Evaluation of Aromatic Compounds by Derivatization Method with GC-MS Analysis in Smokers' Urine

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Abstract

Cigarette smoking is linked to a higher risk of cancer, cardiovascular disease, and early mortality. Aromatic amines (AAs) present in cigarette smoke, such as P-toluidine, 1-aminonaphthalene, and 4-aminobiphenyl, are well-known human bladder carcinogens, and can be tested as exposure biomarkers. Using gas chromatography combined with mass spectrometry and using a new locally manufactured pre-concentration method (evaporator system), many aromatic compounds were diagnosed in the urine of smokers, and perhaps the most important of them are substances that are related to bladder cancer in smokers (as P-toluidine, 1-aminonaphthalene). The sample evaporation system contributed to reducing the sample size to be measured to several times without prejudice to the composition of the analyte. This decrease in the sample size led to an increase in the sensitivity of the method, since the analytes are present in trace concentrations. More than 10 aromatic compounds were diagnosed. The results were compared depending on several factors, including age, smoking period, and the number of cigarettes consumed per day. The fluctuation of the appearance of aromatic compounds in the samples was discussed.

Keywords: Human urine, Aromatic compounds, Derivatization methods, GC/MS

1. Introduction

Aromatic amines are polar chemical compounds formed by attaching nitrogen atoms to the aromatic ring structure of an amine. Anilines range from extremely simple compounds with conjugated aromatic or heterocyclic structures and a large number of substituents to highly complicated molecules with conjugated aromatic or heterocyclic structures and a large number of substituents [1]. Aromatic amines are organic nitrogen compounds that are ammonia derivatives with an aryl group replacing at least one hydrogen atom. The nitrogen must be directly attached to the aromatic ring in order to engage with the aromatic p-electron system. [2] Aniline, toluidines, and other aromatic amines have been found in both indoor and outdoor air recently. [3] Significant amounts of aromatic amines are flushed into household wastewater while washing colored hair. Furthermore, a little amount of aromatic amines deposited on the scalp may irritate the skin. Some of them pass through the skin and are immediately excreted in the urine, while
the rest is carried to other organs through secondary deposition and digested over time. Aromatic amine exposure in humans, on the other hand, is little known. This is due in part to the difficulties in finding suitable measurement techniques for aromatic amines and their metabolites in water. [4] In tobacco smoke, many chemical carcinogens, such as polycyclic aromatic hydrocarbons and aromatic amines, are thought to be significant carcinogens. Aromatic amine 2-aminonaphthalene (2-AN) 4-aminobiphenyl (4-ABP) and 4-aminobiphenyl (4-ABP) are two different types of 4-aminobiphenyl. Cigarette smoke has been discovered in public places [5]. That may bind to tissue macromolecules (proteins) covalently and Aromatic amines' involvement in the genesis of some human malignancies has been well established, and laboratory investigations have shown plausible metabolic activation and detoxification pathways [6]. Smoking is a well-known cause of chronic bronchitis, heart disease, and a variety of cancers, including bladder cancer[7]. Cigarette smoking is a leading cause of lung cancer, as well as malignancies of the bladder, throat, mouth, stomach, liver, and gallbladder, among other human organs. [8]. Although quitting smoking has been suggested as a way to reduce the risk, there is no evidence that the increased risk remains over time [9]. Human milk has also been shown to contain aniline and o-toluidine[10]. The first attempts to test aromatic amines were made in the 1960s. Hoffmann and Masuda utilized gas chromatography to determine 1-naphthylamine and 2-naphthylamine[11]. Discharge of these amines into water or the atmosphere, on the other hand, is considered an environmental contaminant [12]. During the smoking process, aromatic amines travel through the bloodstream, where they are filtered out and discharged into the environment. Because the bladder retains urine, aromatic amines are likely to come into touch with bladder cells, where they will react and potentially induce cancer cell development [13]. Several studies have been conducted to identify the primary aromatic amines and their metabolites in biological materials such as plasma, urine, and hepatic tissue in order to evaluate people's exposure to aromatic amines. Urine analysis, on the other hand, is the most practical technique since it allows for the collection of huge numbers of samples without being invasive. [14]. Teass et al. discovered indicators of occupational exposure to aromatic amines in 1993[15]. For the evaluation of o-toluidine and aniline in worker urine specimens, Riedel et al utilize gas chromatography mass spectrometry (GC-MS) to evaluate o-toluidine, 2-aminonaphthalene, and 4-aminobiphenyl for smokers and nonsmokers by derivatization with pentafluoropropionic anhydride (PFPA) [16]. Grimmer et al detected 1-Amino-naphthalene, 2-Aminonaphthalene, 2-Amino-biphenyl, and 4-Amino-biphenyl in smokers and nonsmokers samples using GC-MS and derivatization with pentafluoropropionyl-imidazol(PFPI) [5]. Weiss 2002 determined Nineteen aromatic amines in smokers and nonsmokers using GC MS/MS and derivatization with PFPA [17]. Then, in 2011, Seyler and Bernert used GC-MS/MS with PFPA derivatization to identify 4-amino-biphenyl in the urine of smokers and nonsmokers[18]. Yu and his colleagues 1-Aminonaphthalene was determined using MIPs-SPE in combination with LC–MS/MS without derivatization. 2-Amino-naphthalene 3-Amino-biphenyl 4-Amino-biphenyl in smokers' and nonsmokers' urine samples [19]. Mazumder et al. 2019 examined the presence of ortho-toluidine, ortho-anisidine, 2,6-dimethylaniline, 1-aminonaphthalene, 2-aminonaphthalene, and 4-aminobiphenyl in smoker and nonsmoker urine samples using GC MS/MS and PFPA[20].The significance of this study is clear in analyzing the sorts of aromatic amines to which people were exposed within the study area because the effect is not confined to smoking cigarettes alone, but
also includes dyes, uncontrolled fuel combustion byproducts, and other environmental pollutants. The significance of this study is in identifying the types of malignant infections to which people in this region have been exposed, as well as determining some causes of cancerous infections and their

2. Experimental part

2.1 Materials

Hydrochloric acid (37%), sodium hydroxide (96.0%), toluene (98%) BDH, and pyridine (99.8%) n – hexane 97 percent, Sigma-Aldrich Honeywell Germany is a company based in Germany. 99 percent pentafluoropropionic anhydride GEEKEE BIO CHINA .

2.2 Gas chromatography mass spectrometry

GC-MS analysis was performed at the Basra Oil Company Laboratory using an Agilent Technologies 7890B GC system USA coupled to an Agilent Technologies 5977A MSD with EI Signal detector, using HP-5ms 5 percent phenyl, 95 percent methyl siloxane (30m*250µm*0.25), the oven temperature was set at 40 °C for 5 minutes, then raised to 10 °C /min to 300 °C for 20 minutes, and the Helium carrier gas flow rate was set. The injection mode was pulsed Splitless, with an injection temperature of 290 °C and a 1 micro letter injection sample volume. The mass spectrometer had a 230 °C Ion Source, a 1562 (N2) scan speed, and a mass range of 44-750 m/z. To identify of substances, the data was run via the NIST 2014 and Wiley 9 Library databases.

2.3 Sample evaporator

The homemade evaporator sample system was manufactured, and its parts collected from a local lab. The manifold system evaporator made by concocting a closed vial and plastic stopper has two holes, one to inlet nitrogen gas and other to inlet needle of the sample syringe. Nitrogen gas flows through the evaporation path and outlets from many holes in the upper to release air. Fig 1 shows the homemade evaporator sample system.
2.4 Acidic hydrolysis of urine sample

The method described by (Weiss 2002)[17] Each 5-ml of urine sample was mixed with 1 ml of 37 percent concentrated hydrochloric acid. To hydrolyze acid-labile conjugates, heat the mixture for 1 hour at 80°C. The urine samples were given 0.60 mL of 10 M NaOH, 3 mL of phosphate buffer, and another 0.55 µl of 10 M sodium hydroxide after chilling in an ice bath. The pH of mixture should be (6.0 - 6.4). The pH of the urine samples was corrected with modest volumes of concentrated acetic acid or 10 M sodium hydroxide if required, and the layers were separated using centrifugation at 2500 rpm for 10 minutes during two extractions with 5 ml n-hexane. Figure 2 shows the acidic hydrolysis of a urine sample.
Fig2. The schematic diagram Acidic hydrolysis for urine sample of urin sample prepration  

A. 5ml of Urine  
B. addition of 1ml of 10 M HCl  
C. water bath at 80 C° 1 hour  
D&E. controlling pH to ( 6.0 - 6.4 ) and Extraction with 5ml of hexane 2time by centrifuge

2.5 Derivatization with PFPA

The layers were separated using n-hexane and centrifugation at 2500 g for 10 minutes. 25 µl of pyridine and 50 µl of pentafluoropropionic anhydride were added to the mixed organic layer. The vials were derivatized by heating them for 1 hour at 80°C in a water bath. The samples were cooled to room temperature to remove any excess pentafluoropropionic anhydride. before being extracted once with 3 ml phosphate buffer (pH 8) for 5 minutes on a laboratory mixer. After that, the layers were separated for 10 minutes using a 2500g centrifuge. The organic layers was poured into a 20-ml vial containing 200 µL toluene,. which was then evaporated using a moderate nitrogen stream to 150µl using evaporator system for quantitative gas (GC–MS) analysis, the residue was put in a micro insert and evaporated to a volume of 40 µl. The sample derivatization method is summarized in Figure 3.
3. Results and Discussion

After conducting the acidolysis process and the derivation process for thirty samples of the urine of smokers and measuring them using gas chromatography coupled with mass spectrometry, a large group of aromatic compounds were obtained and the materials with more appearance times were selected. These aromatic compounds are summarized in Table 1.

Table 1: The aromatic substances observed in the samples and their information

<table>
<thead>
<tr>
<th>NO.</th>
<th>Name</th>
<th>Symbol</th>
<th>m/z</th>
<th>( t_R )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters</td>
<td>M1</td>
<td>191.1</td>
<td>16.9</td>
</tr>
<tr>
<td>2</td>
<td>Ethylbenzene</td>
<td>M2</td>
<td>91.2</td>
<td>5.898</td>
</tr>
<tr>
<td>3</td>
<td>Benzene, 1,1’-(1,2-cyclobutanediyl) bis-, trans-</td>
<td>M3</td>
<td>104.1</td>
<td>19.608</td>
</tr>
<tr>
<td>No.</td>
<td>Compound Description</td>
<td>Formula</td>
<td>Mass (M)</td>
<td>Rf Value</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------------------------------------</td>
<td>---------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>4</td>
<td>Terephthalic acid, piperidide, ethyl ester</td>
<td>M4</td>
<td>127.1</td>
<td>21.869</td>
</tr>
<tr>
<td>5</td>
<td>4a-Methyl-1,2,4a,5,8,8ahexahydronaphthalene</td>
<td>M5</td>
<td>67.1</td>
<td>22.879</td>
</tr>
<tr>
<td>6</td>
<td>1-Cyano-4-(5-hexenyl) benzene</td>
<td>M6</td>
<td>129.1</td>
<td>26.389</td>
</tr>
<tr>
<td>7</td>
<td>3-Iodo-4-methoxybenzylalcohol, pentafluoropropionate</td>
<td>M7</td>
<td>247.2</td>
<td>28.59</td>
</tr>
<tr>
<td>8</td>
<td>(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans</td>
<td>M8</td>
<td>91.1</td>
<td>26.879</td>
</tr>
<tr>
<td>9</td>
<td>Phenol, 2,2’-Methylenebis [6-(1,1-dimethylethyl)-4-methyl</td>
<td>M9</td>
<td>177.1</td>
<td>25.765</td>
</tr>
<tr>
<td>10</td>
<td>1-Naphthalenamine</td>
<td>M10</td>
<td>143.1</td>
<td>17.381</td>
</tr>
<tr>
<td>11</td>
<td>P-aminotoluene</td>
<td>M11</td>
<td>106.1</td>
<td>11.496</td>
</tr>
</tbody>
</table>

Fig4 shows the distribution of these aromatic substances on the spectrum of a gas chromatograph.
After conducting a study of the results of the tests, each substance was distributed according to its repetition in the samples, and the materials with the most repetitions were selected according to the Table 2. The research found that the two compounds M10 and M11 were repeated the most among all compounds, by 30 times each, while M1 and 8 were repeated 9 times and are the two least common substances, and the remainder of the materials were graded in between the two values.

Table 2 aromatic compound repetitions

<table>
<thead>
<tr>
<th>Aromatic compound</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>9</td>
</tr>
<tr>
<td>M2</td>
<td>14</td>
</tr>
<tr>
<td>M3</td>
<td>14</td>
</tr>
<tr>
<td>M4</td>
<td>13</td>
</tr>
<tr>
<td>M5</td>
<td>13</td>
</tr>
<tr>
<td>M6</td>
<td>14</td>
</tr>
<tr>
<td>M7</td>
<td>18</td>
</tr>
<tr>
<td>M8</td>
<td>9</td>
</tr>
<tr>
<td>M9</td>
<td>11</td>
</tr>
<tr>
<td>M10</td>
<td>30</td>
</tr>
<tr>
<td>M11</td>
<td>30</td>
</tr>
</tbody>
</table>

Each donor's information was collected on forms that included their age, gender, smoking history, daily cigarette consumption, occupation, and time spent practicing the profession.

3.1 Influences on the frequency of aromatic components in samples

In the following paragraphs, we will summarize the effect of age, years of smoking and the number of cigarettes consumed per day, on the number of times the material which was viewed in different samples

A. The Age Factor

The following histogram shows that the most frequent occurrence is in the (30-39 )age group. This may be due to the fact that these ages have a relatively active excretory system when compared with the higher age groups, while the lower age groups do not have high concentrations of aromatic compounds according to the lower age, or they need these aromatic
compounds during the growth period. The reason for the decrease in the frequency of the appearance of aromatic compounds in the higher age groups may be due to the decrease in the efficiency of the excretory system due to age, as shown in Fig. 5.

**Fig. 5 Age effect**

![Bar chart showing age effect on repetition percentage](chart.png)

**B. smoking time's effect**

There is a clear increase in the frequency of the appearance of aromatic substances when moving from smokers who smoke for 9 years and less to smokers who smoke between 10-19 years, as this increase may be due to the increase in the accumulation of aromatic compounds with an increase in the smoking period. For those who smoke between 20-29 years and between 30-39 years, there is a decrease in the appearance of the aromatic valley as a result of their increasing age and then the decrease in the efficiency of their excretory system as mentioned previously. It is also noted that both substances M10 and M11, which are closely related to bladder cancer diseases, appear in high rates. This information is summarized in Fig. 6.
C. Effect of daily cigarette use

There is no consistent pattern in the change in the appearance of the samples due to the effect of the number of cigarettes consumed per day, as some substances increase with the increase in consumption, some decrease and others fluctuate between the two cases, but substances M10 and M11 lead the percentage of appearance and these two substances have a clear relationship with bladder cancer as was done explained earlier. This illustrated information by Fig. 7.
Conclusion

Urine samples were collected for smokers of different age groups and for different smoking periods and who consume different numbers of cigarettes per day within the geographical area of the city of Karbala, Iraq. Samples were dealt with based on a pre-existing working method with some modifications with reagents, and a home-designed device was developed to speed up evaporation of samples without the need for heating. The samples were measured after dealing with them using the GS-MS technique and the aromatic compounds were sorted, and their distribution was studied according to the factors affecting the frequency of the appearance of the aromatic compounds.

Acknowledgments

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Conflicts of interest

There are no conflicts to declare.
References


