medical University of Silesia, Poland—A method for the qualitative analysis of aromatic hydrocarbons in air and their various urinary metabolites is presented. The air was sampled in charcoal tubes and extracted with carbon disulfide. The hydrocarbons were identified as being aliphatic hydrocarbons (C$_9$–C$_{19}$), aromatic hydrocarbons and heterocyclic compounds. The urinary metabolites after enzymatic hydrolysis were analyzed by solid-phase extraction with a styrene-divinylbenzene resin, silylation with N,O-bis(trimethylsilyl)acetamide and GC/MS for separation and detection. Satisfactory separation of all compounds investigated was achieved without interference due to matrix peaks. The following compounds were identified in the urine of workers: dimethylphenol isomers, 4-ethyl-1,3-benzenediol, 2-ethoxybenzoic acid and methoxyphenols. Trimethylsilyl derivatives of aromatic hydroxyacids and hydroxymethoxyacids were found in the urine of occupationally exposed workers by means of a silylation procedure.

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Key words: GC/MS analysis, Mixed exposure, Aromatic hydrocarbons, Urinary metabolites

Earlier investigations$^{13}$ of the occupational exposure of coke plant workers to aromatic hydrocarbons allowed us to determine the following compounds in the breathing zone air: benzene, toluene, xylene isomers, naphthalene, polycyclic hydrocarbons and their metabolites in urine: 2-methylphenol, naphthols and m+p-methylhippuric acids. The present study is focused on the identification of different compounds not detected earlier and on the complexity of the exposure.

In environmental and toxicological analyses, chromatographic methods, such as gas-chromatography (GC) or high-performance liquid chromatography (HPLC), play a major role in the detection of chemicals in many materials such as: air$^{2–4}$, cigarette smoke$^5$, blood samples$^6$ and urine$^2, 7–11$. Hoshika and Murayama$^{12}$ performed a simultaneous analysis of lower fatty acids, phenols and indoles in feces. Concentrations of aromatics in air were determined gas chromatographically with flame ionization detection (GC/FID) for passive personal air samples$^{13, 14}$. GC, in combination with mass spectrometry (MS), is by far a more sensitive and selective method than GC/FID, but is more expensive. In effect, the application of GC/MS for environmental analysis has caused a substantial improvement in the quality, and reliability of the identification of organic compounds.

In this study, the application of the GC/MS method to the separation and identification of hydrocarbons in air and their metabolites in the urine of coke plant workers, is presented. The sample preparation procedure was optimized to obtain high quality analytical results.

Methods and Materials

Subjects

Measurements were carried out in a coke plant situated in the industrial area of Poland. The air and urine samples were collected on the same dayshift (from 6:00 a.m. to
Collective urine was divided into 7 identical samples, 10 hydrocarbons were analyzed by GC/MS. Aromatic disulfide in an ultrasonic bath for 15 min. Aromatic hydrocarbons were achieved with an HP 5890 gas chromatograph equipped with a flame-ionization detector. Eighty-Four, PA) and low-flow sampling pump (type AFC Four, PA, USA). Distilled water was used in all analyses. Air sampling Air samples were collected in the breathing zone of workers during the work shift. The sampling system was composed of the charcoal tube (cat no. 226–01, SKC Inc., Phillipsburg, NJ, USA). Charcoal tubes (100 mg/50 mg), (226–01) were purchased from SKC (Eighty Four, PA, USA). Distilled water was used in all analyses. Procedure for SPE The extraction was followed by using a column processing system. The SPE column was preactivated with 6 ml of methanol and 8 ml of distilled water. 10 ml of hydrolyzed sample was loaded onto SPE column and slowly aspirated. The column was washed with 6 ml of distilled water and gently dried by aspiration for 30 minutes. Urinary metabolites were eluted with 1 ml of methanol. The elute was then collected in a 1.8 ml GC vial. The vial was capped and kept at 0°C being analyzed to avoid evaporation. A 2 µl aliquot of sample was injected to GC/MS. Derivatization Methanol extract obtained in the SPE-procedure was evaporated to dryness under a N2 stream and the residue was silylated with N,O-bis(trimethylsilyl)acetamide (25 µl of β-glucuronidase/arylsulfatase at 37°C for 20 h. After cooling to room temperature, samples were loaded onto SPE columns. Gas chromatographic-mass spectrometric identification Identification of the chemical compounds was performed with a Hewlett-Packard gas chromatograph 5890 II (Palo Alto, CA USA) equipped with a HP 5989A MS Engine and HP ChemStation computer system with the NIST/EPA/NICHD Mass Spectral Database. The electron-impact ion source, the quadrupole and GC/MS interface were heated to 176°C, 100°C, and 250°C, respectively. The ionization energy was equal to 70 eV. Prior to MS analysis, separation of the compounds in air samples was performed on a capillary column type Ultra 2 (0.32 mm × 0.17 µm × 50 m). For urine samples, columns type HP-1 MS (0.32 mm × 0.25 µm × 60 m) and HP-5 MS (0.25 mm × 0.25 µm × 60 m) was used. Helium (100 kPa) was used as a carrier gas. Conditions not occupationally exposed. Urinary metabolites were identified by GC/MS after enzymatic hydrolysis7,16, solid-phase extraction on styrene-divinylbenzene resin and after a derivatization procedure17. The GC/FID analyses of 1- naphthol, 2-naphthol, 2-methylphenol, 4-methylphenol, 2,4- and 2,5-dimethylphenol, 3,4-dimethylphenol and 3,5-dimethylphenol were carried out as previously described10. The concentrations of metabolites in urine were corrected against creatinine.

Sample preparation Urine samples were kept at 20°C until analysis. 10 ml of urine was adjusted to pH 5 with 1.0 M HCl, buffered with 6 ml 0.1 M pH 5 acetate buffer and hydrolyzed with 25 µl of β-glucuronidase/arylsulfatase at 37°C for 20 h. After cooling to room temperature, samples were loaded onto SPE columns.

Reagents

All chemicals were of reagent grade quality or better and were used as received without further purification. β-glucuronidase/arylsulfatase from Helix Pomatia containing about 30 U/ml of β-glucuronidase (EC 3.2.1.31) and about 60 U/ml of sulfatase (EC 3.1.6.1) was obtained from Merck (Darmstadt, Germany). Carbon disulfide 99.9+% (Cas registry No 75–15–0) redistilled, industrial hygiene analysis grade, Pyridine 99.9+% (110–86–1) HPLC grade was obtained from Aldrich (Milwaukee, WI, USA). Concentrated hydrochloric acid and 0.1 M (pH 5) acetate buffer were obtained from POCH (Gliwice, Poland). Methyl alcohol 99.9+% (67–56–1) HPLC grade was obtained from Riedel-de Haen (Seelze, Germany). Bakerbond SPE columns packed with styrene-divinylbenzene copolymer (3 ml, 200 mg) (7619–02) were from Baker B. V. (Deventer, The Netherlands). The Baker spe-12G SPE column processing system was from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Charcoal tubes (100 mg/50 mg), (226–01) were purchased from SKC (Eighty Four, PA, USA). Distilled water was used in all analyses.

Air sampling

Air samples were collected in the breathing zone of workers during the work shift. The sampling system was composed of the charcoal tube (cat.no.226–01, SKC Inc., Eighty-Four, PA) and low-flow sampling pump (type AFC 123 Casella Ltd, London). The sampling flow rate was 0.5 l/min for 6 h (180 l). The analytes were released from the charcoal by treatment with 1 ml of carbon disulfide in an ultrasonic bath for 15 min. Aromatic hydrocarbons were analyzed by GC/MS.

Gas chromatographic separation of aromatic hydrocarbons was achieved with an HP 5890 gas chromatograph equipped with a flame-ionization detector under conditions previously described in a paper by Bieniek16.

Urinalysis

5 ml urine samples were collected after the work shift from 15 workers occupationally exposed, and than mixed together to obtain 75 ml of the collective urine sample. Collective urine was divided into 7 identical samples, 10 ml each, in order to test different conditions of the chromatographic analysis and find the optimal separation. The same procedure was applied to the urine of 10 subjects not occupationally exposed. Urinary metabolites were identified by GC/MS after enzymatic hydrolysis7,16, solid-phase extraction on styrene-divinylbenzene resin and after a derivatization procedure17. The GC/FID analyses of 1- naphthol, 2-naphthol, 2-methylphenol, 4-methylphenol, 2,4- and 2,5-dimethylphenol, 3,4-dimethylphenol and 3,5-dimethylphenol were carried out as previously described10. The concentrations of metabolites in urine were corrected against creatinine.

Sample preparation

Urine samples were kept at 20°C until analysis. 10 ml of urine was adjusted to pH 5 with 1.0 M HCl, buffered with 6 ml 0.1 M pH 5 acetate buffer and hydrolyzed with 25 µl of β-glucuronidase/arylsulfatase at 37°C for 20 h. After cooling to room temperature, samples were loaded onto SPE columns.

Procedure for SPE

The extraction was followed by using a column processing system. The SPE column was preactivated with 6 ml of methanol and 8 ml of distilled water. 10 ml of hydrolyzed sample was loaded onto SPE column and slowly aspirated. The column was washed with 6 ml of distilled water and gently dried by aspiration for 30 minutes. Urinary metabolites were eluted with 1 ml of methanol. The elute was then collected in a 1.8 ml GC vial. The vial was capped and kept at 0°C being analyzed to avoid evaporation. A 2 µl aliquot of sample was injected to GC/MS.

Derivatization

Methanol extract obtained in the SPE-procedure was evaporated to dryness under a N2 stream and the residue was silylated with N,O-bis(trimethylsilyl)acetamide (0.2 ml) and pyridine (0.2 ml). The reaction mixture was heated at 60°C for 1 h and after cooling to room temperature was used for analysis by GC/MS.

Gas chromatographic-mass spectrometric identification

Identification of the chemical compounds was performed with a Hewlett-Packard gas chromatograph 5890 II (Palo Alto, CA USA) equipped with a HP 5989A MS Engine and HP ChemStation computer system with the NIST/EPA/NIH Mass Spectral Database. The electron-impact ion source, the quadrupole and GC/MS interface were heated to 176°C, 100°C, and 250°C, respectively. The ionization energy was equal to 70 eV. Prior to MS analysis, separation of the compounds in air samples was performed on a capillary column type Ultra 2 (0.32 mm × 0.17 µm × 50 m). For urine samples, columns type HP-1 MS (0.32 mm × 0.25 µm × 60 m) and HP-5 MS (0.25 mm × 0.25 µm × 60 m) was used. Helium (100 kPa) was used as a carrier gas. Conditions
The GC/MS analyses are shown in Table 1. Compounds shown in Figs 1–3 were identified by comparing their spectra with the spectra in the NIST/EPA/NIH mass spectral database, stored in the HP ChemStation and with a match quality parameter >90. GC/MS analyses were performed according to the standard specifications, which include electron impact ionization- 50 pg of hexachlorobenzene yields a signal-to-noise ratio of 20:1 rms (4:1 peak-to-peak) at the molecular ion m/z 283.8 when scanned at 350 amu/second between m/z 60 and m/z 310.

Results

A typical GC-MS chromatogram of air samples collected in the breathing zone of operators at the time of the naphthalene oil distillation process is shown in Fig. 1. The hydrocarbons are identified as aliphatic hydrocarbons (C9–C19), benzene and naphthalene homologues, heterocyclic compounds and other aromatic hydrocarbons. This result confirms the mixed exposure to aromatic hydrocarbons at the coke plant. GC/MS conditions are shown in Table 1 as Method 1.

Only benzene, toluene, 1,2-dimethylbenzene, 1,3- and 1,4-dimethylbenzene and naphthalene have been quantitatively determined. The time weighted average concentrations of aromatic hydrocarbons in the breathing-zone air vary in range: 86.2–563.7 ppb for benzene, 60.4–483.5 ppb for toluene, 36.2–235.6 ppb for 1,2-dimethylbenzene, 80.4–436.7 ppb for 1,3- and 1,4-dimethylbenzene and 120.5–828.4 ppb for naphthalene. For benzene, toluene, o-xylene, m+p-xylene and naphthalene the detection limits are in the range 0.47–1.02 ppb.

Figure 2 shows a typical chromatogram obtained for the urine samples subjected to the enzymatic hydrolysis and solid-phase extraction without using styrene-divinylbenzene resin. GC/MS conditions are collected in Table 1 as Method 2. The following compounds have been identified in urine of occupationally exposed workers: 4-ethyl-1,3-benzenediol, dimethylphenol isomers, 2-ethoxybenzoic acid and methoxyphenols (Fig. 2a). In urine of non-exposed subjects but smokers the 2,2′-methylenebis[6-(1,1-dimethylthyl)-4-methylphenol] and bis(2-ethylhexyl)phthalate have been found (Fig. 2b). Using an extract ion chromatogram method, we were able to identify the 4-methyl-1,2-benzenediol (m/z 124, 132, 177, and 209).

Table 1. Three methods of GC/MS analysis. Conditions of experiment for CS2 extract (1), SPE-extract (2) and trimethylsilyl derivatives of hydroxyacids (3) samples. Compound were separated: I-a capillary column HP Ultra 2 (0.32 mm × 0.17 μm × 50 m), II-HP-5 MS (0.25 mm × 0.25 μm × 60 m), III-HP-1 MS (0.32 mm × 0.25 μm × 60 m).

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample</th>
<th>Column type</th>
<th>Sampling conditions</th>
<th>Spitless time [s]</th>
<th>Temperature programme conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CS2 extract</td>
<td>I</td>
<td>250</td>
<td>30</td>
<td>35°C for 1 min, increase by 3°C/min to 80°C, increase by 5°C/min to 150°C and hold for 3 min, and then increase by 20°C/min to 240°C and hold for 5 min</td>
</tr>
<tr>
<td>2</td>
<td>SPE-extract</td>
<td>II, III</td>
<td>270</td>
<td>12</td>
<td>50°C for 1 min, increase by 3°C/min to 150°C and increase by 10°C/min to 290°C, and hold for 20 min</td>
</tr>
<tr>
<td>3</td>
<td>TMS derivative</td>
<td>II</td>
<td>270</td>
<td>12</td>
<td>130°C for 1 min, increase by 2°C/min to 200°C, and increase by 10°C/min to 290°C, and hold for 15 min</td>
</tr>
</tbody>
</table>

Fig. 1. Gas chromatogram of the air sample after charcoal enrichment.

Numbered peaks were identified as follows:
1 = benzene, 2 = toluene, 3 = 1,1,3-trimethylcyclohexane, 4 = ethylbenzene, 5 = 1,3- and 1,4-dimethylbenzene, 6 = styrene, 7 = 1,2-dimethylbenzene, 8 = nonane, 9 and 14 = ethylmethylbenzene isomers, 10 and 11 = trimethylbenzene isomers, 12 = benzofuran, 13 = decane, 15 = indan, 16 = indene, 17 = undecane, 18 = 2,3-dihydro-4-methylindene, 19 = 2,3-dihydro-1-methylindene, 20 = naphthalene, 21 = benzo[b]thiophene, 22 = dodecane, 23 = 1-methylnaphthalene, 24 = 2-methylnaphthalene, 25 = biphenyl, 26 = 28 = dimethylnaphthalene isomers, 29 =acenaphthene, 30 = pentadecane, 31 = dibenzofuran, 32 = fluorene, 33 = hexadecane, 34 = heptadecane, 35 = nonadecane, 36 = octadecane, 37 = nonadecane.
78) with a retention time of 31.311 min. The observed differences in retention times result from the different types of capillary columns.

Concentrations of urine metabolites expressed in units µmol/mol creatinine vary in the ranges: 40.6–486.4 for 1-naphthol, 30.4–376.5 for 2-naphthol, 12.6–87.3 for 2-methylphenol, 22.4–56.8 for 2,4- and 2,5-dimethylphenol, 14.6–40.6 for 3,4-dimethylphenol and 18.4–28.6 for 3,5-dimethylphenol. For urinary phenolic compounds detection limits are in the range 0.09–0.13 µmol/mol creatinine.

The GC/MS analysis of the compounds adsorbed on styrene - divinylbenzene resin from hydrolyzed urine reveals the presence of different metabolites. GC conditions are shown in Table 1 as Method 3. A gas -
chromatographic separation of the methyl estertrimethylsilyl derivatives of the acidic metabolites of hydrocarbons isolated from urine is shown in Fig. 3. The following compounds have been found in the urine of occupationally exposed workers: naphthol isomers, benzenedicarboxylic acid, hydroxy- and dihydroxybenzoic acid and hydroxybenzenepropanoic acid (Fig. 3a). In the urine of non-exposed subjects the abovementioned compounds have not been identified, whereas peaks from the 3-hydroxybenzeneacetic acid, 3-methoxy-4-hydroxybenzeneacetic acid, citric and hippuric acids are shown in Fig. 3b. Separation of N-methylbenzamide at a retention time of 47 min (match quality 80–87) did not succeed.

Discussion

The time weighted average concentrations of aromatic hydrocarbons in the breathing zone air showed that the exposure level of the coke plant workers was rather low\(^{15}\), but the GC/MS analysis of the compounds adsorbed from air on active carbon revealed the presence of many different compounds not determined earlier in literature, e.g.: 1,1,3-trimethylcyclohexane, benzoeturan, dibenzofuran and biphenyl. The specific setting of the chromatography parameters presented in Table 1, allowed us to separate and identify 37 compounds. These GC/MS conditions can be useful for environmental and toxicological studies of hydrocarbons in air. Benzene homologues, biphenyl, benzoeturan, dibenzofuran and aliphatic hydrocarbons were identified. The results obtained confirm the presence of mixed exposure to hydrocarbons of the coke plant workers and can be useful for future investigations regarding the assessment of occupational exposure.

According to the earlier literature data, phenols\(^{18}\) and naphthols\(^{19}\) were determined quantitatively in the urine of coke plant workers. In our study, we made an effort to identify compounds not detected earlier. In our experiment, a four-step process consisting of hydrolysis, extraction, evaporation and derivation was developed and applied to the isolation of urinary metabolites. Methods for conjugate hydrolysis and ether extraction of simple phenols in urine was described by Dirmikis and Darbre\(^{7}\). Application of the styrene-divinylbenzene resin dimethylphenol isomers allowed us to identify in urine the following compounds: 4-ethyl-1,3-benzenediol, 2-ethoxybenzoic acid and methoxyphenols. Trimethylsilyl derivatives of aromatic hydroxyacids and hydroxymethoxyacids were found in the urine of occupationally exposed workers (Fig. 3a) but they were not identified in the urine of non-exposed subjects (Fig. 3b). In the urine of the non-exposed subjects, 3-hydroxybenzeneacetic acid, 3-methoxy-4-hydroxybenzeneacetic acid, citric and hippuric acids, were found, possibly because of the metabolic process, as well as because of environmental exposure.

Conclusions

The GC/MS analysis is useful for the environmental and toxicological studies. Operators at the time of the tar and naphthalene oil distillation processes are simultaneously exposed to the mixture of different aromatic hydrocarbons. GC/MS separation after enzymatic hydrolysis and solid-phase extraction with a styrene-divinylbenzene resin dimethylphenol isomers allowed us to identify in urine the following compounds: 4-ethyl-1,3-benzenediol, 2-ethoxybenzoic acid and methoxyphenols. Trimethylsilyl derivatives of aromatic hydroxyacids and hydroxymethoxyacids were found in the urine of occupationally exposed workers by using the silylation procedure.

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