

# Analytical Methods

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## Analytical Methods

## Paper

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# A Versatile Probe for Chemoselective Capture and Analysis of Carbonyl Compounds in Exhaled Breath

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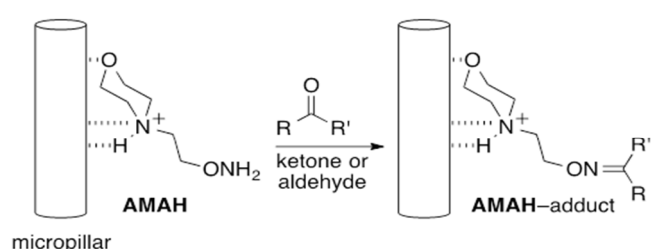
**ABSTRACT:** We describe an aminoxy reagent for the capture of trace aldehyde and ketone volatile organic compounds (VOCs) in exhaled breath. The reagent, 4-(2-aminoxyethyl)-morpholin-4-ium chloride (AMAH), when coated onto micropillars within a silicon microreactor, chemoselectively and covalently retains carbonyl VOCs from exhaled breath. The AMAH-carbonyl adducts are then eluted from the microreactor with methanol and directly analyzed by Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry (MS), where the aminium ion of the reagent enhances the sensitivity for high mass accuracy. We also outline a protocol for treatment of the AMAH-carbonyl adducts with poly(4-vinylpyridine) to afford the corresponding volatile carbonyl adducts that now can be analyzed by gas chromatography-mass spectrometry (GC-MS). This convenient protocol imparts flexibility for the identification and quantification of isomeric VOCs using both FT-ICR-MS and GC-MS. Representative breath analyses are given to illustrate this applicability of AMAH.

## Introduction

We have previously reported the use of *N*-(2-(aminoxy)ethyl)-*N,N,N*-trimethylammonium iodide (ATM) to chemoselectively capture volatile aldehydes and ketones from exhaled human breath and ambient air.<sup>1-4</sup> Some carbonyl compounds in exhaled breath have been identified as lung cancer markers.<sup>1, 5-10</sup> Reaction of ATM with these volatile organic compounds (VOCs) was achieved by first coating ATM on the surface of micropillars within a silicon microreactor. The microreactor (preconcentrator) contains thousands of micropillars that function to distribute air flow through channels and provide surface area for efficient capture of the VOCs by the reactive coating. Aldehydes and ketones from exhaled breath then are selectively and covalently preconcentrated in the microreactor using a click chemistry reaction (oximation). The resultant ATM-carbonyl adducts are eluted from the microreactor using a small volume (*ca.* 100  $\mu$ L) of methanol, and analysis of the breath analytes then is performed using rapid direct-infusion Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry (MS) to determine the concentrations of exhaled carbonyl VOCs.

Though convenient for rapid identification of biomarkers, FT-ICR-MS is often incapable of distinguishing structural

isomers (e.g., constitutional isomers), especially as compound molecular weights increase. One method for confirming an isomer assignment is to use gas chromatography-mass spectrometry (GC-MS) in conjunction with a reference standard of the compound. Unfortunately, the adducts of ATM are not suitable for analysis by GC-MS since ATM is non-volatile as a consequence of its quaternary ammonium moiety. The use of ammonium ions to enhance the MS signal intensity at low concentrations is a well-established technique,<sup>11</sup> but since such ions typically are non-volatile a new strategy is required to conjunctively use the speed and accuracy of FT-ICR-MS with the isomeric differentiation afforded by GC-MS. We describe herein an approach to address this challenge by introducing a versatile aminoxy reagent, 4-(2-aminoxyethyl)-morpholin-4-ium chloride (AMAH, Scheme 1), a chemoselective probe that contains a titratable ammonium salt for enhancing [+]-ion electrospray MS analysis. Moreover, AMAH-carbonyl adducts can be made volatile for analysis by GC using a straightforward basification procedure we have developed. By applying AMAH onto the micropillars of the silicon microreactor, we illustrate this dual capability of AMAH by detection and structural assignment of carbonyl VOC adducts from exhaled breath using both FT-ICR-MS and GC-MS.



**Scheme 1.** AMAH oximation covalently traps aldehydes and ketones in the microreactor.

## Methods

**Materials.** All reagents and solvents, including 4-(2-hydroxyethyl)morpholine, deuterated acetone (acetone- $d_6$ ), acetone, 2-butanone, 2-pentanone, propanal, *n*-hexanal, 3-hydroxy-2-butanone, *tert*-butyldimethylsilyloxyacetaldehyde, *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoro-acetamide (MTBSTFA) with 1% *tert*-butyldimethylchlorosilane, acetonitrile and methanol, were purchased from Sigma-Aldrich. Poly(4-vinylpyridine) 2% cross-linked, *ca.* 60 mesh, 8.0 meq/g, was purchased from Alfa Aesar. The progress of reactions was monitored by thin-layer chromatography (TLC, silica gel 60 Å F-254 plates). The plates were visualized first with UV illumination followed by staining with iodine and/or a *p*-anisaldehyde solution. Column chromatography was performed using silica gel (230-400 mesh).<sup>12</sup> NMR spectra were obtained using a Varian/Agilent 400-MR NMR spectrometer equipped with a 5mm z-axis gradient AutoX probe operating at the nominal <sup>1</sup>H frequency of 399.66 MHz and <sup>13</sup>C frequency of 100.49 MHz. All spectra are reported in parts per million (ppm) relative to the residual solvent peak in <sup>1</sup>H NMR and the deuterated solvent peak in <sup>13</sup>C NMR.

**Synthesis of AMAH.** By analogy to our published method<sup>13</sup> for synthesis of quaternary ammonium aminoxy compounds as well as to a literature procedure,<sup>14</sup> we prepared AMAH as follows: diisopropyl azodicarboxylate (DIAD) (3.60 mL, 18.3 mmol) was added dropwise to a stirred solution of 2-(4-morpholinyl) ethanol (2.00 g, 15.3 mmol), *N*-hydroxyphthalimide (2.98 g, 18.3 mmol), and triphenylphosphine (4.80 g, 18.3 mmol) in dry THF (95 mL) at 0 °C under nitrogen. The resulting solution was allowed to warm to rt and then stirred. After 16 h, the THF was removed *in vacuo* and the resulting oil was dissolved in EtOAc (150 mL) and washed with sat. NaHCO<sub>3</sub> (3 x 50 mL) and brine (50 mL). The organic phase was reduced to 50 mL *in vacuo* followed by the addition of cold 5% HCl to pH 3. The aqueous phase was separated, washed with Et<sub>2</sub>O (3x20 mL), and then basified with sat. NaHCO<sub>3</sub> to pH 7.5. The aqueous phase then was extracted with CHCl<sub>3</sub> (3x50 mL). The combined organic extract was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and the solvent was removed *in vacuo* to afford 2-(2-morpholinoethoxy)isoindoline-1,3-dione as a light yellow solid (3.67 g, 87%); mp, 78-79 °C; TLC, *R<sub>f</sub>* 0.37 (EtOAc); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.55 (s, 4H), 2.84 (t, *J* = 4.8 Hz, 2 H), 3.60 (t, *J* = 3.8 Hz, 4 H), 4.36 (t, *J* = 5 Hz, 2 H), 7.74-7.76 (m, 2 H), 7.82-7.84 (m, 2 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 53.8, 57.2, 66.9, 74.2, 123.7, 129.2, 134.7, 163.7.

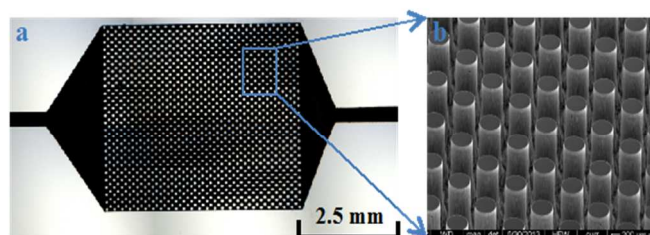
Hydrazine monohydrate (527 μL, 10.9 mmol) was added to a stirred solution of 2-(2-morpholinoethoxy)isoindoline-1,3-dione (735 mg, 2.66 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (11 mL) at rt. After stirring for 20 h, the suspension was filtered through a fritted glass funnel and the cake was washed with ample CH<sub>2</sub>Cl<sub>2</sub>. The filtrate and combined CH<sub>2</sub>Cl<sub>2</sub> washes were concentrated *in vacuo* and the residue was distilled using a Kugelrohr apparatus (10 Torr, 150 °C). The

distillate was dissolved in Et<sub>2</sub>O (11 mL) and gaseous HCl was bubbled into the solution. The acidic solution was stirred at rt for 1 h, and then dried *in vacuo* to provide 4-(2-aminoxyethyl)morpholin-4-ium chloride (AMAH, 486 mg, 100% yield) as a white solid; mp, 148-151 °C; IR ν (cm<sup>-1</sup>) 902, 1030, 1444, 2022, 2640, 3418; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 3.29 (s, 4 H), 3.47 (t, *J* = 4.4 Hz, 2 H), 3.89 (t, *J* = 4.4 Hz, 4 H), 4.47 (t, *J* = 4.6 Hz, 2 H), 11.18 (br s, 1H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) δ 51.2, 53.5, 63.1, 68.2.

**Procedure for basification of AMAH-adducts.** A 40 μL aliquot of the methanol AMAH-adduct mixture was transferred to a 200 μL insert containing 2 mg of poly(4-vinylpyridine) (PVP) for the neutralization of AMAH-adducts to AMA-adducts. After shaking the vial for 30 seconds, the vial was centrifuged at 1000 rpm for 5 minutes to settle the PVP. A 2 μL aliquot was taken from the supernatant and directly injected into the GC-MS.

**Silicon microreactor chip fabrication.** The design and fabrication process for the microreactor chips mirrored the micro-electromechanical system procedures described previously.<sup>15-17</sup> Deep reactive ion etching (DRIE) was used to create microfluidic channels and cylindrical micropillars with a height of roughly 350 μm on a silicon wafer. The silicon microreactors have a 7 x 5 mm microfluidic channel consisting of over 2500 micropillars with a surface area of about 130 mm<sup>2</sup>. The microreactor channels and micropillars were thermally oxidized to form a 50 nm SiO<sub>2</sub> thin film in an O<sub>2</sub> and H<sub>2</sub>O atmosphere.

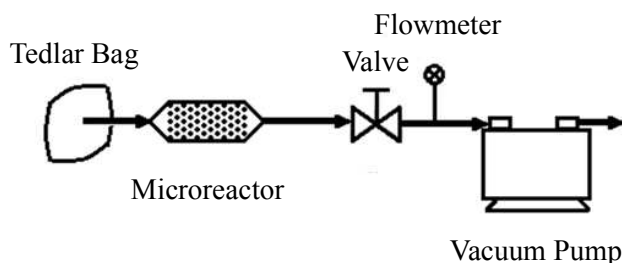
After thermal oxidation, the wafer was bonded with a Pyrex glass wafer using an anodic wafer bonding process. Each wafer was subsequently diced, and the connection ports were opened for connecting fused silica tubes to the microreactors. The surfaces of the micropillars in the microreactor were coated with AMAH by infusing a solution of AMAH in methanol into the microreactors with a microliter size syringe followed by evaporation of the solvent under vacuum. The slightly negative surface charge of SiO<sub>2</sub> surfaces of the micropillars enforces the close association of AMAH with the solid support. Finally, fused silica tubes with 340 μm o.d. and 200 μm i.d. were connected to the inlet and outlet ports of the microreactor, respectfully, with a silica-based bonding agent.



**Figure 1.** Silicon microreactor for the capture of carbonyl VOCs in exhaled breath. (a) Optical micrograph of the microreactor before bonding with a glass wafer. (b) SEM micrograph of the micropillar array within the microreactor.

**Determination of AMAH capture efficiency.** To test AMAH coated microreactor for capturing trace levels of carbonyl compounds, a solution of formaldehyde, acetaldehyde, acetone or 2-butanone in methanol ( $7.67 \times 10^{-7}$  to  $7.67 \times 10^{-10}$  mol) was injected into a 1 liter air Tedlar bag. The vapor-filled Tedlar bag then was connected to the preconcentration setup as shown in Figure 2 before a vacuum pump was used to pull the gaseous vapor from the Tedlar bag through the microreactor at a flow rate of 3.5 mL/min. A flow

rate higher than 3.5 mL/min significantly decreases the capture efficiencies. The microreactor then was disconnected from the system after the air sample in the bag had been completely evacuated.



**Figure 2.** Schematic flow diagram of the preconcentration set-up.

The reacted AMAH adduct and unreacted AMAH were eluted from the microreactor by flowing 100  $\mu\text{L}$  methanol from one slightly pressurized vial through the microreactor, and into an empty collecting sample vial. An internal reference for FT-ICR-MS analysis was established by adding a known amount of AMAH-acetone- $d_6$  adduct in methanol to each sample of eluent. The resulting solutions were directly injected into the FT-ICR-MS for analysis without any further process.

**Collection and analysis of exhaled breath samples.** To test the chip for capture of trace carbonyl compounds, exhaled breath samples were collected. After approval by the Internal Review Board of the institution and after having obtained written informed consent, exhaled breath samples were collected from 10 current smoking (smokers) and 10 never-smoking healthy (non-smokers) subjects. The subjects exhaled 1L of breath into 1-L Tedlar® bags. In this way, mixed alveolar breath and non-alveolar breath was collected. This simple protocol for collection of breath samples has been used for identification of lung cancer markers in breath.<sup>1,6, 19</sup> The Tedlar bags were purchased from Supelco (Bellefonte, PA, USA). The bags were tested free of ketone and aldehyde contamination. After collecting exhaled breath, the sample bags were connected to the inlet of the microreactor through septa and fused silica tube. The outlet of the microreactor was connected to a vacuum pump as shown in Fig.2. Each microreactor was loaded with  $7.67 \times 10^{-7}$  mol AMAH. During this process, the carbonyl compounds in the exhaled breath react with the AMAH coating and are retained in the microreactor while the rest of the breath sample flows through the microreactor. The AMAH-carbonyl adducts and unreacted AMAH were eluted from the microreactor by flowing 100  $\mu\text{L}$  methanol as described above for the determination of AMAH capture efficiency. A solution of  $5.44 \times 10^{-9}$  mol of AMAH-acetone- $d_6$  adduct in methanol was added to each sample of eluent as the internal reference for FT-ICR-MS analysis. The resulting solutions were directly injected into the FT-ICR-MS for analysis without any further processing.

**FT-ICR-MS Instrumentation.** The methanol-eluted mixtures of AMAH-VOC adducts were analyzed on a hybrid linear ion trap- FT-ICR-MS instrument (Finnigan LTQ-FT, Thermo Electron, Bremen, Germany) equipped with a TriVersa NanoMate ion source (Advion BioSciences, Ithaca, NY) with an electrospray chip (nozzle inner diameter 5.5  $\mu\text{m}$ ). The TriVersa NanoMate was operated in positive ion mode by applying 2.0 kV with no head pressure. Initially, low-resolution MS scans were acquired for 1 min to ensure the stability of ionization, after which high mass accuracy data were collected using the FT-ICR analyzer where MS scans were acquired for 8.5 min and at the target mass resolution of 100,000 at 800 m/z. AMAH and AMAH-adduct species were assigned on the basis of their

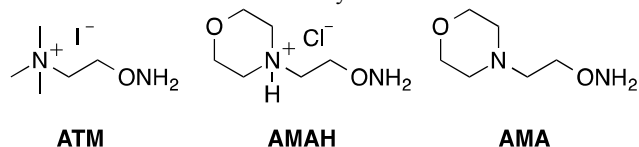
accurate mass by first applying a small (typically <0.0005) linear correction based on the observed mass of the internal standard.

**GC-MS Instrumentation.** A Thermo Scientific GC/MS instrument equipped with an AI 1310 automatic sampler, a TRACE 1310 GC with a split/splitless injector and an ITQ 1100 series ion trap MS was used for analysis. The GC was fitted with an Agilent J&W DB-17ms column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  film thickness). The carrier helium flow rate was set to 1.5 mL/min. The initial column temperature was 50  $^{\circ}\text{C}$  for 1 minute, then the temperature was increased by 12  $^{\circ}\text{C}/\text{min}$  up to 290  $^{\circ}\text{C}$  and was held at 290  $^{\circ}\text{C}$  for 5 minutes. The total running time was 26 minutes. The samples were split injected with split flow 15 ml/min and split ratio 10.

Carbonyl adduct standards for retention time determination were prepared by individually reacting excess AMA ( $5.1 \times 10^{-7}$  mol) with commercially available aldehydes or ketones ( $3.5 \times 10^{-7}$  mol) in a 1:1 mixture of acetonitrile:MTBSTFA (v/v) (100  $\mu\text{L}$ ). MTBSTFA reacts with the AMA-3-hydroxy-2-butanone adduct to form AMA-3-(*tert*-butyldimethylsilyloxy)-2-butanone (AMA-3-TBSO-2-butanone) for identification of 3-hydroxy-2-butanone in exhaled breath samples. To identify hydroxyacetaldehyde in exhaled breath samples, *tert*-butyldimethylsilyloxyacetaldehyde was also added to the solution to react with AMA to form AMA-*tert*-butyldimethylsilyloxyacetaldehyde (AMA-TBSO-acetaldehyde) as a standard.

## Results and discussion

We have previously shown how a silicon microreactor coated with first generation reagent ATM (Figure 3) was used to capture and accurately identify metabolic tags for the early detection of lung cancer.<sup>1</sup> ATM was specifically designed with several features that would optimize analyses of its adducts by FT-ICR-MS. The quaternary ammonium salt allowed for electrostatic interaction between ATM and the negatively charged silica surface of the micropillars. Secondly, the aminoxy moiety allowed for the rapid, chemoselective capture of gaseous aldehyde and ketone metabolites in exhaled breath samples with >90% capture efficiency.<sup>4</sup> Finally, the permanent positive charge of the quaternary ammonium salt enhanced the signal intensity of the metabolite adducts at low concentrations when analyzed by FT-ICR-MS.<sup>11</sup> In addition to cost and availability considerations, a limitation of analyzing ATM-adducts using FT-ICR-MS is the inability to differentiate constitutional isomers for a given molecular mass. MS-MS techniques can be applied to address this issue; however, use of a more widely available and affordable analytical platform, such as GC-MS, would improve the accessibility of the microreactor approach for identifying metabolites. Unfortunately, GC-MS cannot be used to analyze ATM-carbonyl metabolite adducts since these are non-volatile. To address this issue, we developed a second-generation reagent that allows for both FT-ICR-MS and GC-MS identification with isomeric accuracy.

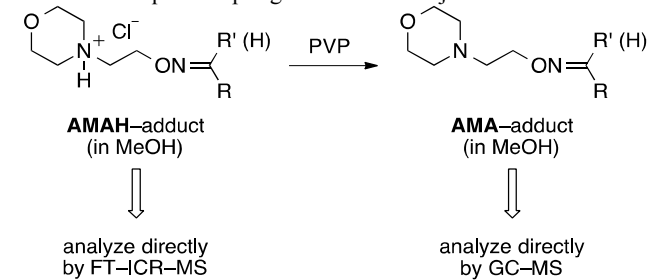


**Figure 3.** Structures of ATM, AMAH and AMA.

By slightly expanding the structural framework of ATM, we have engineered a second-generation reagent, AMAH (Figure 3), that features a titratable aminium nitrogen instead of a quaternary ammonium salt. The morpholino bridge, as opposed to *N,N*-

dimethyl, imparts an order of magnitude greater acidity for ease of basification, a subsequent key step, to generate neutral species, such as AMA or neutral AMA-carbonyl adducts.<sup>18</sup> Upon introduction to the microreactor, the aminium salt of AMAH electrostatically bonds to the silica surface of the micropillars while the morpholino oxygen and aminic proton also provide hydrogen bonding opportunities with surface silanols to improve the availability of the aminoxy moiety for carbonyl capture. Finally, the tertiary ammonium ion also enhances the MS signal intensity for analyses using FT-ICR-MS so that high mass accuracy can be determined for even low abundance carbonyl metabolites.

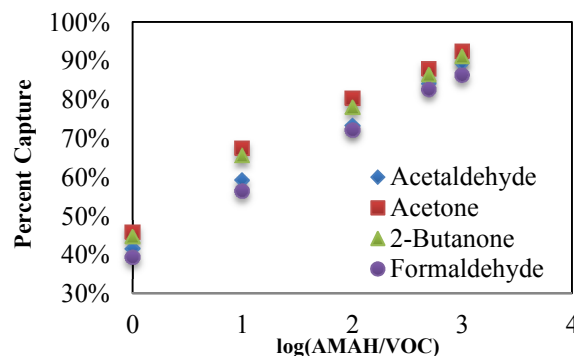
The key advantage in using AMAH over ATM for metabolite identification is its ability to be volatilized through a basification procedure, thus making it applicable for both FT-ICR-MS and GC-MS analyses. To ensure that the basification of AMAH-carbonyl adducts proceeds to give an analyte mixture that can be injected directly into a GC instrument, we developed a salt-free neutralization procedure that does not require liquid-liquid extraction or other lengthy handling processes (Scheme 2). First, the eluted methanolic AMAH-carbonyl adduct mixture can be directly analyzed by FT-ICR-MS. An aliquot can then be prepared for GC-MS analysis by reaction with poly(4-vinylpyridine) (PVP), an acid scavenging polymer. We were gratified to find that when a 40  $\mu$ L sample of AMAH-adducts in methanol was added to 2 mg of PVP followed by shaking for 30 seconds, neutralization of the adducts was achieved. This procedure is particularly appealing in that after basification using PVP the polymer quickly sediments to allow convenient aliquot sampling for direct GC injection.



**Scheme 2.** Use of AMAH for both FT-ICR-MS and GC-MS analyses. PVP = poly(4-vinylpyridine)

To examine the reactivity of AMAH with volatile carbonyl compounds, we performed a calibration experiment with formaldehyde, acetaldehyde, acetone and 2-butanone in a microreactor. Each carbonyl ( $7.67 \times 10^{-7}$  to  $7.67 \times 10^{-10}$  mol) was injected into a 1-L air Tedlar bag and connected to the preconcentration setup (Figure 2). A vacuum pump pulled the vaporized contents of the bag through the AMAH-functionalized microreactor at a flow rate of 3.5 mL/min. After the Tedlar bag had been fully evacuated, the microreactor was removed from the preconcentration assembly and the analytes were eluted from the chip using methanol. A solution of AMAH-acetone- $d_6$  adduct was added to the analyte solution to serve as an internal standard. For both aldehydes and ketones, the linear capture efficiency of AMAH peaks at a  $\sim 90\%$  capture rate at a  $10^3$  AMAH/analytes molar ratio (Figure 4, Table 1). Some loss of carbonyl compounds occurs due to instability and/or adsorption within the Tedlar bag<sup>19</sup> as well as uncaptured compounds flowing through the microreactor during the evacuation process.<sup>3,4</sup> The reason that the higher capture efficiency occurred at higher AMAH/analyte molar ratios is due to the elevated reaction probability for the capture of aldehyde and ketone VOCs. Due to the trace concentrations of carbonyl VOCs in exhaled breath, for example: formaldehyde, acetaldehyde and acetone reported in

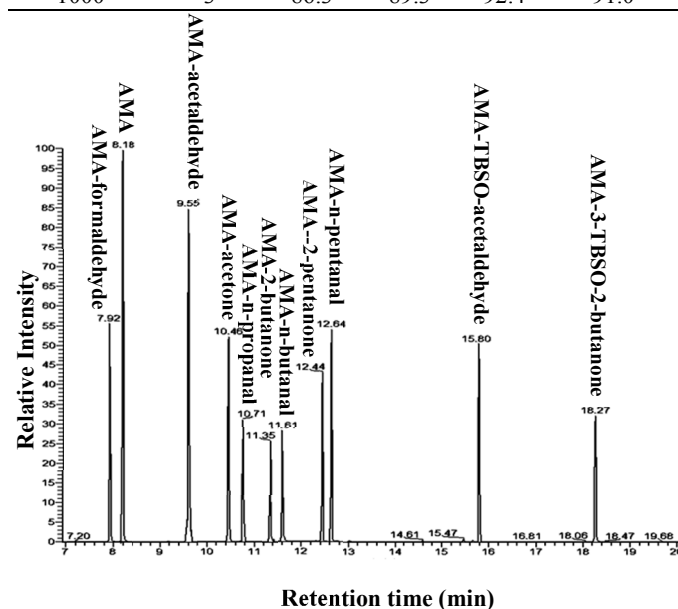
ppb to ppt range or less than a few nmol/liter,<sup>20-23</sup> AMAH should capture nearly all of VOCs of interest and provide an accurate concentration of each metabolite. Having confirmed that AMAH was highly reactive and applicable for both FT-ICR-MS and GC-MS, we analyzed a panel of AMA-C<sub>1</sub>-C<sub>5</sub> aldehyde and ketone adducts (Figure 5, Table 2) on GC-MS to establish reference retention times for the adducts.



**Figure 4.** Graphical relationship between VOC capture efficiency and log(AMAH/VOC molar ratio).

**Table 1.** Tabulated relationship between VOC capture efficiency and AMAH/VOC molar ratio.

AMAH/ VOC molar ratio	log(AMAH/ VOC ratio)	Capture Efficiency (%)			
		Formal dehyde	Acetald ehyde	Acetone	2-Butanone
1	0	39.3	41.5	45.8	44.7
10	1	56.5	59.3	67.4	65.5
100	2	72.2	73.3	80.3	78.0
500	2.699	82.6	84.1	87.9	86.4
1000	3	86.3	89.3	92.4	91.0



**Figure 5.** GC-MS chromatogram of reference AMA-carbonyl adducts; TBSO = *t*-butyldimethylsilyloxy.

**Table 2.** Retention times of AMA–C<sub>1</sub>–C<sub>7</sub> adducts  
<sup>a</sup>TBSO = *t*-butyldimethylsilyloxy

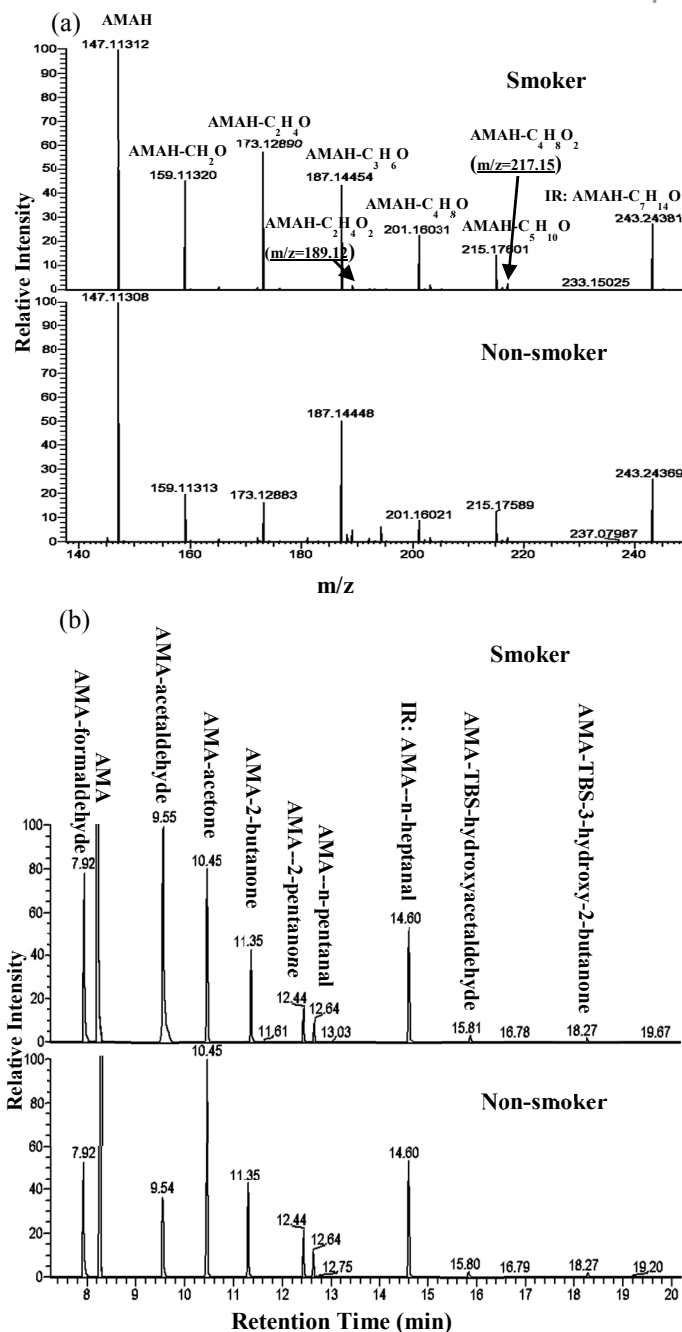
Adduct	<i>t</i> <sub>R</sub> (min)	Adduct	<i>t</i> <sub>R</sub> (min)
AMA–formaldehyde	7.92	AMA– <i>n</i> -butanal	11.61
AMA	8.18	AMA–2-pentanone	12.44
AMA–acetaldehyde	9.55	AMA– <i>n</i> -pentanal	12.64
AMA–acetone	10.46	AMA–TBSO–acetaldehyde <sup>a</sup>	15.80
AMA– <i>n</i> -propanal	10.71	AMA–3-TBSO–2-butanone <sup>a</sup>	18.27
AMA–2-butanone	11.35		

To verify the conversion percentage from AMAH to AMA, an aliquot (50 μL) of a panel of 5 × 10<sup>−9</sup> mol AMAH–C<sub>1</sub>–C<sub>5</sub> aldehyde and ketone adducts was added to PVP. After neutralization, the PVP was filtered and the methanol filtrate was evaporated. To the residue was added a 1:1 mixture of acetonitrile:MTBSTFA (*v/v*) (30 μL). AMA–*n*-heptanal (5 × 10<sup>−9</sup> mol) was added as an internal reference. The resultant silylated AMA-adduct mixture was then analyzed by GC-MS. Figure 5 shows GC-MS chromatogram of reference AMA-adducts. The results indicated 94.2 ± 2.5% AMAH adducts were neutralized to AMA adducts.

Following above calibration experiments, exhaled breath samples from 10 non-smokers and 10 smokers were preconcentrated by the microreactors and the eluted solutions were analyzed by GC-MS. Figure 6 shows that the exhaled breath samples can be analyzed by both FT-ICR-MS and GC-MS. Smokers have the highest abundance of acetaldehyde while non-smokers have the highest abundance of acetone. AMA–*n*-heptanal was added to all samples as an internal reference for GC-MS analysis. The concentrations of detected carbonyl compounds were calculated from the calibration curves of the ratios of the area under peak of AMA–*n*-heptanal to that of other compounds from GC-MS spectra. The estimated mean concentrations and standard deviation of captured carbonyls in the breath samples of smokers and non-smokers measured by GC-MS are given in Table 3. The smokers have considerably more formaldehyde, acetaldehyde and acetone in breath than non-smokers because these compounds are abundant in cigarette smoke. The standard deviations in Table 3 are relatively large in comparison with the mean values likely due to collection of a mixed alveolar and tidal breath.

**Table 3.** Estimated mean and standard deviation of carbonyl concentrations of gaseous breath samples of 10 non-smokers and 10 smokers determined by GC-MS. (Unit: nmol/L)

Entry	Carbonyl VOC	Non-smoker	Smoker
1	Formaldehyde	0.327±0.253	0.559±0.296
2	Acetaldehyde	0.514±0.329	0.971±0.471
3	Acetone	0.827±0.329	1.055±0.528
4	<i>n</i> -Propanal	0.011±0.006	0.015±0.009
5	2-Butanone	0.246±0.171	0.259±0.144
6	2-Pentanone	0.182±0.168	0.174±0.155
7	<i>n</i> -Pentanal	0.140±0.112	0.152±0.130
8	Hydroxyacetaldehyde	0.020±0.008	0.022±0.011
9	3-Hydroxy-2-butanone	0.017±0.006	0.018±0.006



**Figure 6.** Analyses of a never smoker and a current smoker subject via (a) FT-ICR-MS and (b) GC-MS; IR = internal reference.

Propanal, 2-butanone, *n*-pentanal, hydroxyacetaldehyde, and 3-hydroxy-2-butanone are detected by GC-MS in all breath samples of the 20 healthy subjects. Lung cancer patients have been reported to have higher concentrations of these compounds than healthy controls in breath.<sup>1, 6, 7, 9-10</sup> The concentrations of 2-butanone, hydroxyacetaldehyde, and 3-hydroxy-2-butanone presented in Table 3 are in the ranges of previously reported values of exhaled breath samples from healthy controls using ATM as the coating phase of silicon microreactors and FT-ICR-MS for analysis.<sup>1</sup> In our previous work of using open silicon chip microreactor coated with AMAH placed in a small vial for concentration carbonyl compounds in exhaled breath, it could only identify isomers of 2-pentanone and

1 pentanal by GC-MS<sup>24</sup>. The method is unable to do quantitative  
2 analysis because of unknown volume of exhaled breath and  
3 undetermined capture efficiencies of carbonyl compounds. Also in  
4 this work, *tert*-butyldimethylsilyloxyacetaldehyde, *N-tert*-butyl-  
5 dimethylsilyl-*N*-methyltrifluoro-acetamide (MTBSTFA) derivatizing  
6 agents enabled quantitative analysis of hydroxyl-acetaldehyde and 3-  
7 hydroxy-2-butanone by GC-MS. It should be noted that 2-  
8 butanone<sup>6</sup>, 3-hydroxy-2-butanone<sup>6,10</sup> *n*-pentanal<sup>6,7</sup> in breath were  
9 previously analyzed by GC-MS with a solid phase microextraction  
10 (SPME) method which is based on physical adsorption of carbonyl  
11 compounds on SPME. Thus, AMAH/AMA functionalized  
12 microreactor may provide a new method for quantitative analysis of  
13 these reported markers in gaseous breath by GC-MS. A major  
14 advantage in using AMAH/AMA is the ability to accurately  
15 determine marker molecular formulae as well as to detect the  
16 isomeric differences by GC-MS that FT-ICR-MS does not provide.  
17 For example, 2-pentanone and *n*-pentanal are constitutional isomers  
18 that would register as a single *m/z* (215.17601) on FT-ICR-MS (Fig.  
19 6a), but the AMA adducts of these VOCs are readily separated by  
20 GC-MS to reveal an abundance of one over the other (Fig. 6b). In  
21 addition, due to the chemoselectivity of aminoxy compounds for  
22 reacting with aldehydes and ketones,<sup>25</sup> interference of other  
23 abundant volatiles in exhaled human breath, such as O<sub>2</sub>, CO<sub>2</sub>, H<sub>2</sub>O  
24 and numerous VOCs, is avoided and this greatly facilitates carbonyl  
25 compound calibration as well as peak identification.

## 26 Conclusions

27 We have disclosed a second-generation aminoxy probe, AMAH,  
28 which efficiently captures trace aldehyde and ketone VOCs from  
29 exhaled breath when applied to a silicon microreactor containing  
30 thousands of micropillars. The microreactor chemoselectively and  
31 covalently concentrates carbonyl VOCs through an oximation  
32 reaction. The capture efficiencies of the AMAH functionalized  
33 microreactors have achieved higher than 90% for acetone and 2-  
34 butanone at a AMAH/analyte molar ratio of 10<sup>3</sup>. The positive  
35 charge of the AMAH-VOC adducts enhances sensitivity for the high  
36 mass accuracy and ultra-high resolution of FT-ICR-MS to enable  
37 determination of molecular formulae. The conversion from positive  
38 charged AMAH-VOC to neutral AMA-VOC was 94.2 ± 2.5% for a  
39 protocol whereby the AMAH-VOC adduct solution is added to a  
40 small amount of the acid-scavenging polymer PVP to produce  
41 volatile AMA-VOC adducts that can be analyzed by GC-MS. A  
42 derivatization method was developed to enable analysis hydroxyl-  
43 acetaldehyde and 3-hydroxy-2-butanone in exhaled breath by GC-  
44 MS. This versatile attribute of AMAH enables the identification and  
45 quantification of isomeric metabolites of trace carbonyl compounds  
46 in exhaled breath and air by GC-MS.

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## 51 Notes and references

- 52 1 X.-A. Fu, M. Li, R. J. Knipp; M. H. Nantz and M. Bousamra,  
53 *Cancer Med.*, 2014, **3**, 174-181.
- 54 2 M. Li, S. Biswas, M. H. Nantz, R. M. Higashi and X.-A. Fu, *Sens.*  
55 *Actuators B Chem.*, 2013, **180**, 130-136.
- 56 3 Fu, X.-A.; Li, M.; Biswas, S.; Nantz, M. H.; and Higashi, R. M.  
57 *Analyst*, 2011, **136**, 4662-4666.
- 58 4 M. Li, S. Biswas, M. H. Nantz, R. M. Higashi, X.-A. Fu, *Anal.*  
59 *Chem.*, 2012, **84**, 1288-1293.

- 60 5 M. Hakim, Y. Y. Broza, O. Barash, N. Peled, M. Phillips, A. Amann,  
H. Haick, *Chem. Rev.*, 2012, **112**, 5949-5966.
- 6 A. Bajtarevic, C. Ager, M. Pienz, M. Klieber, K. Schwarz, M. Ligor,  
T. Ligor, W. Filipiak, H. Denz, M. Fiegl, W. Hilbe, W. Weiss, P.  
Lukas, H. Jamnig, M. Hackl, A. Haidenberger, B. Buszewski, W.  
Miekisch, J. Schubert, A. Amann, *BMC Cancer*, 2009, **9**, 348-363.
- 7 P. Fuchs, C. Loeseke, J. K. Schubert, W. Miekisch, *Int. J. Cancer*,  
2010, **126**, 2663-2670.
- 8 D. Poli, M. Goldoni, M. Corradi, O. Acampa, P. Carbognani, E.  
Internullo, A. Casalini, A. Mutti, *J. Chromatography B*, 2010, **878**,  
2643-2651.
- 9 M. Phillips, N. Altorki, J. H. M. Austin, R. B. Cameron, R. N.  
Cataneo, R. Kloss, R. A. Maxfield, M. I. Munawar, H. I. Pass, A.  
Rashid, W. N. Rom, P. Schmitt, J. Wai, *Clin. Chim. Acta*, 2008, **393**,  
76-84.
- 10 G. Song, T. Qin, H. Liu, G. B. Xu, Y. Y. Pan, F. X. Xiong, K. S. Gu,  
G. P. Sun, Z. D. Chen, *Lung Cancer*, 2010, **67**, 227-231.
- 11 S. J. Mattingly, T. Xu, M. H. Nantz, R. M. Higashi, T. W.-M. Fan,  
*Metabolomics*, 2012, **8**, 989-996.
- 12 W. C. Still, M. Kahn, A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923-2925.
- 13 S. Biswas, X. Huang, W. Badger, M. H. Nantz, *Tetrahedron Lett.*,  
2010, **51**, 1727-1729.
- 14 C. Deraeve, Z. Guo, R. S. Bon, W. Blankenfeldt, R. DiLucrezia, A.  
Wolf, S. Menninger, E. A. Stigter, S. Wetzel, A. Choidas, K.  
Alexandrov, H. Waldmann, R. S. Goody, Y.-W. Wu, *J. Am. Chem.*  
*Soc.*, 2012, **134**, 7384-7391.
- 15 B. Alfeeli, D. Cho, M. Ashraf-Khorassani, L. T. Taylor, M. Agah,  
*Sens. Actuators*, 2008, **133**, 24-32.
- 16 B. Alfeeli, M. Agah, *IEEE Sens. J.*, 2009, **9**, 1068-1075.
- 17 I. Voiculescu, M. Zaghoul, N. Harasimhan, *Trends Anal. Chem.*,  
2008, **27**, 327-343.
- 18 M. R. Crampton, I. A. Robotham, *J. Chem. Res. (S)*, 1997, 22-23.
- 19 P. Mochalski, J. King, K. Unterkofler, A. Amann, *Analyst* 2013, **138**,  
1405-1418.
- 20 C. Deng, J. Zhang, X. Yu, W. Zhang, X. J. Zhang, *Chromatogr., B:*  
*Anal. Technol. Biomed. Life Sci.*, 2004, **810**, 269-275.
- 21 I. Ueta, Y. Saito, M. Hosoe, M. Okamoto, H. Ohkita, S. Shirai, H.  
Tamura, K. Jinno, *J. Chromatogr., B: Anal. Technol. Biomed. Life*  
*Sci.*, 2009, **877**, 2551-2556.
- 22 P. Cap, K. Dryahina, F. Pehal, P. Spanel *Rapid. Commun. Mass*  
*Spectrom.* 2008, **22**, 2844-2850.
- 23 C. Turner, P. Spanel, D. Smith *Rapid. Commun. Mass Spectrom.*  
2006, **20**, 61-68.
- 24 M.X. Li, PhD Dissertation, University of Louisville, December 2013,
- 25 A. Dirksen, P. E. Dawson, *Bioconjugate Chem.*, 2008, **19**, 2543-  
2548.