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Complex mixture analysis by two-dimensional mass spectrometry using a miniature ion trap

Lucas J. Szalwinski, R. Graham Cooks*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, United States

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ABSTRACT

Quadrupole ion trap mass spectrometers are robust instruments that can perform tandem mass spectrometry (MS/MS) while being operated under moderate vacuum conditions. These features make quadrupole ion traps an attractive option as miniature mass spectrometers, particularly because the sensitivity and selectivity gained from employing MS/MS does not decrease with reduced instrument size. Recent developments in scan methodology have allowed quadrupole ion traps to be used to perform two-dimensional MS/MS (2D MS/MS) experiments which identify all precursor ions and their dissociation products. This study describes the implementation of this single scan in a miniature mass spectrometer where sample amount is limited, and time is prioritized. Remarkably, the miniature instrument 2D MS/MS experiment demonstrated greater sensitivity than a benchtop ion trap mass spectrometer utilizing the 2D MS/MS scan. Even more notably, the 2D MS/MS experiment performed using the miniature instrument achieved higher mass resolution than the benchtop instrument in both precursor and product ion dimensions. The miniature instrument's performance was tested on mixtures of drugs of abuse in the presence of a matrix as well as on a mixture of phospholipids.

Introduction

Mass spectrometers have become indispensable in analytical research labs. In order to cater to many different application areas, commercial mass spectrometer development has centered around combining different instrumental components to create integrated platforms [1,2]. These innovations prioritize mass resolution and sensitivity over instrument simplicity, speed, and size. On the other hand, miniature mass spectrometers are typically built for a specific type of analytical problem [3–6]. The size requirements are stringent, only allowing for relatively simple instrumentation. The instruments are designed to have sensitivity and selectivity appropriate for designated problems. Because of the design requirements, innovations demonstrated in commercial or modified commercial benchtop mass spectrometers are typically achieved with significant performance loss when transferred to miniature mass spectrometers. For example, high-resolution ($R > 10^5$) benchtop instruments have become commonplace in part due to improvements in both time-of-flight and orbitrap mass analyzers [7,8]. Neither of these mass analyzers transfers well to miniature instruments due to their high vacuum requirements.

Quadrupole ion traps do not require precise machining tolerances or high vacuum, making them an attractive option for miniature mass spectrometers [9–12]. Another feature that has made miniature quadrupole ion traps popular is their ability to perform tandem mass spectrometry (MS/MS). Traditionally, quadrupole ion traps require ion isolation in order to acquire interpretable MS/MS data. The efficiency in terms of both sample and time in which MS/MS data is obtained by this method is relatively poor because a new ion population must be introduced for each scan. Previously, our group demonstrated a method of avoiding ion isolation yet obtaining MS/MS data, so allowing for the implementation of precursor and neutral loss scan modes [13,14]. MS/MS was performed by mass selectively fragmenting precursor ions while simultaneously ejecting the product ions orthogonally into the detector. The improved efficiency, and thus speed, of these scan modes was due to the ability to interrogate many different precursor ions after their trapping in a single ion injection event. This improvement was expected to be particularly valuable when using miniature instruments that utilize a discontinuous atmospheric pressure interface (DAPI) [15] because of the long dwell time required to achieve operating vacuum after each ion injection. Remarkably, the miniature instrument implementation of precursor and neutral loss scans showed improved sensitivity over that of commercial benchtop instruments [16].

Although the implementation of precursor and neutral loss scans marked a substantial improvement of MS/MS efficiency, following on work by Gäumann and coworkers [17], we recently demonstrated that the entire 2D MS/MS data domain, containing two dimensions of mass and one of intensity, can be recorded in a single ion introduction event [18–20]. The 2D MS/MS scan is the most efficient way to obtain MS/MS

* Corresponding author.
E-mail address: cooks@purdue.edu (R.G. Cooks).

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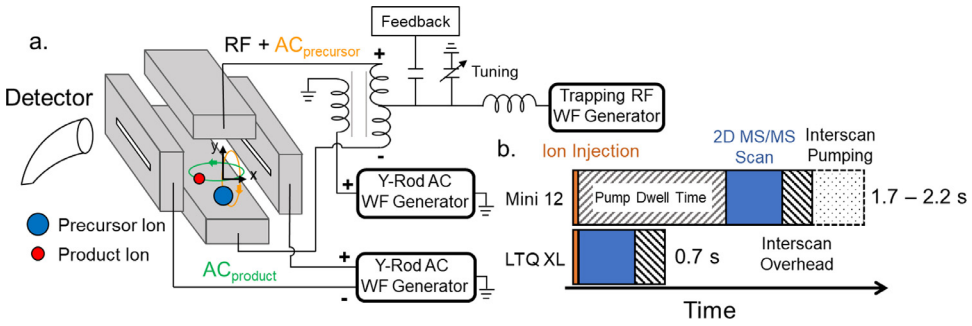

Fig. 1. (a) Schematic of applied waveforms on the Mini 12 mass spectrometer. (b) Typical 2D MS/MS scan table for both the Mini 12 and LTQ XL mass spectrometers.

data on a complex sample because of the high mass scan rate and the absence of ion isolation. In this study, the 2D MS/MS scan was implemented in a miniature quadrupole ion trap and the performance was compared to its benchtop counterpart for the detection of fentanyl and related fentanyl analogs (fentalogs). Then, the 2D MS/MS scan on the miniature mass spectrometer was used to interrogate a sample containing a high concentration of matrix. Finally, a sample containing many lipids was interrogated to determine the method's feasibility for providing a miniature lipidomics platform capable of determining lipid profiles.

Experimental

Chemicals

Fentanyl analogs and other pharmaceuticals were purchased from Sigma Aldrich (St. Louis, MO) and diluted in methanol. For the fentanyl quantitation and resolution comparison, fentanyl was diluted to 1000, 500, 300, 100, 50, 25 ppb (ng/mL) with acetyl fentanyl as an internal standard held at 250 ppb. The 13 fentalog mixture was made by diluting each fentalog from a standard solution so that each fentalog had a final concentration of 100 ppb. A 100 μL solution of Differential Ion Mobility System Suitability Lipidomix (Avanti Polar Lipids, Inc, Alabaster, Alabama) was dried and redissolved in 400 μL of acetonitrile/methanol/300 mM ammonium acetate (3:6.65:0.35, v/v/v) so that each lipid, besides 14:1 PI, had a final concentration of 250 ppm (PI was at 62.5 ppm).

Instrumentation

A previously modified Finnigan LTQ mass spectrometer (San Jose, CA) utilizing the 2D MS/MS scan was used for all benchtop mass spectrometry experiments [18,19]. A modified Mini 12 mass spectrometer developed at Purdue University was used for all miniature mass spectrometry experiments [16,21]. The 2D MS/MS scan utilizes a constant trapping RF voltage as well as the capability to apply auxiliary waveforms individually to the x- and y-rod pairs. The RF trapping voltage of the modified LTQ mass spectrometer was controlled using an external waveform generator as described previously [14]. The Mini 12 has user-controlled RF voltages accessible within the user interface, so no modification was needed. The Mini 12 uses capacitively coupled feedback circuitry to minimize errors in the RF trapping voltage. In both instruments, auxiliary waveforms were applied to the x-rod pair without any other modification; however, both instruments required modification in order to apply auxiliary waveforms to the y-rod pair [16]. The Mini 12 was improved by further optimization of the secondary transformer using a new iron core toroidal transformer (Laird Technologies LFB180100-000, Earth City, MO, USA) and wrapping the transformer in glass wool to avoid discharge across the toroid. This resulted in a change in the resonant frequency from 1.0 MHz to 1.1 MHz. Fig. 1a details how the trapping RF and auxiliary AC were inductively coupled to the x- and y- rod pairs.

Scan time

Fig. 1b depicts the difference in scan time between the benchtop and miniature mass spectrometers. The scan table for the 2D MS/MS scan on the modified benchtop instrument has been described previously [18]. The scan table used in the Mini 12 is similar except for one key difference. The Mini 12 requires approximately one second after sample introduction before mass analysis can be performed in order to remove neutral molecules to achieve appropriate vacuum [22]. A period of 100 milliseconds was added to the end of each scan on both instruments in order to ensure all electronics are ready for the next scan. However, additional time is added to the Mini 12 scan to ensure that the vacuum is at steady state. This time was made sufficiently long during optimization of scan parameters and no further changes are required once optimization is complete. The additional time allocated for achieving appropriate pressures during the scan is the main reason for the decreased duty cycle of miniature instruments utilizing DAPI interfaces. The effect of decreased duty cycle is felt most when performing product ion scans as each additional scan requires re-introduction of a new precursor ion population as the precursors are removed during ion isolation in order to correlate product ions to each activated precursor ion in turn. The 2D MS/MS scan avoids ion isolation and re-introduction of analytes by fragmenting each precursor ion sequentially while continuously monitoring for product ions. The decrease in duty cycle is one motivation for implementing the 2D MS/MS scan as it provides more MS/MS data per ion introduction event than do other MS/MS scans. Another improvement is that no precursor ions are wasted during ion isolation as all precursor ions are fragmented in each 2D MS/MS scan.

Results and discussion

Comparison of performance for the detection of fentanyl

In order to characterize sensitivity and resolution, fentanyl was interrogated using a 2D MS/MS scan on the miniature and modified commercial instruments. In both instruments, the trapped precursor ion was held at the same q-value, 0.357, and because m/z and q-values are proportional, the product ions for both instruments occur at the same q-value. Both the miniature and benchtop 2D MS/MS scan covered the same range of q-values over the same scan time in order to minimize differences caused by different scan rates. The resulting three-dimensional data for fentanyl collected by the 2D MS/MS scan in both instruments is shown in Fig. 2. The peak widths at half of the maximum amplitude (FWHM) for the benchtop are 5.9 and 5.4 Thomson (Th) in the precursor (337 Th) and product ion (188 Th) dimensions, respectively. Surprisingly, the miniature instrument shows the better resolution with the FWHM decreasing to 3.5 and 3.0 Th for the precursor (337 Th) and product ion (188 Th), respectively. Previous comparisons of performance between the two instruments demonstrated that the benchtop LTQ outperformed the Mini 12 in terms of resolution in full mass scans [23] (LTQ: 0.7, Mini 12: 3 Th) and in single-analyzer precursor ion/neutral loss scans [16] (LTQ 0.5 Th, Mini 12 1 Th). The resolution obtained using

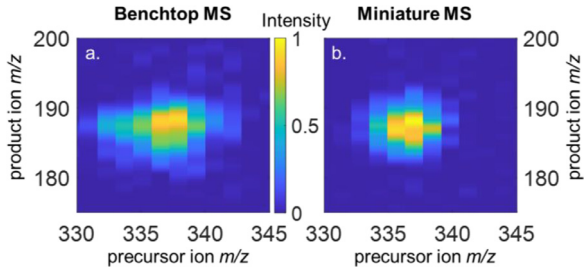

Fig. 2. Comparison of the 2D MS/MS data for fentanyl showing the characteristic precursor/product ion (Pre/Pro, 337/188 Th) using 2D MS/MS scans in a modified (a) benchtop LTQ XL mass spectrometer and (b) Mini 12 mass spectrometer.

the LTQ 2D MS/MS scan is unusually poor compared to the resolution usually obtained when the instrument is operated conventionally due to the higher scan rates used in 2D MS/MS. The improved miniature system resolution is likely due to contributions from higher order fields and the higher operating pressure. The miniature instrument is operated at a higher pressure and has more significant contributions from higher order fields [24,25]. The effect of higher order fields on resonance ejection for mass analysis has been extensively studied using numerical simulations [26,27]. However, the current study combines the fragmentation and ejection processes so comparison to previously explored systems is difficult.

Evaluation of the sensitivity of each system was performed by constructing calibration curves for each instrument. These calibration curves (SI Fig. S1) showed adequate quantitative performance across the ppb range for fentanyl. The limit of detection for fentanyl was determined as the concentration that would give S/N of 3. The miniature instrument yielded a limit of detection of 3 ppb compared to 37 ppb for the LTQ benchtop instrument. A similar more favorable performance of the miniature instrument was previously demonstrated for neutral loss/precursor ion scans on the same systems [16]. Previously, it was shown that moving from helium to nitrogen/air was a major factor for improved sensitivity in that experiment. However, it is unlikely that the difference in bath gas composition is the only factor affecting sensitivity; the greater contribution from octopolar fields in the Mini in improving the fragmentation efficiency is also involved.

Identification of fentalogs by mini 2D MS/MS

To better compare the performance of both systems, a mixture of 13 fentalogs (Fig. S5) was analyzed. The 2D MS/MS spectrum for each system is shown in Fig. 3. The first notable difference between the two spectra is that the systems have different mass-dependent transmissions. The miniature instrument performs better in detecting low mass product ions from low mass precursor ions while the benchtop instrument has a higher transmission of higher mass product ions arising from higher mass precursor ions. This result can be explained by the intra-scan pressure changes within in each instrument. The pressure in the benchtop instrument is constant throughout the whole scan. The pressure in the miniature instrument decreases throughout the scan due to pumping following discontinuous sample introduction. The decrease in collisional cooling with decreasing pressure affects the 2D MS/MS experiment in two major ways. The precursor ions are more likely to be ejected before they fragment, and the fragment ions produced are not sufficiently cooled to the center of the trap thereby increasing the chance of off-resonant excitation from the auxiliary waveform used for fragmentation opposed to the ejection waveform. In either case, the competition between ejection and fragmentation can cause a decrease in transmission of the unfragmented precursor ion and/or product ions with small differences in m/z relative to the precursor ion. The competition between the two process can be optimized to avoid the decrease of transmission as is discussed later.

This effect is present as the second notable difference in Fig. 3 where peaks at precursor ion m/z 377 and 395, corresponding to remifentanil and carfentanil, respectively, are absent in the mini 2D MS/MS spectrum. It would appear that the ejection of the precursor before sufficient fragmentation is the cause of the absence of these peaks as indicated by the fact that even low mass product ions are not observed in either of the extracted product ion scans (data not shown). The presence of low mass product ions for alfentanil, precursor ion m/z 417, indicates that product ions generated at sufficient well-depth can be trapped and ejected at the reduced pressure reached near the end of the scan. This off-resonance excitation of the precursor ions could be mitigated by reducing the amplitude of the fragmentation waveform. The fragmentation waveform amplitude is limited by the amplitude required in the ejection waveform. This ejection waveform must perform mass analysis on the millisecond time scale. This fact, in combination with higher operating pressure and the use of nitrogen as the bath gas, increases the amplitude required for product ion ejection.

In many cases it becomes convenient to extract slices of the 2D MS/MS spectrum into a 1D MS/MS spectrum to facilitate comparisons, e.g. study of a conserved functional group. For example, previous work has divided the fentalogs into two categories separated by whether or not the conserved moiety in the analog was phenylpiperidine or N-phenylpropanamide. [18] Any analog with the unmodified phenylpiperidine should produce a charged fragment at m/z 188 whereas any analog with an unmodified N-phenylpropamide should produce a neutral fragment with a mass of 149 Da. The absolute abundance of these fragments is analyte dependent, but this general trend allows analogs to be identified by using only these two scans. An advantage of the 2D MS/MS scan is that in addition to identifying which of the analytes is a potential fentalog, the product ion scan for every potential analog has already been captured. Extracted precursor and product ion scans obtained from the 2D MS/MS spectra for both instruments are shown in Fig. 4. Fig. 4a demonstrates that all fentalogs containing phenylpiperidine produce a fragment ion at m/z 188. Fig. 4b shows that an extracted product ion scan for precursor ion m/z 323 indicates the presence of two isomeric fentalogs. Benzylfentanyl and acetylfentanyl differ by the location of a methyl group and are separated by their fragmentation behavior in both instruments. Examples of extracted neutral loss spectra can be found in SI Figure S2 where 5 fentalogs are identified by the neutral loss of phenylpropamide.

Mixture analysis for a heroin matrix

An important aspect of mixture analysis by mass spectrometry is the ability to detect particular analytes in the presence of substantial matrix. Matrix effects can affect the ionization of the analyte by either sequestering charge away from the analyte or by forming adducts with the charged analyte thereby changing its m/z value. These effects are traditionally mitigated by relying on separation before ionization or by using ionization methods that are matrix independent. The matrix can also generate background ions that are isobaric with the analyte ion, resulting in a false positive. This is mitigated in an MS/MS experiment by choosing product ions that are unique to the analyte. In order to determine how miniature 2D MS/MS performs when a substantial amount of matrix is present, heroin was chosen as the matrix to determine how the detection of seven drugs would be affected. First, the analytes were analyzed as a mixture without heroin. The resulting 2D MS/MS spectrum is shown in Fig. 5. The same analytes were then examined with heroin at 100 and 1000 times the concentration of the analytes. The analytes' concentrations were held at 100 ppb and from the resulting data a limit of detection was calculated for the concentration that gives a S/N of 3. The decision to express the results as a LOD instead of S/N at a given concentration was made to emphasize the relatively low concentrations detectable using the miniature instrument.

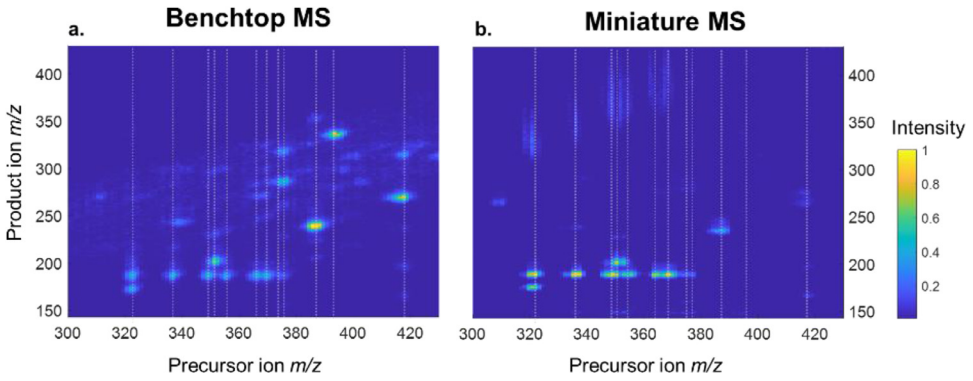

Fig. 3. Mixture of 13 fentanyl analogs analyzed by 2D MS/MS using modified (a) benchtop LTQ XL mass spectrometer and (b) Mini 12 mass spectrometer. White dotted lines indicate expected precursor ion *m/z* for each analyte. There are two isomeric fentalogs at *m/z* 323. Each spectrum was normalized relative to the highest peak.

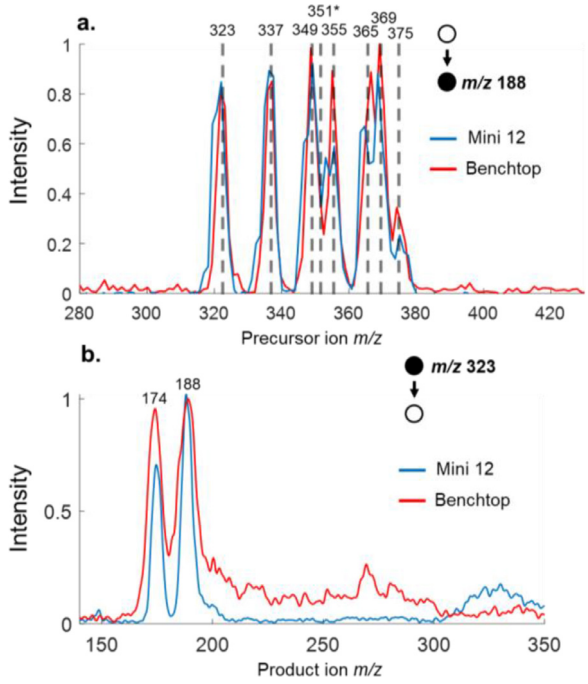

Fig. 4. Comparison of extracted (a) precursor ion scan and (b) product ion scan on both miniature and benchtop ion trap mass spectrometers. Ion intensity is relative to largest peak in each individual spectrum.

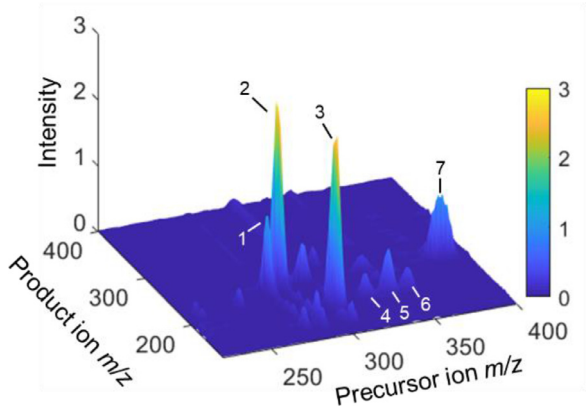

Fig. 5. 2D MS/MS mass spectrum of seven analytes at 100 ppb. The indicated peaks are the most intense product ions observed for each expected preucrsor ion *m/z*. The identities of each analyte can be found in Table 1.

The limit of detection of an analyte encompasses effects due to both the reduction of signal caused by ion suppression as well as increased signal in the blank caused by interfering peaks overlapping with the analyte peak. In a traditional ion trap MS/MS experiment, ion suppression can be partially compensated for by altering the injection time so that the number of trapped analyte ions is kept constant. This solution is not feasible in the 2D MS/MS scan as all precursor ions are interrogated simultaneously, so the total precursor ion population limits the ion storage capacity for any particular analyte. This is not the case in the traditional ion trap methodology because the number of trapped ions is reduced during the precursor ion isolation step so that only the analyte ion population's charge is present during mass analysis. Finally, if background peak overlap is expected, which would result in a false positive, a more unique transition can be chosen, or additional qualifier transitions can be measured.

The limits of detection determined in Table 1 show interesting trends. The most notable is how different the fentalogs' responses are to the matrix. Fentanyl's limit of detection increases from 6 ppb to 18 ppb whereas acetyl fentanyl goes from 10 pbb to 95 ppb in the presence of 1000x heroin. The difference could be attributed to an interfering peak overlapping with the transition used to detect acetylfentanyl and not to decreased fentanyl signal. Without peak overlap, the difference in LOD trends could either be caused by differences in ionization or fragmentation efficiencies. It can be seen from SI Figure S3 that neither the fentanyl or acetyl fentanyl suffers from peak overlap at the transition used for detection, so the change must be caused by either fragmentation or ionization differences between the molecules. Since fragmentation efficiency should be independent of the matrix, the ratio between the product ion peaks with and without the matrix demonstrates how the matrix affects the ionization of the molecule. The advantage of this technique is that all of the analytes are sampled from the source at the exact same time. This is possible because the whole ion population is utilized in the 2D MS/MS scan whereas traditional product ion scans would require different ion injections for each analyte and thus be susceptible to drift in the ion source for changes in sample.

It can be seen that none of the analytes or heroin ionizes to give isobaric ions. However, the introduction of ions into the miniature ion trap, using air as a bath gas, can cause the protonated molecules to fragment. These ions are still considered precursor ions because they are not fragmented mass selectively. The charged fragments of heroin can be isobaric with one of these analyte ions. The overlap of interfering background ions with analyte precursor ions does not significantly affect any of the analytes tested besides morphine. Morphine and heroin are very similar in structure except that the former's two alcohol groups have been acetylated to produce the latter. Fragmentation by deacetylation (loss of neutral ketene) from protonated heroin is seen at high concentrations of heroin. The extracted product ion spectra of *m/z* 286 from protonated morphine, deacetylated protonated heroin, and a mixture of the two drugs is shown in SI Figure S4. There is a product ion at *m/z* 155 seen in the mixture and the morphine sample, but not in the heroin

Table 1
Detection of seven analytes at 100 ppb in the precence of 10^3 and 10^4 times greater concentration of heroin.

Analyte # - Name	Quantifier Transition (Pre → Pro)	LOD Analytes only (ppb)	LOD with 100x Heroin (ppb)	LOD with 1000x Heroin (ppb)
1 - Amitriptyline	278 → 233	8	10	30
2 - Morphine	286 → 155	15	20	81
3 - Cocaine	304 → 185	4	5	13
4 - Acetyl Fentanyl	323 → 188	10	34	95
5 - Fentanyl	337 → 188	6	16	18
6 - Cyclopropyl Fentanyl	349 → 188	9	39	52
7 - Sufentanil	387 → 238	12	35	30

spectrum. The differences in the product ion spectra indicate a difference in ion structure. It might be assumed that neutral morphine and the neutral product of a deacetylated heroin would be the same neutral structure and thus ionize in a similarly. The difference in ion structure is due to how the precursor ions are generated. The ability to differentiate ionic species is a hallmark of MS/MS. This difference could also be observed by traditional product ion scans; however, the data shown is just a sliver of that obtained in a single 2D MS/MS scan. By being sensitive to all possible precursor/product ion relationships, it is possible to detect analytes affected by the confounding matrix. The 2D MS/MS scan is the only scan that is sensitive to changes during ionization thereby either altering precursor ion identity or abundance as well as the fragmentation of the individual precursor ion *m/z* thus producing different product ions from a single precursor ion *m/z* as shown above.

Future potential use for lipid profiling

The detection of individual analytes is a key aspect of mixture analysis as discussed above. For many practical applications, it is only important to identify the complex sample as such rather than identifying the individual analytes within the complex sample. In rare cases a single biomarker can identify the sample type even if it contains a complex mixture, and then the experiment can be simplified to only detect that marker. This was shown where genomic and immunohistological analysis were replaced by analysis using a miniature mass spectrometer when the determination of isocitrate dehydrogenase (IDH) mutation status was enabled by the measurement of a single biomarker, 2-hydroxyglutarate [28]. However, a single biomarker is typically less reliable in identifying a sample than a combination of biomarkers. It has previously been demonstrated that many different biological systems can be identified by their lipid profile [29–31]. Specifically, by observing the relative abundance of many lipids, different samples can be characterized by type, e.g. heathy or diseased. A lipid profile is then a more robust identification technique than is the monitoring of absolute abundances of individual analytes. This can be done without chromatographic separation as ion suppression affects the lipids similarly. Lipid profile information has been commonly obtained by 1D MS (full scan), 1D MS/MS (precursor/product/neutral loss scan), and dimensionless MS/MS (multiple reaction monitoring). These methods increase in their specificity per scan. An ideal 2D MS/MS scan lies at the extreme of specificity per scan as all lipids are identified in a single scan (although the sensitivity falls relative to the more specific scans). In order to determine the feasibility of the 2D MS/MS scan in lipid profiling, a mixture of known lipids was analyzed using the miniature instrument. Fig. 6 demonstrates that many lipid classes can be detected using the 2D MS/MS scan. A specific example of how this could be used to identify a particular class is seen for lipids containing a choline group. A few common product ions are seen for the three different lipid headgroups containing the same acyl chain length. Although the analytes are individually identified in this experiment, this is only done to demonstrate that many different lipid class are detected in a single scan. It is possible to use 2D MS/MS to guide targeted MS/MS methods and the combination is likely to be useful in future experiments. In the profiling experiment, the exact identity of these lipids would not be required in order to identify a difference of the lipid profile between two samples.

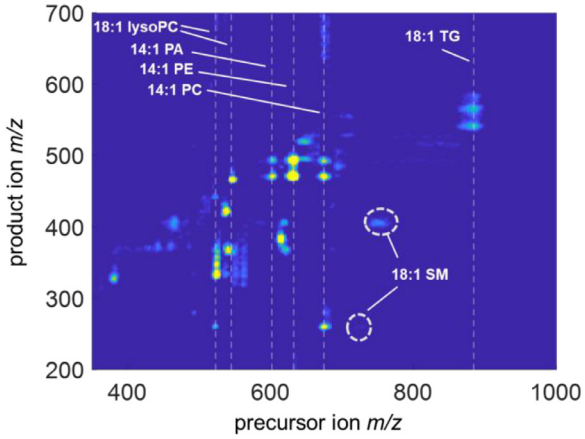

Fig. 6. 2D MS/MS scan of a mixture of lipids. The two precursor lines related to 18:1 PC and 18:1 SM are from the $[M+H]^+$ and $[M+Na]^+$.

Conclusions

The 2D MS/MS experiment was successfully implemented using a miniature rectilinear ion trap. The scan demonstrated here is particularly useful for experiments where time and/or sample is limited because both are utilized efficiently. The miniature instrument outperformed a commercial benchtop ion trap instrument in terms of sensitivity and resolution for a single analyte. This was not true when analyzing a wide range of analytes as the miniature instrument's performance degraded at high precursor ion *m/z*. This is potentially due to the time-varying pressure profile, an effect that might be mitigated in future modifications to the instrumentation. Importantly, the capability of using a miniature mass spectrometer utilizing the 2D MS/MS scan on mixtures was demonstrated on mixtures including fentalogs, drugs of abuse, and phospholipids.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2020.100028.

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