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A novel microreactor approach for analysis of ketones and aldehydes in breath

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We report a fabricated microreactor with thousands of micropillars in channels. Each micropillar surface is chemically functionalized to selectively preconcentrate gaseous ketones and aldehydes of exhaled breath and to enhance ultra-trace, rapid analysis by direct-infusion Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry (MS). The micropillar reactive coating contains the quaternary ammonium aminooxy salt 2-(aminooxy)ethyl-*N*,*N*,*N*-trimethylammonium iodide (ATM) for capturing trace carbonyl VOCs by means of an oximation reaction. We demonstrate the utility of this approach for detection of C_1 to C_{12} aldehydes and ketones in exhaled breath, but the approach is applicable to any gaseous sample.

Analysis of exhaled breath has been elevated to an international research frontier because of its potential and applicability in noninvasive health diagnosis, metabolite bioinformatics, and drug discovery.[1–5] The gaseous portion of breath is a complex mixture of atmospheric gases, water vapor, and trace volatile organic compounds (VOCs). In 1971, Pauling reported the first gas-chromatographic analysis of breath, and the study revealed the presence of a large number of VOCs in human breath.[6] A number of recent publications suggest that the analysis of exhaled breath promises to be a non-invasive diagnosis for early detection of particulate cancers since some VOCs in exhaled breath represent metabolic output of cancer tissues and cells.[7–13]

There are several critical challenges for the analysis of VOCs in exhaled breath, including ultra-trace concentrations of VOCs and interference of complex gas mixtures. Exhaled breath contains more than 200 VOCs. Recently, several reports indicate that some ketones and aldehydes in exhaled breath could be used for the diagnosis of lung cancer in its early stage.[4,5,7,12,13]

However, so far there is no established protocol for the analysis of all ketones or aldehydes in exhaled breath, in part due to their highly reactive nature. These carbonyl metabolites are produced in biochemical pathways as intermediates and some can be unique to a given pathway or process. However, even common carbonyl metabolites can be attributed to specific processes when stable isotope labeled substrates are metabolized (*e.g.* by human subjects) and detected by mass spectrometry or NMR.[14,15] Volatile ketones and aldehydes are also generated from damaging oxidative reactions, such as lipid peroxidation.[16–18] Therefore, development of a new method for analysis of ketone and aldehyde VOCs in exhaled breath is a crucial first step to fulfilling the potential that breath analysis promises.

Ketones and aldehydes can be detected by proton transfer reaction mass spectrometry (PTR-MS)[7,19–21] or selected ion flow tube mass spectrometry (SIFT-MS) without any preconcentration process.[22–25] Most recently, solid phase microextraction (SPME) with adsorbed *O*-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) has been used for analysis of aldehydes in exhaled breath by gas chromatography-mass spectrometry (GC-MS).[4,5] SPME is a popular preconcentration method introduced a decade ago as a rapid extraction technique for analysis of volatile compounds from a variety of matrices.[26] However, the surface area of the SPME polymer extraction phase is small and aldehyde capture requires physical adsorption of PFBHA onto SPME first. In most cases, it is also extremely difficult to improve upon, or even determine the volume of air that is actually sampled by the SPME fiber. The quality of the fibers depends on the manufacturer, and the performance varies from batch to batch. Derivatization of ketones and aldehydes by reaction with 2,4-dinitrophenylhydrazine has also been used for analysis of carbonyl compounds in exhaled breath,[16,27] which has the advantage of converting volatile aldehydes and ketones into stable, easy-to-handle non-volatile analytes. However, this class of reagents was not designed to aid in detection by modern ultra-sensitive MS ion sources such as nanoelectrospray.

In this work, we describe a microreactor approach for chemoselective capture of gaseous ketones and aldehydes from gaseous samples such as exhaled breath. The microreactor was fabricated on a silicon wafer and charged with an aminooxy-functionalized quaternary ammonium salt. The high selectivity of the aminooxy moiety ($R\text{-}ONH_2$) for reaction with ketone and aldehyde carbonyl groups suggested that an oximation reaction could be used to selectively capture ketone and aldehyde metabolites directly from the air, such as exhaled breath. For rapid analysis and identification of VOC adducts, we additionally turned to direct-infusion nanoelectrospray FT-ICR-MS analysis. This microscale sampling technique matched the samples sizes of the microreactor, and the ultra-high resolution FT-ICR-MS enables simultaneous analysis of the ketone and aldehyde adducts, while avoiding analytical interference from the capture

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phase. The latter, in turn, allows complete elution of the preconcentrator phase along with the analytes. Thus, coupling the strategies of oximation capture chemistry and FT-ICR-MS analysis with microreactors fabricated on silicon wafers benefit from design flexibility in microstructure. We highlight these novel preconcentration features by reporting here the results of ketone and aldehyde capture from breath using our uniquely designed microreactors.

The microreactors were fabricated on 4″-silicon wafers using standard microelectro-mechanical systems (MEMS) fabrication technology.[28] Fig. 1 shows an optical micrograph of fabricated microreactor with inserted a SEM micrograph of the micropillars. The micropillars have high-aspect-ratio with dimensions of 50 μm × 50 μm × 250 μm created by dry reactive ion etching silicon. The distances from center to center of the micropillars are 100 μm. The flow channel size is 7 mm × 5 mm. The total empty space in the microreactor is about 5 μL. We calculate that there are more than five thousand square micropillars within the microreactor corresponding to a total micropillar surface area of about 260 mm^2. The inlet and outlet of the microreactor were connected with 190 μm O.D., 100 μm I.D. deactivated fused silica tubes using a silica-based bonding agent.

The aminooxy-based reactive coating, 2-(aminooxy)ethyl-*N*,*N*,*N*-trimethylammonium iodide (ATM, Scheme 1) was synthesized according to a recently published method from the authors Biswas and Nantz.[29] In addition to the aminooxy group for oximation of the carbonyl VOCs, the coating was also designed with the quaternary ammonium group for greatly enhancing detection by the nanoelectrospray FT-ICR-MS. Overall, these features also make the VOC adducts non-volatile and stable to degradation, which allows for simple and routine elution, sample handling such as concentration to dryness, storage, and analysis.

The surface functionalization of the channels and micropillars by ATM ions was performed by infusing a known amount of the ATM-iodide salt in methanol solution into the microreactor channel from one connection port followed by evaporation of the solvent under vacuum. The slightly negative surface charge of silicon oxide micropillars enforces the close association of ATM with the solid support, yet another benefit of including the quaternary ammonium group in the design.

To test the capture efficiency of the microreactor, measured amounts of deuterated acetone (acetone-*d6*), acetone or propanal in the microlitre range or lower (diluted in methanol) were injected into a high purity (99.9999%) helium gas flow stream passing through the

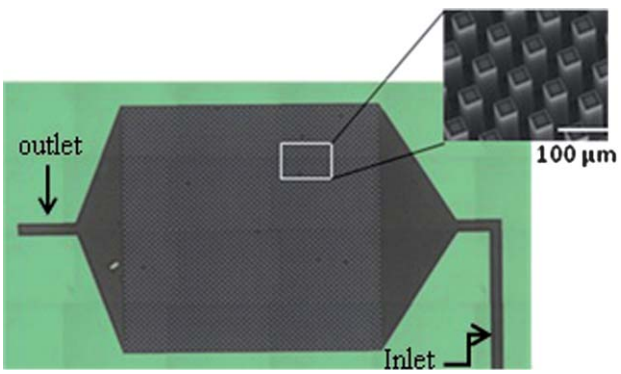
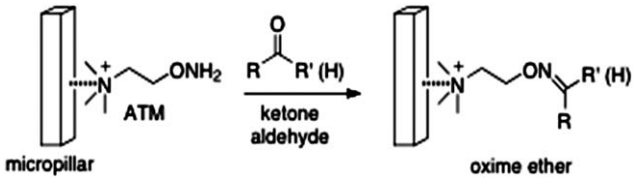


Fig. 1 Optical micrograph of a fabricated microreactor with thousands of internal micropillars. The insert is an SEM micrograph of the micropillar array.



Scheme 1 ATM oximation traps ketones and aldehydes in the microreactor.

microreactor. As illustrated in Scheme 1, the adsorbed ATM on the micropillars react with gaseous ketones and aldehydes *via* oximation to capture the analytes as oxime ether adducts. The adducts subsequently are collected by flowing methanol from one slightly pressurized vial into one sample collection vial, fully eluted in typically 10 μL, well-scaled for the nanoelectrospray ion source of the FT-ICR-MS. The overall process is one of high efficiency preconcentration and lossless analyte transfer for analysis—target ketone and aldehyde gases are derivatized and transferred into solution in microlitre scale volumes after this preconcentration process. The collected liquid samples are directly analyzed by nanoelectrospray FT-ICR-MS for rapid analysis, yet providing superior VOC-adduct identification compared to other MS techniques due to the accurate-mass, ultra high resolution capability. The advantages of direct infusion FT-ICR-MS can be found in a published paper.[30]

Fig. 2 shows schematically the test setup for preconcentration of ketones and aldehydes by the microreactor. For testing the capture efficiency of acetone, various quantities of deuterated acetone, from 0.271 nmole to 0.271 μmole in methanol, were added to the 20 mL vial to evaporate into helium flowing through the vial and then the microreactor. The vial had a Teflon lined silicone septum connected to both helium source and the microreactor by the silica capillary tubing. Acetone-*d6* was used in these tests to avoid bias from any environmental trace acetone contamination. The microreactor operated at an inlet helium pressure from 15 to 25 psi. The flow rate of helium through the microreactor was 2 to 5 mL/min. After adding acetone-*d6* to the vial, helium flowed for at least 30 min to allow completion of the acetone-*d6* transfer into the microreactor. After preconcentration, the microreactor was disconnected from the setup. Then, the microreactor was eluted by flowing 10 μL methanol portions through the microreactor into a septum-sealed glass vial; each aliquot was analyzed separately to gauge elution efficiency *via* mass balance. The collected elution solutions were directly analyzed by nanoelectrospray FT-ICR-MS for 5 min, using calibrations and parameters reported previously[15,30] except the spectral region was selected to be from 50–500 *m*/*z*. Fig. 3 shows a typical FT-ICR-MS spectral region that has oximation product of ATM with acetone-*d6* (165.18688 *m*/*z* ion) as well as unreacted ATM cations

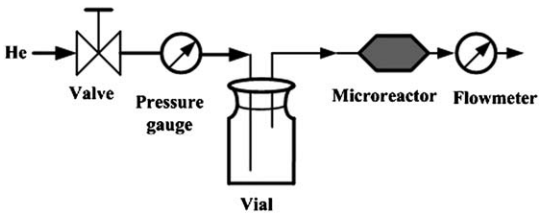


Fig. 2 Schematic flow diagram of the preconcentration setup.

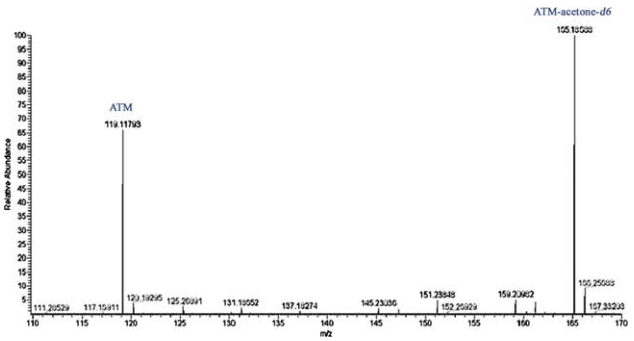


Fig. 3 Positive-ion nanoelectrospray FT-ICR-MS spectrum of pre-concentrated deuterated acetone from the microreactor.

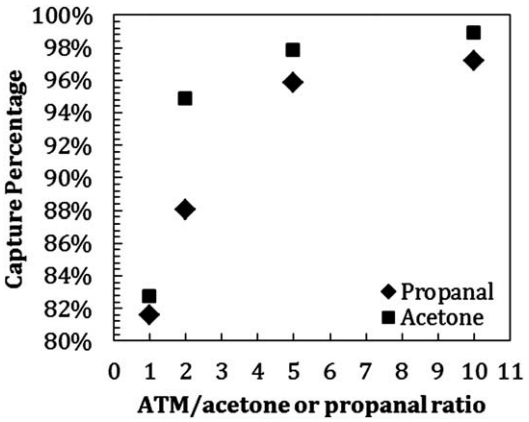


Fig. 4 The effect of ATM/acetone or ATM/propanal ratio on capture percentage of acetone and propanal by the microreactor.

(119.11793 *m/z* ion). Greater than 98% of total reacted ATM and unreacted ATM was collected from the microreactor with the first 10 μL methanol aliquot. There was no trace detectable ATM and ATM-acetone-*d6* after flushing a total 40 μL methanol through the microreactor. Thus, the transfer efficiency exceeded 98% for a rapid 5 min, 10 μL elution at the benchtop, requiring no specialized equipment other than septum-capped vials and a syringe, The total analytical recovery of the acetone-*d6* dosage was >98%. Fig. 2 also indicates that trace acetone contamination from ambient air was very low. Previously reported microfabricated MEMS preconcentrating devices rely on physical adsorption and thermal desorption mechanisms.[28,31] Therefore, the present microreactor approach for preconcentration based on chemical reaction and liquid elution is a fundamentally different class of device from previous MEMS preconcentrators. Furthermore, the preconcentration efficiency is improved by avoiding the current practices of thermal desorption for analyte detection. Rather, in the present study we have found that solvent elution is effective for recovery of more than 98% of the oxime ether adducts. These chemistries combine to make the present microreactor approach attractive for analysis of carbonyl compounds.

Now that the analytical recovery was determined to be >98% for acetone-*d6*, the next task was to study the effect of the ATM/ketone or ATM/aldehyde ratio on the capture efficiency of the microreactor. A series of experiments preconcentrating acetone and propanal with varied ATM/acetone and ATM/propanal ratios was performed by varying the amount of acetone or propanal added into the helium flow through the microreactors in Fig. 2. Each microreactor was loaded a constant amount of 365 nmole ATM. The experimental procedures were the same as described above for preconcentrating acetone-*d6*. After preconcentration, unreacted ATM and reacted ATM adduct were eluted by methanol from the microreactor to a sample vial. Then, a 5μL solution containing 11.4 nmole acetone-*d6* and 117 nmole ATM in methanol was added into each eluted sample for analysis by FT-ICR-MS as internal reference in order to calculate the capture percentage. The reason for high ATM/acetone-*d6* molar ratio was to ensure that acetone-d_6 was completely reacted with ATM so that ATM-acetone-*d6* adduct could be used as the internal reference.

Fig. 4 shows that as ATM/acetone and ATM/propanal molar ratios increase from 1 to 10, the capture percentages also increase. As the ATM/acetone or ATM/propanal molar ratios increased to 100 or higher, 100% capture efficiency has been achieved (not shown in the figure). These results indicate that the microreactor is highly efficient at capturing trace ketones and aldehydes. To capture ketones and aldehydes passing through the microreactor with larger than 98% capture efficiency, a load of ATM with ATM/ketone or ATM/aldehyde molar ratios higher than 10 is required. This requirement can be easily met since VOCs in exhaled breath are in nmol L^{-1} (or ppbv) concentration range.[4,5] Fig. 4 also shows that the capture percentage of acetone is slightly higher than propanal at the same ATM/acetone and ATM/propanal molar ratio. It is likely that the reaction kinetics of ATM with ketones or aldehydes affects the capture percentage of the microreactor.

Further testing was then performed using exhaled breath as a prelude to future non-invasive health diagnosis methods. After approval by the Internal Review Board at the University of Louisville and after having obtained written informed consent, exhaled breath samples were collected from nine voluntary healthy non-smoking subjects in the age range from 20 to 40. The subjects breathed 1L breath air into one liter Tedlar® bags. A mixed alveolar breath and non-alveolar breath was collected. 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 bag was connected to the inlet of the microreactor through septa and the silica tube. The outlet of the microreactor was connected to a Teflon diaphragm dry vacuum pump. Each microreactor was loaded with 365 nmole ATM. After the breath air in the sample bag was evacuated, the microreactor was disconnected from the flow line and then eluted with methanol as described above. A 8μL solution containing 2.32 nmole acetone-*d6* completely reacted with ATM in methanol was added into each eluted sample for analysis by FT-ICR-MS. The ATM-acetone-*d6* adduct served as the internal reference to estimate captured amounts of ketones and aldehydes. Fig. 5 shows a typical FT-ICR-MS spectrum of exhaled breath. C_1 to C_{12} ketones and aldehydes were detected. ATM-acetone-*d6* and excess ATM were also observed. It should be noted that these ketones and aldehydes have the same molecular formulae as those typically measured by the cryogenic condensation method.[16] Table 1 shows estimated minimum, median and maximum values of ketone and aldehyde concentrations in healthy subject breath. CH_2O is formaldehyde and C_2H_4O is acetaldehyde. The mean concentrations of formaldehyde and acetaldehyde are in close agreement with the results measured by SIFT-MS.[23] Constitutionally isomeric

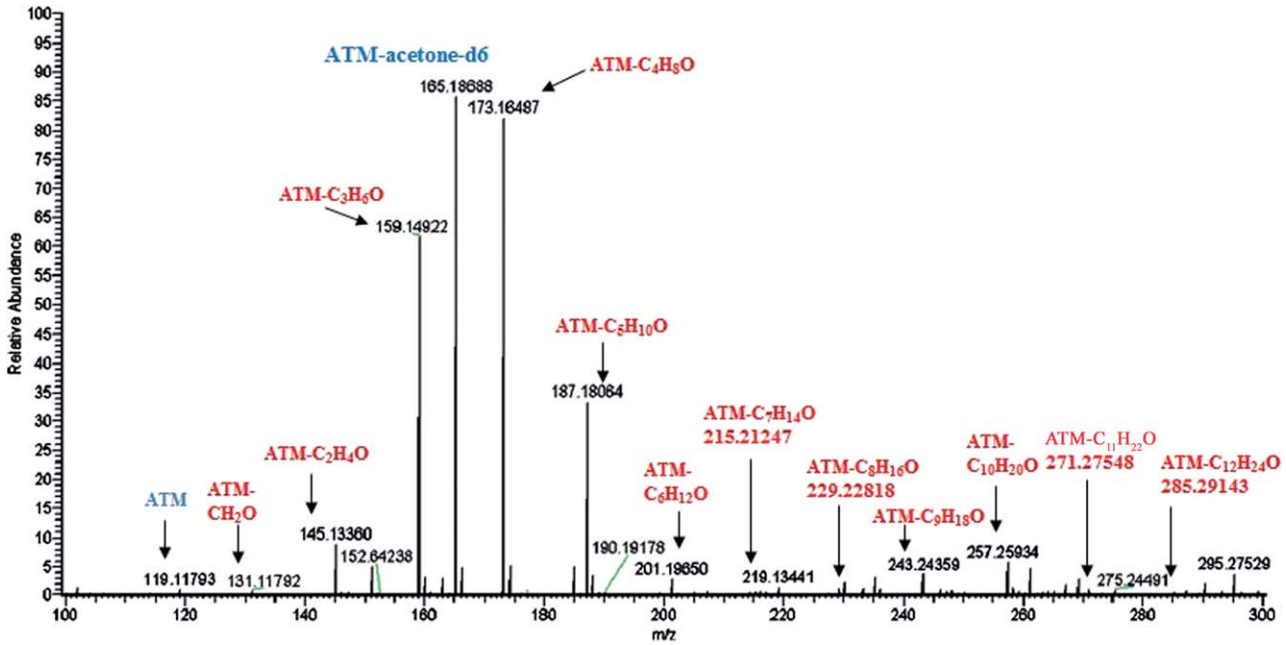


Fig. 5 A typical FTICR-MS spectrum of an exhaled breath preconcentrated by the ATM-coated microreactor.

Table 1 Acetone and aldehyde estimated concentrations of exhaled breath from healthy subjects

Ketone/Acetone	Min. (nmol L^{-1})	Max. (nmol L^{-1})	Mean (nmol L^{-1})	Median (nmol L^{-1})
CH_2O	0.024	0.107	0.057	0.049
C_2H_4O	0.104	0.359	0.219	0.187
C_3H_6O	0.939	2.48	1.536	1.385
C_4H_8O	1.541	8.808	4.022	3.40
$C_5H_{10}O$	1.073	6.615	2.523	1.428
$C_6H_{12}O$	0.065	0.406	0.157	0.135
C_7H1_4O	0.009	0.039	0.016	0.013
$C_8H_{16}O$	0.012	0.181	0.074	0.055
$C_9H_{18}O$	0.014	0.547	0.284	0.325
$C_{10}H_{20}O$	0.167	0.959	0.526	0.471
$C_{11}H_{22}O$	0.025	0.579	0.22	0.197
$C_{12}H_{24}O$	0.019	0.262	0.074	0.065

ketones and aldehydes are indistinguishable by direct infusion one--dimensional FT-ICR-MS, however, the measured molecular weight at a resolving power of 200,000 provides accurate chemical formulas. Since the FT-ICR-MS is also capable of tandem-MS analyses in the same run, this mode can be used to distinguish isomers.

The propanal concentration in healthy human breath is known to be much lower than the acetone concentration.[4,5] Thus, if we assume that the mean concentration of C_3H_6O in Table 1 is principally acetone, the observed value is lower than reported acetone concentrations in exhaled breath of healthy subjects as measured by gas chromatographic methods.[32–34] We are currently using other synthesized aminooxy compounds in combination with GC-MS to separate ketone and aldehyde isomer adducts and to further verify the results listed in Table 1. A commercial aminooxy compound PFBAH in combination with GC-MS has been used to identify ketone and aldehyde adducts in exhaled breath.[4,5,32] The results of this communication demonstrate that the microreactor approach can be used to preconcentrate and analyze C_1 to C_{12} ketones/aldehydes in breath samples rapidly with the aid of accurate-mass assignments, extremely high recovery efficiency (>98% for acetone), non-volatile adducts for convenient sample handling, and utilizing far smaller air sample volumes.

Conclusions

The results of this work show that a microreactor-based aminooxy coating efficiently oximates gaseous carbonyl species for practical analysis of ketone and aldehyde VOCs. The designed microreactor features thousands of micropillars that uniformly distribute gas flow to maximize the interactions with ketones and aldehydes in the gas flow, resulting in capture efficiencies higher than 98%. Moreover, the key inclusion of a quaternary ammonium group in the design of the coating of the micropillar surface greatly enhances electrospray MS analyses. Simple elution of the entire microreactor coating including all captured analytes eliminates sample transfer problems that have plagued previous preconcentration approaches. This lossless elution scheme is enabled by the use of direct infusion nanoelectrospray FT-ICR-MS which has the resolution to avoid interference of analytes by the capture phase, while achieving rapid and highly sensitive analysis of carbonyl VOCs in breath with high-confidence peak assignments

due to the accurate-mass capability. The micro-scale of this approach should be attractive for a broad range of other applications.

Acknowledgements

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