**Biochemistry Method: Simultaneous determination of formaldehyde and methyl tert-buthyl ether in environmental and human biological matrices using static headspace gas chromatography mass spectrometry**

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**A B S T R A C T**

The present study describes a method based on static headspace extraction (HS) followed by gas chromatography/mass spectrometry (GC/MS) for the qualitative and quantitative analysis of methyl tert-buthyl ether (MTBE) and formaldehyde (HCHO) in human blood and water samples. Cytochrome P4502A6 has important role for converting of MTBE to tert-butyl alcohol (TBA) and HCHO. To enhance the extraction capability of the HS, extraction parameters such as extraction temperature, extraction time, the ratio of headspace volume to sample volume and sodium chloride concentration have been optimized. Wide linearity range was verified in a range of 5-10000 µgL⁻¹ for MTBE ($r^2=0.9998$), while those for HCHO was 5-500 µgL⁻¹ ($r^2=0.9996$). Detection limits for MTBE and HCHO was 1.0 µgL⁻¹ and 1.3 µgL⁻¹, respectively. Best results were obtained when the analyzed oily water samples were heated to 70 °C for 20 min, with the sample volume 10 mL in 20 mL vial, and NaCl 30% (w/v) was used to saturate the samples. The proposed analytical method was successfully used for the quantification of analytes in human biological samples and wastewater samples.

**1. Introduction**

Formaldehyde (HCHO) is the most widespread carbonyl compound in the atmosphere. It enters the environment from natural sources (including forest fires) and from direct human sources such as fuel combustion, industrial on-site uses, off gassing from building materials and consumer products. Although formaldehyde is a gas at room temperature, it is readily soluble in water. Formaldehyde is very active, and is transported in air, water and contaminted soils. In aqueous systems, atmospheric deposition is a significant source of formaldehyde [1], and in drinking water formaldehyde arises mainly from the oxidation of natural organic matter during ozonation [2] and degradation of oxygenates such as methyl tert-buthyl ether (MTBE) and dimethyl carbonate (DMC) [3]. It also enters drinking water via leaching from polyacetal plastic fittings in which the protective coating has been broken [4]. Formaldehyde is a very toxic compound and has been classified as a human carcinogen by the international agency for research on cancer (IARC), and also as a probable human carcinogen by the US. Environmental Protection Agency [5].

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DOI: https://doi.org/10.24200/amecj.v2.i01.40
national institute for occupational safety and health (NIOSH) considered formaldehyde as immediately dangerous to life and health at 24 mgm⁻³ (20 µgmL⁻¹) [6]. It can damage the person’s nerve system, lung and liver, and cause irritation of eyes, nose, throat and skin. Therefore, formaldehyde is one of the analytical interesting substances as a marker of fuel additive degradation. Its determination becomes also a hot spot of the research especially in oily wastewater matrices. A variety of methods for the determination of formaldehyde have been reported, including spectrophotometry [7-13], flow-injection catalytic method [14], high performance liquid chromatography [15], gas chromatography [16, 17], isotope dilution mass spectrometry [18], fluorimetry [19, 20], chemiluminescence [21,22], polarography [23], Fourier Transform Infrared Absorption [24] and sensors [25-28]. MTBE is also a volatile organic compound (VOC) produced from natural gas. It is primarily used for the oxygenation of fuel to enhance octane number and to improve the combustion process, in order to reduce carbon monoxide emissions [29]. MTBE readily dissolves in water, and moves rapidly through soils and aquifers. It is resistant to microbial decomposition and difficult to remove in water treatment. Its occurrence in the environment is of a great concern because of the toxicity of MTBE and its degradation products [30]. Since MTBE is highly volatile and very soluble in water, it can be easily found both as airborne pollutants of living and working environments and as contaminants of drinking water [31]. To date limited data are available on the effects of MTBE on health. Notwithstanding this, USEPA has concluded that at high doses, MTBE is a potential human carcinogen and recommended that MTBE levels in drinking water be kept below a range of 20-40 ppb [32].

MTBE and other oxygenates in ground waters are frequently measured using standard US EPA approved methods (e.g., EPA 8021B, EPA 8260B, ASTM D 4815). These procedures usually perform gas chromatographic separation coupled with photo ionization detector (PID), flame ionization detector (FID) or mass detector (MS). The introduction of analytes in the chromatographic apparatus is performed either via direct injection of water samples (DAI) [33,34], or using sampling techniques as dynamic headspace (P&T), static headspace [35], solid phase microextraction (SPME) [36-44], and solvent microextraction (SME) [45,46]. The DAI technique presents some difficulties to be coupled with capillary GC, due to the large expansion volume of water. Direct water injections are prone to back flush in the injector port, which can cause loss of analyte response as well as injection port contamination. MTBE oxidation can generate tert-buthyl alcohol (TBA) and formaldehyde (Fig.1). Our previous study demonstrated that human cytochrome P450 2A6 is able to metabolize MTBE to tert-butyl alcohol (TBA) and formaldehyde, a major circulating metabolite and markers for exposure to MTBE [3]. CYP2A6 plays a significant role in metabolism of gasoline ethers in liver tissue. The purpose of this present study is to develop a simple, sensitive and selective method for simultaneous determination of trace amounts of formaldehyde and MTBE in environmental and human biological matrices. To our knowledge, no method was found in the literature for this case.

2. Experimental

2.1. Chemicals and Standard Solutions
In this work, analytical grade of chemicals and reagents were purchased from Merck, Germany.
Double distilled water (DDW) was used for preparation and dilution of samples. Helium and nitrogen (ultrapure carrier grade) were obtained from Roham gas Company (Tehran, Iran). An aqueous formaldehyde stock solution, 1000 g mL⁻¹, was prepared by diluting 2.5 mL of 37% w/v stock formaldehyde solution (Merck) to 1 L with deionized distilled water (DDW) and was standardized by the sulfite method [47]. Working solutions of formaldehyde were subsequently prepared by appropriate dilution of the stock solution with DDW. MTBE Calibration stock solutions were prepared by adding 10 µL of pure MTBE (99.5%, Merck) to 10 ml of MeOH (Merck) in a 10ml vial with a PTFE-silicon septum. The mixture was manually agitated for 5 min. The first dilution steps were performed with methanol whereas further preparation of the standard solutions was carried out with DDW. The standard solutions used within 4 weeks. All sample and standard vials were completely filled to eliminate headspace. Individual and cumulative working standard solutions were obtained by appropriate dilution of the stock in 50 ml of methanol and further diluted in ultrapure Milli-Q water to prepare solutions containing MTBE and formaldehyde at the nanogram per milliliter level. The method was optimized with MTBE and formaldehyde solutions of 50 µg/L⁻¹ concentration. It should be noted that in this work Methyl ethyl ketone (MEK) (50 ng mL⁻¹) was used as internal standard in environmental and human biological samples.

2.2. Apparatus and Procedure

Static headspace analysis was performed using a CTC-CombiPAL autosampler (Bender and Holbein, Zurich, Switzerland) mounted on top of a GC-MS system. The autosampler was equipped with a heatable CTC agitator for incubation and shaking, and a robotic arm. To prevent the carry over of analytes, we used a heated flushing station for conditioning of the HS needle and reconditioning after each analysis. Both the gas station and the heated flushing station were flushed with nitrogen. The syringe body was held in the syringe adapter heater. 20 mL vials sealed with screw top caps with PTFE/silicon septa were used. Parameters of the instrument are shown in Table 1. A salt content of 30% (w/v) was chosen for the quantitative determination of target analytes in environmental and human biological samples.

The GC–MS analysis was performed using a Varian (CP-3800 series) gas chromatograph equipped with a mass-selective detector (Varian, quadrupole 1200) and a factor-four, VF-5ms fused-silica capillary column with a 30m × 0.25 mm i.d. and 250 um film thickness (Varian) was used. The GC conditions were as follows: inlet temperature, 250 ºC; inlet mode, split operation with split ratio 1:25. The oven temperature was set at 50 ºC and raised to 100 ºC at 5 ºC/per min, and raised to 275 ºC at 20 ºC per minute. The final temperature was maintained for 1.75 min and the total run time was 20 min. Helium, at a constant flow rate of 1.5 ml/min was used as the carrier gas. Mass spectra were obtained at 70eV in the electron impact ionization mode; the spectrometer was operated in the full scan mode over the mass range from 75 to 110(m/z). The source, transfer line and quadrupole temperatures were maintained at 200ºC, 250 ºC and 200 ºC, respectively. Total ion current chromatograms were acquired and processed using Workstation data analysis software (Varian). To increase sensitivity, the selected ion monitoring (SIM) mode was applied in quantitative analysis. The most abundant ion was used as the quantified ion. In Table 2, some

<table>
<thead>
<tr>
<th>Table 1. Headspace conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe Temperature : 71ºC</td>
<td>Plunger fill speed: 100 µLper sec</td>
</tr>
<tr>
<td>Agitator Temperature : 70ºC</td>
<td>Pre-injection delay: 4 sec</td>
</tr>
<tr>
<td>Sample incubation time: 20 min</td>
<td>Plunger injection speed: 250 µLper sec</td>
</tr>
<tr>
<td>Agitator speed: 500 rpm</td>
<td>Syringe flush time: 120 sec</td>
</tr>
<tr>
<td>Agitation cycle: 2 sec on, 4 sec off</td>
<td>sample volume, 10 ml in 22 ml vial</td>
</tr>
</tbody>
</table>
analytical conditions of MTBE, formaldehyde and methyl ethyl keton by GC-MS with SIM mode are shown. All quantifications made in this study were based on the relative peak area of analytes to the internal standard from the average of three replicate measurements in environmental and human biological samples.

3. Results and discussion
Various parameters were evaluated during the method development. In the present study, the evaluation of individual parameters was carried out while all other method parameters were kept constant.

3.1. Extraction temperature
The temperature of sample affects on evaporation of analyte into the headspace. We expected that an increase in sample temperature will result in improved the extraction efficiency, because of the increased evaporation of the analyte concentration in the headspace. The effect of sample temperature was studied by changing the sample temperature from 40 to 80 °C. As can be seen in figure 2, the amount of extracted analyte (into the headspace) increases with increasing temperature up to 80 °C. In headspace analysis, it is recommended not to use high temperatures (in order to avoid the overpressurization of the vial sample, and so avoid accidents) and, therefore, an extraction temperature of 70°C was selected in environmental and human biological samples. The syringe temperature of 5 °C above vial temperature was selected to avoid the analytes condensation.

3.2. Extraction time
The time required for the extraction process was an important parameter to be investigated. The most adequate time for the HS extraction was considered to be the time reaching the equilibrium of the analytes between the vapor phase and aqueous phase. Extraction time between 5 and 30 min were tested for the samples of 50 µg L⁻¹ at 70°C, and the heating-time profile for the MTBE and formaldehyde mixture is shown in figure 3. An increasing efficiency was observed for both

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Retention time (min)</th>
<th>Quantification ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>30</td>
<td>1.39</td>
<td>30</td>
</tr>
<tr>
<td>MTBE</td>
<td>88</td>
<td>1.45</td>
<td>73</td>
</tr>
<tr>
<td>Methyl ethyl keton</td>
<td>73</td>
<td>1.90</td>
<td>43</td>
</tr>
</tbody>
</table>

Table 2. Analytical conditions of MTBE, formaldehyde and methyl ethyl ketone by GC-MS with SIM method

Fig. 2. Influence of the extraction temperature on the relative peak areas of 50 µg L⁻¹ MTBE and formaldehyde in water.

Fig. 3. Effect of extraction time on peak areas of 50 µg L⁻¹ MTBE and formaldehyde in water at 70 °C.
compounds when the longer extraction time was used until 20 min, and then an increase in extraction time caused a decrease in the efficiency. A reason for this phenomenon was the transfer of water molecules to headspace which diluted the gas phase and decreased extraction amounts. So the extraction time of 20 min was considered for the subsequent experiments.

3.3. Ionic strength influence
Because the ionic strength of the solution influences the partition coefficient between the gas and liquid phase (K) the effect of salt amount on extraction efficiency was also checked. The effect of the salt on the extraction efficiency was investigated by comparing the extraction efficiency of samples which contained different amounts of sodium chloride (NaCl) from 0 to 40 (%w/v), and its influence, as the salting out agent, on the ion abundance of GC-MS chromatogram for MTBE and formaldehyde is shown in figure 4. As can be seen the addition of salt does not have the same effect for both target analytes: the addition of NaCl led to better results in the case of MTBE, while for the HCHO no favorable, and sometimes unfavorable effects (when more than 30% (w/v) of sodium chloride were employed) were observed. In human blood, the effect of different ions on extraction of Formaldehyde and MTBE based on proposed procedure was investigated. The interference of some coexisting ions in blood, serum and urine samples on the recovery of Formaldehyde and MTBE was studied under optimized condition. The proposed procedure was performed using a 10 mL sample containing 5-500 µgL⁻¹ of formaldehyde and MTBE and 2 mg L⁻¹ of different concentration of matrix ions such as, Zn²⁺, Cu²⁺, Mn²⁺, Na⁺, K⁺, Ca²⁺ and Mg²⁺. The tolerate amounts of important ions and biological matrix (albumin and proteins) were tested that caused less than 6% of the head space extraction alteration. In optimized conditions, the ions and biological matrix do not interfere to formaldehyde and MTBE extraction by procedure (less than 5%). The results showed us, the most of the probable human matrix concomitant have no considerable effect on the recovery efficiencies of formaldehyde and MTBE.

For MTBE the headspace extraction efficiency is increased with increasing concentration of salt in environmental samples and it reached the peak yield when NaCl (30%, w/v) was used to saturate the samples. The reason was considered to be the increase of ionic strength in aqueous samples by adding salt, therefore the solubility of analytes was decreased and more analyte was released into the headspace. For HCHO the observed behavior could be explained on account of its high solubility in water (37%) and strong interaction by solvent molecules (water) through hydrogen bonding that cause a greater affinity for water samples. Therefore, 30 % (w/v) salt content was chosen for the quantitative simultaneous determination of both target analytes.

3.4. Sample volume
The ratio of sample volume to headspace volume is an important parameter that affects the extraction efficiency of HS. An increase in sample volume and, consequently, a decrease in headspace volume enhance the extracted amount of analyte, which improves the sensitivity. The optimal ratio of the aqueous volume to the headspace volume for headspace analysis in 20 mL vials was determined by varying the sample volume and measuring the peak area for each sample volume.
volume from 5 mL (1/4 vial volume) to 15 mL (3/4 vial volume). The results are also shown in figure 5. The extracted amounts of analytes increase continuously with increasing sample volume reach a maximum at an aqueous volume of 10 ml and then decrease because of the decreased volume of the headspace. In the work, sample volume of 10.0 mL (in 20.0 mL vial) was used.

3.5. Evaluation of the method performance

Fig. 6 shows a typical total ion chromatogram (TIC) of a standard solution containing, 100 µg L\(^{-1}\) of MTBE and HCHO after its headspace extraction under optimal experimental conditions in human biological and environmental samples. The linearity, limits of detection and precision were calculated when the optimum conditions for the HS-GC–MS procedure were established. The linearity of the method was examined by spiking DDW with MTBE and HCHO in a concentration range from 5 to 10000 µg L\(^{-1}\) in water samples and 5 to 500 µg L\(^{-1}\) in human biological samples. Each solution was submitted to the HS-GC-MS analysis three times. The figures of merit of the calibration graphs are summarized in Table 3. A plot of the peak areas against the concentrations of standards was obtained (Fig. 7). Lack-of-fit test was performed to check the goodness of fit and linearity [48]. Lack-of-fit test demonstrated that the linear models were adequate because the whole p values were more than 0.05 at significance level of 95%. (Table 4). The linear range experiments provided the necessary information to estimate LODs, based on the signal that differed three times from the blank average signal, was 2 and 5 µg L\(^{-1}\) for MTBE and HCHO, respectively. Analytical accuracy was assessed from the recovery of analyte spiked to various of human biological and environmental samples (Table 5). The repeatability expressed as the relative standard deviation (R.S.D.) was obtained by carrying out five replicate assays on each water samples (Table 2), and gave a value less than 4.8% and 2.6% in human biological and environmental samples, respectively. Therefore, this method is deemed acceptable for determining of trace level of µg L\(^{-1}\) in water and human biological matrix.

![Fig. 5. Effect of solution volume in 20 ml vial on peak areas of 50 µgL\(^{-1}\) MTBE and formaldehyde in water produced by HS for 20 min at 70 °C.](image)

![Fig. 6. Total ion chromatogram (TIC) in SIM mode of an ultrapure water solution contaminated with MTBE (50 µgL\(^{-1}\)) and formaldehyde (50 µgL\(^{-1}\)), extracted using static headspace. Extraction conditions: Extraction time: 20 min, Extraction temperature: 70 °C, sample volume 10 mL in 20 mL vial and NaCl 30% (w/v).](image)
3.6. HS-GC–MS of real samples

The proposed method was firstly used to quantify MTBE and HCHO in wastewaters related with Tehran oil refinery and human blood and urine of petrochemical workers. The obtained results in Table 5, all samples showed good recoveries, and the method was ideally suited for these matrices. The synthetic biological samples were also analyzed by the develop method (Table 6). Here, blank is containing 500 µl mixture of 50 mM tris-HCl buffer (pH=7.4), 1mM NADPH (as inducer), 10 mM MgCl\textsubscript{2} and 150 mM KCl (as electrolytes). Synthetic sample 1 is prepared by addition of 5.02 µg ml\textsuperscript{-1} MTBE in the blank solution. Synthetic samples 2 and 3 are also the same synthetic sample 1 that are treated by 20 picomol of human cytochrome P450 (2A6), prepared from Sigma-Aldrich Co., at 37 °C for 13 and 30 minutes, respectively. Cytochrome P450 (2A6) is known as one of the most effective enzymes in metabolism alkoxyethers. In order to control enzyme activity and termination of reaction time, it was need to a deactivator such as 100 µl of 0.10 M perchloric acid. Formaldehyde was also a mainly byproduct of enzymatic degradation reaction of MTBE and was detected by developed method as given in Table 6. In the case of formaldehyde, although the calculated values can be estimated stoichiometrically, but a

![Fig. 7. Standard calibration curves of peak areas against the concentrations of MTBE (●) and HCHO (■). MTBE: y = 14.90x + 28.32 (r = 0.996), HCHO: y = 61.07x + 88.88 (r = 0.998).](image)

**Table 3** Analytical figures of merit of the determination of MTBE and HCHO (µg L\textsuperscript{-1})

<table>
<thead>
<tr>
<th>Compound</th>
<th>Regression Equation</th>
<th>Linear Range</th>
<th>LOD</th>
<th>RSD (%)</th>
<th>n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTBE</td>
<td>y =513.24x + 0.319</td>
<td>5-10000</td>
<td>2</td>
<td>4.8</td>
<td>6.8</td>
</tr>
<tr>
<td>formaldehyde</td>
<td>y = 1.759x + 27.53</td>
<td>5-500</td>
<td>5</td>
<td>1.9</td>
<td>7.8</td>
</tr>
</tbody>
</table>

\textit{a} y: analyte area-to-internal standard area, \textit{x}: concentration (µg L\textsuperscript{-1}).

**Table 4.** Evaluation of the goodness of fit and linearity of calibration graphs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Correlation coefficient, r</th>
<th>Determination coefficient, R\textsuperscript{2}</th>
<th>Lack-of-fit, p\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTBE</td>
<td>0.9998</td>
<td>0.9993</td>
<td>0.089 &gt; 0.05</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>0.9996</td>
<td>0.9984</td>
<td>0.078 &gt; 0.05</td>
</tr>
</tbody>
</table>

\textit{a} Confidence interval, 95%.
standard method based on derivatization with 2, 4-dinitrophenylhydrazine and HPLC detection was used to assay the values[49]. An average recovery of 65.5 and 91.7 % was obtained for degradation process of MTBE after passing 13 and 30 min. from course of the reaction, respectively. The standard deviation of measurements at ppm levels was not greater than 2.6%.

4. Conclusions
An automated and simple method has been developed for simultaneous determination of MTBE and formaldehyde in water and human matrices. It was based on the use of HS device coupled with a GC–MS instrument. The no necessity of consumables or reagents for sample treatment made HS-GC–MS to be considered as the best extraction option of the studied ones. The analysis required 20 min of sample incubation or extraction time and less than 5 min for chromatographic determination programming the MS detector in SIM acquisition mode. Good precision and the simple sample preparation enable to use this procedure for routine investigations. This proposed method was then applied to the analysis of human biological and environmental samples (synthetic biological samples).

5. References

Table 5. Determination of HCHO and MTBE in water and Human samples at optimum extraction conditions (µg L⁻¹)

<table>
<thead>
<tr>
<th>Water</th>
<th>Conc.</th>
<th>Added</th>
<th>Found</th>
<th>Recovery (%)</th>
<th>Conc.</th>
<th>Added</th>
<th>Found</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCHO</td>
<td>ND²</td>
<td>25.0</td>
<td>25.2 ± 2.8</td>
<td>100.8</td>
<td>ND²</td>
<td>25.0</td>
<td>23.9 ± 4.2</td>
<td>95.6</td>
</tr>
<tr>
<td>MTBE</td>
<td>ND²</td>
<td>25.0</td>
<td>24.4 ± 4.8</td>
<td>97.6</td>
<td>12.0</td>
<td>25.0</td>
<td>36.2 ± 3.1</td>
<td>97.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human blood</th>
<th>Conc.</th>
<th>Added</th>
<th>Found</th>
<th>Recovery (%)</th>
<th>Conc.</th>
<th>Added</th>
<th>Found</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCHO</td>
<td>22.1</td>
<td>20.0</td>
<td>41.4 ± 2.3</td>
<td>96.5</td>
<td>12.8</td>
<td>10.0</td>
<td>22.5 ± 4.2</td>
<td>97.0</td>
</tr>
<tr>
<td>MTBE</td>
<td>16.3</td>
<td>20.0</td>
<td>36.9 ± 1.5</td>
<td>103.1</td>
<td>9.7</td>
<td>10.0</td>
<td>19.3 ± 3.1</td>
<td>96.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urine</th>
<th>Conc.</th>
<th>Added</th>
<th>Found</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCHO</td>
<td>22.1</td>
<td>20.0</td>
<td>41.4 ± 2.3</td>
<td>96.5</td>
</tr>
<tr>
<td>MTBE</td>
<td>16.3</td>
<td>20.0</td>
<td>36.9 ± 1.5</td>
<td>103.1</td>
</tr>
</tbody>
</table>

a Mean of triplicates with percent R.S.D (n=5).
² Not found.
³ Well water nearby Tehran oil refinery.

Table 6. Simultaneous determination of MTBE and Formaldehyde in synthetic biological samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>MTBE / µg ml⁻¹</th>
<th>Formaldehyde / µg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calcd.</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>Synthetic sample 1</td>
<td>5.02</td>
<td>4.91</td>
</tr>
<tr>
<td>2</td>
<td>Synthetic sample 2</td>
<td>-</td>
<td>1.73</td>
</tr>
<tr>
<td>3</td>
<td>Synthetic sample 3</td>
<td>-</td>
<td>0.415</td>
</tr>
</tbody>
</table>
Determination of MTBE and HCHO in human; Ali Akbar Miran Beigi, et al


[28] L. Campanella, M. Battilotti, R. Dragone, I. Mevola, Suitable solid state chemical sensor for HCHO


