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ARTICLE

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High-throughput reaction screening by mass spectrometry (MS) enables detection of multiple analytes simultaneously without tagging, permitting the starting materials, intermediates, and products to be detected and identified concurrently. Presented here, desorption electrospray ionization (DESI) was coupled with MS to accelerate and screen the formation of highly desired precursors to energetic cage compounds structurally similar to 2,4,6,8,10,12-hexabenzyl-2,4,6,8,10,12-hexaazaisowurtzitane (HBIW) with a variety of amine analogs and acid catalysts. Organic reactions are accelerated in the microdroplets generated from DESI source, due to the evaporation of solvent, the increase in reagent concentration, and higher surface-to-volume ratios, amongst other phenomena. To increase the throughput and create reproducible reaction spots, a low-cost commercial-off-the-shelf automated pipetting robot was used to prepare and spot the reaction solutions. Once spotted, the reaction mixtures were analyzed using a low-cost and homebuilt DESI stage coupled to a linear ion trap MS. The expected cage products, HBIW and analog cages, were successfully formed using benzylamine, bromo-benzylamine, and methoxy-benzylamine as the amine starting material, demonstrating this budget-friendly setup as a rapid and high-throughput screening set-up for exploring alternative complex cage structures. Several acids were also screened for alternative acid catalyst options. Compared to other expensive automated pipetting systems and DESI setups, this design has reduced throughput (6000 vs 100 samples per hour), but it provides a budget-friendly, open-source option.

Introduction

High-throughput screening (HTS) has been applied in the fields of drug discovery,[1] biological assays,[2] and reaction screening[3] to identify compounds with potential pharmacological or biological activity, discover new reaction pathways, or optimize reaction conditions.[4] Methods that incorporate HTS typically utilize optical detection methods such as luminescence or fluorescence[5] but require analytes to be tagged with molecules that are optically responsive. In addition, tagging often involves additional steps that contribute to the overall analysis time. Mass spectrometry (MS) can be used for HTS without labeling or tagging target analytes and it provides the versatility of monitoring multiple targets.[6] Liquid-chromatography mass spectrometry (LC-MS),[7] microfluidics,[8] ambient ionization,[9] and many other techniques[6a, 10] have been used for HTS.[6b, 6c]

Desorption electrospray ionization (DESI) is an ambient ionization technique that utilizes charged microdroplets which are pneumatically directed towards a surface.[11] These primary microdroplets collide with analyte molecules, (*e.g.* reaction mixtures that are deposited onto the surface) and desorb these analytes as secondary droplets to be analyzed by a mass spectrometer.[12] Microdroplets formed by DESI[13] or other electrospray ionization techniques have been used extensively as microreactors for reaction acceleration.[14] As a result, there have been reports that product formation is achieved orders of magnitude faster in microdroplets than the same reaction performed in bulk.[15] These interfacial microdroplet reactions are proposed to have faster rate constants and reaction rates, due to the larger surface-to-volume ratios and air-liquid interfaces compared to conventional bulk synthesis. Additionally these systems have been reported to have increased reagent concentration from solvent evaporation, pH extremes, undergo rapid mixing, and have partially solvated reagents with a decreased energy barrier.[16] Mechanistic and fundamentals studies are on-going, but there have been reports that under these unique conditions, reactions that require catalysts in bulk can proceed without catalysts.[17]

Applications for DESI reaction acceleration have included several organic reactions with pharmaceutical relevance to help screen through reaction conditions and reagents,[18] including the Suzuki cross-coupling, reductive aminations, aldol reactions, N-alkylation, nucleophilic aromatic substitutions, and screening enzymatic bioassays.[19] Cooks *et al*. developed an automated, high-throughput setup based on the Beckman Biomek liquid handling system for spotting and DESI for reaction screening.[19c-f, 20] The Beckman system is fully automated and interfaces with the DESI stage. The liquid handling system can also transfer slides from the robot to the mass spectrometer and can screen over 6000 samples per hour. Using DESI-MS for small-scale syntheses and reaction screening provides quick spectral data on whether the product was formed that can be simplified to yes/no responses.[20-21] These responses save time,

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money, and reduce waste by determining if a reaction will work on the small scale before performing the reaction in bulk.[19g] Small-scale syntheses are especially useful tools for determining if novel reactions form the desired products before scale-up. They reduce waste and enhance safety for potentially hazardous syntheses (*e.g.* energetic materials).

Energetic materials research is geared towards developing explosives and propellants with higher performance and reduced sensitivity. Heterocycles, specifically cyclic nitramines, are of particular interest due to their higher heats of formation, densities, and oxygen balance when compared to carbocyclic compounds.[22] 2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (HNIW), also known as CL-20, is a very powerful high-energy and high-density explosive material due to its strained, heavily-nitrated cage structure.[23] Traditional synthesis of CL-20 involves the condensation of benzylamine and glyoxal to form 2,4,6,8,10,12-hexabenzyl-2,4,6,8,10,12-hexaazaisowurtzitane (HBIW), which acts as the basic cage structure.[24] The reaction mechanism of this condensation involves the formation of several intermediate structures containing dicarbinolamine or diamine functional groups.[24c, 25] Formic acid is used as the acid catalyst for the condensation reaction. Acetonitrile is typically used as a solvent. Once HBIW is formed, catalytic hydrogenation occurs, resulting in the removal of the benzyl group and subsequent nitration.[26]

Most of the development of novel energetics are performed under traditional synthetic methodologies (round bottom flask chemistry) but HTS microdroplet chemistry provides the ability to examine a plethora of starting materials in a compressed timeline without the generation of excess of hazardous and energetic waste. Additionally, screening reactions using smaller volumes provides an inherent safety aspect to performing novel small-scale reactions with unknown hazards. In this work, the synthesis of HBIW was utilized as a model reaction to demonstrate the screening capabilities of the DESI setup for novel energetic synthesis. Herein, we combine a low-cost pipetting robot with a home-built DESI source for reaction acceleration and to demonstrate high-throughput screening of substituted amines and acid catalysts that form a variety of energetic precursors analogous to HBIW. The pipetting robot provides an automated workflow and low-cost alternative to prepare the DESI target with the various reactions mixtures to be tested, compared to the expensive and potentially cost-prohibitive alternatives previously reported.[21] Subsequent DESI reaction acceleration and mass spectrometry analysis provides quick intermediate and product determination and can rapidly determine which amines can be used to form the cage structure. Furthermore, DESI screening can be utilized to optimize reaction conditions for interesting reactions.

Experimental

Materials

Glyoxal (40% wt in H_2O), benzylamine, 4-methoxybenzylamine, trifluoroacetic acid, glacial acetic acid, formic acid (LiChropur) and methanol were purchased from Millipore Sigma (Burlington, MA). 4-bromobenzylamine was purchased from Oakwood Chemical (Estill, SC). Acetonitrile (HPLC LC-MS grade) was purchased from VWR chemical (Radnor, PA). Solutions were stored in 1.5 mL Eppendorf microcentrifuge tubes (Enfield, CT) and 15 mL Falcon Conical Centrifuge tubes (Fisher Scientific, Waltham, MA). PTFE coated glass slides, purchased from SPI Supplies (West Chester, PA), were used as the DESI substrate.

Automated Pipetting Robot

An Opentrons (New York, NY) OT2 robot was equipped with a P1000 Single Channel Pipette (Gen2) and a P20 Single Channel Pipette (Gen2). A 4-in-1 Tube rack set from Opentrons was used for bulk sample storage, specifically the 10-tube rack with Falcon 4 x 50 mL, 6 x 15 mL conical tube slots and the 24-tube rack with Eppendorf 1.5 mL slots for diluted reaction mixtures. Pipette tips, 20 µL and 1000 µL with tip rack base that locks into the robot deck, were purchased from Opentrons. A custom labware definition (.json file) for the slide holder was also created in collaboration with Opentrons and has been provided in the Supporting Information. The .json file is used within the protocol designer to add the custom labware to the list of labware to be used in the protocol being created. The **"*DESI_Prep*"** and **"*DESI_Spot*"** protocols designed for these experiments are detailed below and the corresponding files can be found in the **Supporting Information**. After the reactions were spotted onto the DESI substrate, the substrate was manually transferred to the DESI automated stage. Automation of this step has been demonstrated by more sophisticated robotic setups but to keep in-line with the scope of low-cost automation, the manual transfer step was deemed sufficient human interaction.

3D-Printing Parameters

A custom labware DESI slide holder was printed using a MakerGear M2 3D-printer (Beachwood, OH). EcoMax PLA filament from 3DXSTAT (Grand Rapids, MI) was used to print the holder. In collaboration with Opentrons, the custom slide holder was designed based on the dimensions of the slide and the size of the slots on the robot deck. The 3D rendering (.STEP file) has been provided in the **Supporting Information**. The .STEP file was used to 3D-print the custom holder by converting it to an STL file and sliced using Simplify3D (Cincinnati, OH). The glass printing platform was prepared with Kapton Tape and heated to 60 °C. A 0.35mm stainless steel extruder nozzle was utilized and was held at 215 °C for the entire print. **Figure S1A** shows a schematic of the slide holder including the dimensions in mm. Photographs of the 3D-printed holder and the Teflon coated slide are shown in **Figure S1B-S1C**.

Desorption Electrospray Ionization Mass Spectrometry (DESI-MS)

A custom DESI source was constructed using a Swagelok tee union, NanoTight™ sleeves, a PEEK union assembly, a fused silica capillary (ID 100 µm OD 200 µm) for spray solvent, and a fused-silica capillary (ID 255 µm OD 360 µm) for sheath gas. A photograph of the connections can be seen in **Figure S2**. Methanol, the spray solvent, was pumped through the fused

silica capillary using a syringe pump (PHD Ultra, Harvard Apparatus) at 2.5 µL/min. The N_2 gas pressure was 100 psi. High voltage (4.5 kV) was applied to the syringe using the high voltage power supply from the mass spectrometer. The emitter was positioned 1 mm from the surface at a 54° angle and 4 mm from the MS inlet.

The DESI source was mounted on a custom, automated stage. The aluminum breadboard base and the connection pieces used to attach the stage to the LTQ frontend were based on schematic drawings from the University of Washington's Proteomics resource for building a custom nanospray ionization source.[27] Pictures of the full stage setup with part numbers are included in the supplemental **Figure S3**. A full list of parts, vendor, part number, and cost of each part at the time of publication is provided in the **Table S1**. The xy translational stage is controlled using Thor Labs Kinesis software. For each line scan, the stage velocity was 0.05 mm/s. Due to the constant velocity of the stage, each set of scans could be correlated to an individual spot. Each spot was analyzed for the targeted *m/z*.

Mass spectra were collected using a Thermo LTQ (San Jose, CA) instrument. The instrument's front end was modified to enable ambient ionization by defeating the interlock and adding an extended cable to the instrument's voltage power supply. A 2 cm extended ion transfer capillary (Scientific Instrument Services; Palmer, MA) was also used to increase the coverage of the sampling area on the DESI slide. Mass spectra were collected in positive-ion mode and the spray voltage was set to 4.5 kV. The capillary temperature was set to 300 °C. Other notable instrument settings include 100 ms injection time and 3 microscans.

Results and Discussion

Opentrons Protocol Development

Two automated pipetting protocols were designed to prepare and spot the appropriate reaction mixtures onto DESI substrates. The reaction scheme for the condensation of benyzlamine and glyoxal to form HBIW is shown in **Scheme 1**. The "***DESI_Prep"*** and ***"DESI_Spot"*** protocols were designed using the online protocol designer (https://designer.opentrons.com/) from Opentrons. Stock solutions of Glyoxal (200 mM) in water, acetonitrile, formic acid (FA), trifluoroacetic acid (TFA), glacial acetic acid (AA) and amines 1-3 (200 mM) in acetonitrile were needed to setup the starting deck state seen in **Figure 1A**. Opentrons 24 tube rack in slot 2 was used to hold the starting stock solutions and 3 empty 1.5 mL Eppendorf tubes for the 50 mM glyoxal solution with 1% acid that will be prepped using the robot. Tip racks for 20 µL and 1000 µL pipette tips are placed in slot 1 and 4 respectively. Slot 3 holds the custom slide holder created with Opentrons to hold four PTFE coated glass slides. **Figure 1B** shows the final deck state after the **"DESI_*Prep*"** protocol has completed. In slot 2, the gray circles represent the glyoxal solutions that was made using the robot. These solutions along with the three stock amine solutions were used for the "***DESI_Spot***" protocol to prepare the DESI slide. **Figure 1C** represents the final deck state after the "**DESI_Spot**" protocol has finished, and the gray circles show that the DESI slides have been spotted. A picture of the complete deck setup is shown in **Figure 1D**. A zoomed in view of the Eppendorf holder in slot 2 is shown in **Figure 1E**. In column 1, the Eppendorf tubes contain amines 1-3, column 2 contains the stock acid solutions, and column 3 contains the 50mM glyoxal 1% acid solutions made during the protocol.

After the working glyoxal acid solutions were made, 1 µL of the amine solution was co-spotted with 2 µL of the glyoxal solution onto the respective DESI spot. **Figure 2** shows the resulting slide layouts for the two slides spotted using this protocol. Slide 1 was spotted with benzylamine (BA) and two substituted benzylamines, bromo- (BrBA) and methoxy-benzylamine (mBA). This slide was spotted to test the reproducibility of the DESI signal across a single line scan. Slide 2 contains each amine co-spotted with FA, TFA, or AA to screen acid catalyst. The blank spots were used for DESI setup and slide alignment.

DESI Assembly and Setup

A custom, low-cost DESI stage was designed and constructed for accelerated reactions and screening of the formation of traditional HBIW, HBIW analogs and acid catalysts. Several iterations were constructed until the signal and positioning was optimized. Updates to the stage and emitter construction included adding a rotational mount, replacing the LTQ ion transfer capillary with an extended bent capillary, and reducing the size of the capillary used for the sheath gas. Progression photos of the emitter and stage construction are shown in **Figure S4**.

The rotational mount was incorporated into the spray emitter holder such that the angle of the DESI emitter could be precisely controlled. The commercial LTQ ion transfer capillary was replaced with an extended ion transfer capillary to help cover more area of the DESI slide surface. The commercial extended capillary was modified in-house (bent ~10°) to increase ion collection and transmission. The stainless-steel tubing originally used to provide sheath gas around the silica solvent capillary was replaced with a 255 µm ID silica capillary to improve the spot size and improve reproducibility.

DESI Reproducibility for Cage Formation Screening

To determine the reproducibility of DESI's signal intensity for reaction acceleration, the same reaction mixture was spotted on the DESI substrate across each row. Line scans were collected for each row, each collecting in full scan mode to monitor the starting material, intermediates, and product of each reaction. Line 1 recorded the traditional HBIW reaction of BA with glyoxal and 1% FA. Lines 2 and 3 recorded mass spectral data for BrBA and mBA, respectively. **Figure 3** contains the total ion chronogram (TIC) of the line scans for each reaction and the extracted ion chronogram (EIC) for each product observed. The full scan mass spectra can be viewed in **Figure S5**.

Each TIC shows that overall signal increased when traversing a spot that contained the reaction mixture. Most of the signal is stable across the entire spot. However, some spots the signal decreases in the middle consistent with "coffee-ring" effects observed when spotting onto glass and the analytes drying in

the outer ring of the spot. The signal is reproducible across the entire row for each reaction, showing no bias in one section of the slide or certain spots. The EIC for each product are also shown to demonstrate that the product signal is associated with the spot where the reaction mixture was deposited, and no carry-over is observed after the spray leaves the spot. Analogous HBIW products were observed for each substituted amine indicating that DESI can be used to screen new reagents and quickly get a simple confirmatory answer for novel reactions. Replicates are suggested for all studies to ensure that no spotting issues occurred with the robot, nor any issues with the DESI-MS. The reproducible signal observed at the larger spot sizes (µL vs. nL) compared to more costly robotic systems reported may aid in ensuring that sample is analyzed in every scan.

DESI Acid Catalyst Screening

Using DESI reaction acceleration is useful for screening substituted starting materials, but it can also be utilized to screen acid catalysts for this reaction. Traditional HBIW synthesis uses formic acid as the acid catalyst. However, other acids could help increase product yield. The robot was used to spot reaction mixtures containing the three substituted amines as well as three different acid catalyst. Formic acid, trifluoroacetic acid and acetic acid were screened using the DESI setup. In **Figure 4**, the TIC for each line scan is shown as well as the EIC for the respective product formed. For BA, FA showed the highest amount of product, with AA forming some product, and TFA not forming product at all. The full scan mass spectra can be seen in **Figure S6**. For both BrBA and mBA, all three acids form product, with FA and TFA being equivalent and AA forming slightly less product based on relative signal intensity. This setup provides an approach to screen acids with varying pKa values to determine optimal conditions for product formation.

Conclusions

A custom DESI stage was constructed and coupled with an automated pipetting robot to serve as a low-cost alternative to high-end robots and commercial DESI imaging stages. The Opentrons robot cost of ~$6k, including pipettes and various accessory options and the DESI stage built with commercial readily available parts for an additional ~$6k is order of magnitudes lower than other reported systems. Although the throughput is diminished compared to these larger and more complex robotic systems, as a low-cost option, this DESI and robot combination can accelerate the reactions of interest and provide reproducible results at a low entrance fee. Of similar importance DESI can be utilized to screen complex cage structures in a high throughput fashion. Product was observed for all three substituted amines, confirming the formation of HBIW, and two HBIW analogues. This system was also useful for screening acid catalyst, supporting the previous literature that FA is the best catalyst for the traditional HBIW reaction. However, all three catalysts form the desired product when used to catalyze the condensation of benzylamine alternatives, demonstrating this low-cost system can be a rapid and environmentally friendly precursory screening technique for novel molecules prior to bench scale synthesis. The scope of the low-cost platform can be easily scaled to explore a wide combination of reagents, solvents, catalysts, pH, and even temperature through the optional Opentrons Temperature Module for a plethora of target compounds. The reactions that show product formation can then be down selected and scaled at the bench, saving time on exploratory syntheses.[19c, 28]

Author Contributions

HMB contributed to data curation, investigation, methodology, and writing the manuscript for this work. PWF contributed to conceptualization, funding acquisition, project administration, and writing the manuscript for this work.

Conflicts of interest

There are no conflicts to declare.

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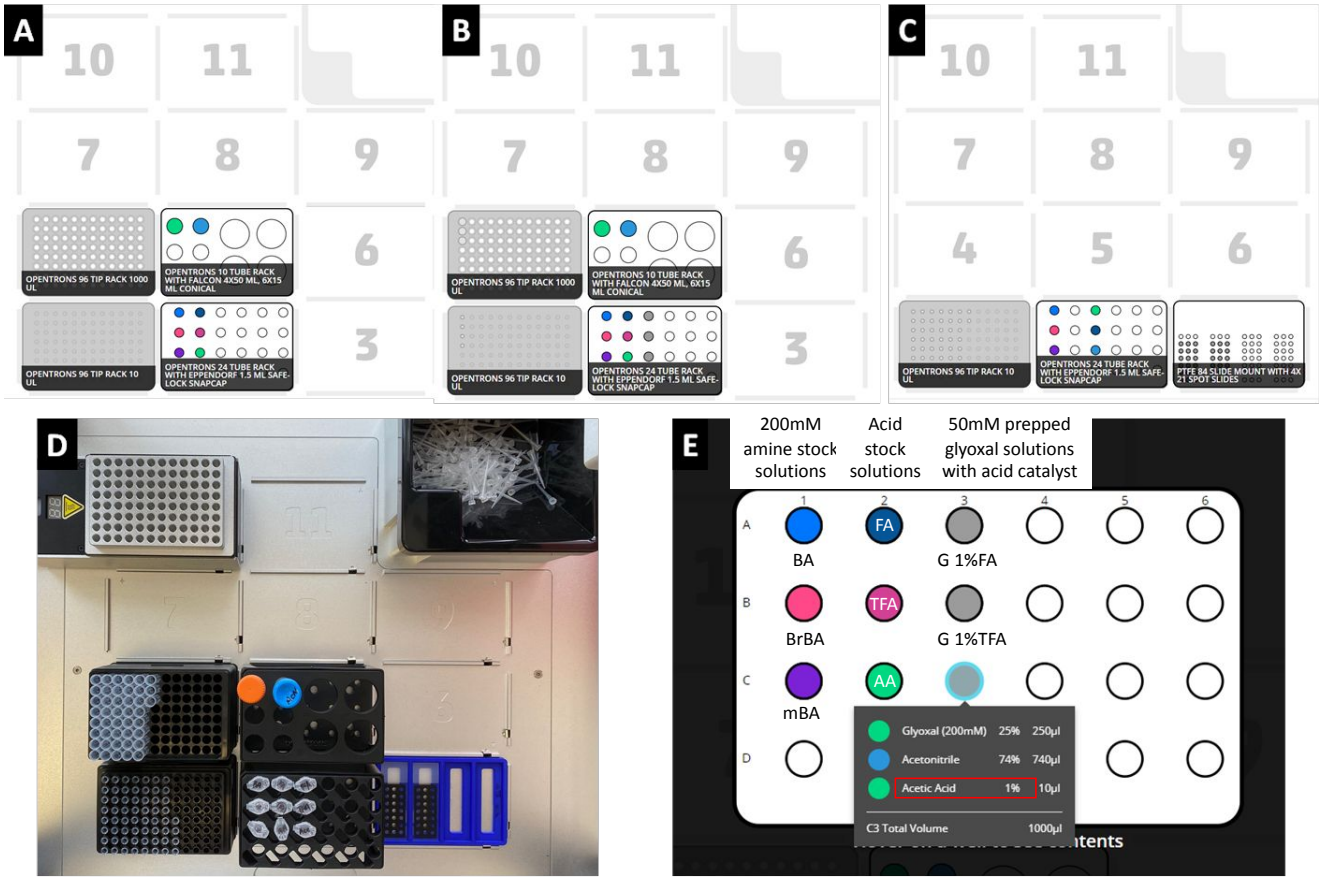


Figure 1. (A) Starting deck state of the Opentrons robot for the *"DESI*_Prep*"* protocol (B) Final deck state of the Opentrons robot for the *"DESI*_Prep*"* protocol (C) Final deck state of the Opentrons robot for the *"DESI*_Spot*"* protocol (D) Picture of the deck layout (E) Zoomed view of Opentrons 24 Tube Rack from slot 2 in final deck state of "DESI_Prep" protocol.

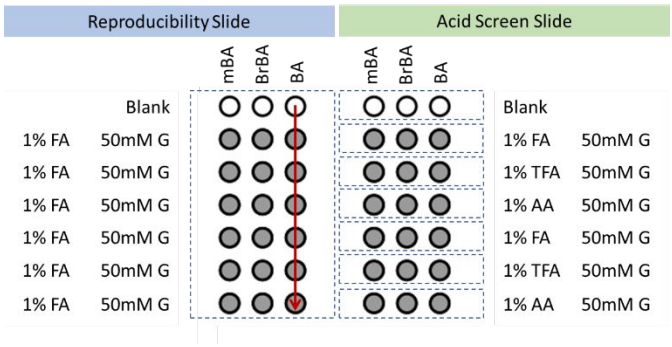
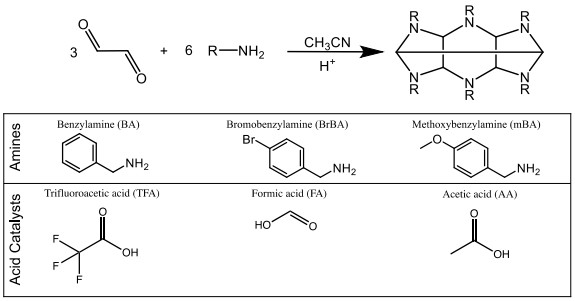


Figure 2. Representation of spotted slides after the "DESI_Spot" protocol has completed. One µL of 200 mM amine and two µL 50 mM glyoxal with 1% acid were added to each well, resulting in three µL per well. The final concentration was 67 mM amine and 33 mM glyoxal with 0.67% acid. The red arrow indicates the direction in which data was collected, starting with the blank spot. The left depicts the reproducibility screening for methoxy-benzylamine (mBA), bromo-benzylamine (BrBA), and benzylamine (BA) reacting with glyoxal (G) and formic acid (FA) as the acid catalyst. The right illustrates the screening of acid catalysts FA, trifluoracetic acid (TFA), and acetic acid (AA).



Scheme 1. Condensation reaction step to form HBIW intermediate. Traditional HBIW formation is carried out with benzylamine and formic acid. Bromo- and methoxy-benzylamine were substituted in this reaction to form HBIW analogs. Formic acid was also substituted with trifluoroacetic acid and acetic acid to screen acid catalysts.

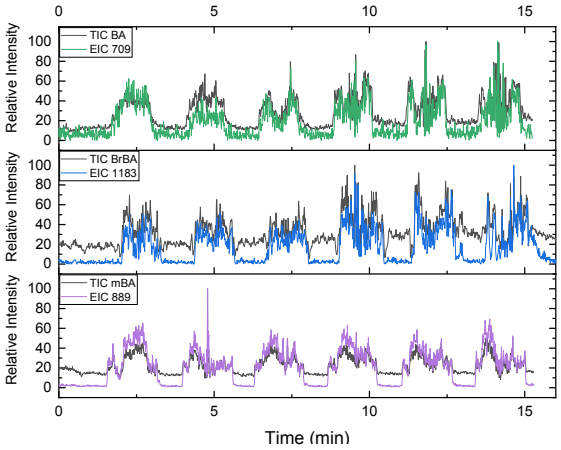


Figure 3. Total ion chronogram (TIC) and extracted ion chronograms (EIC) are shown for the reproducibility DESI slide. The line scan includes six spots with the same reaction mixture across the slide. The EIC for benzylamine product (*m/z* 709) is in green, blue for the bromo-benzylamine product (*m/z* 1183), and the methoxy benzylamine (*m/z* 889) in purple.

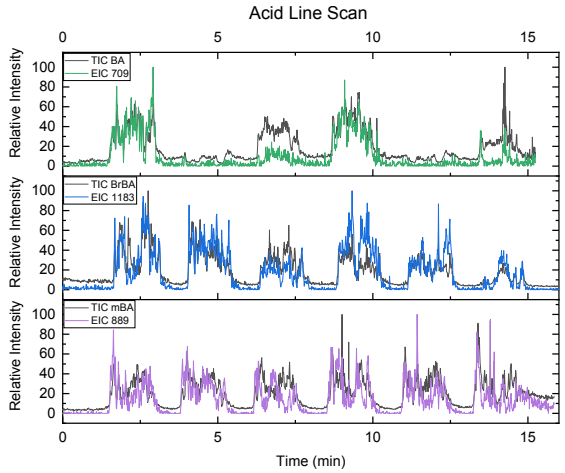


Figure 4. Total ion chronogram (TIC) and extracted ion chronograms (EIC) are shown for the acid screening DESI slide. The line scan includes six spots with formic acid spotted in the 1st and 4th position, trifluoroacetic acid spotted in the 2nd and 5th position, and acetic acid spotted in the 3rd and 6th position. The EIC for benzylamine product (*m/z* 709) is in green, blue for the bromo-benzylamine product (*m/z* 1183), and the methoxy benzylamine (*m/z* 889) in purple.