Manuscript for submission to:
Analytical Chemistry

High-resolution Twin-Ion Metabolite Extraction (HiTIME) Mass Spectrometry: Non-targeted identification of unknown drug metabolites by isotope labelling, liquid chromatography mass spectrometry, and automated high-performance computing

Michael G. Leeming,⁽¹⁾ Andrew P. Isaac,⁽²⁾ Bernard J. Pope,^(2,3) Noel Cranswick,⁽⁴⁾ Christine E. Wright,⁽⁴⁾ James Ziogas,⁽⁴⁾ Richard A. J. O'Hair,^{(1,5)*} and William A. Donald^{(6)*}

- (1) School of Chemistry and Bio21 Institute of Molecular Science and Biotechnology, University of Melbourne, Victoria 3010, Australia.
- (2) Victorian Life Sciences Computation Initiative, University of Melbourne, Victoria 3010, Australia
- (3) Department of Computing and Information Systems, University of Melbourne, Victoria 3010, Australia
- (4) Department of Pharmacology and Therapeutics, University of Melbourne, Victoria 3010, Australia.
- (5) ARC Centre of Excellence for Free Radical Chemistry and Biotechnology
- (6) School of Chemistry, University of New South Wales, Sydney, NSW 2052, Australia.

*Corresponding Authors:

Prof. Richard A. J. O'Hair
School of Chemistry
Bio21 Research Institute
University of Melbourne
Melbourne, Victoria, 3010
Tel: +61 3 83442452
E-mail: rohair@unimelb.edu.au
Fax: +61 3 9347-5180

Dr. William A. Donald
School of Chemistry
University of New South Wales
Dalton Building
Sydney, NSW, 2052
Tel: +61 2 9385 8827
E-mail: w.donald@unsw.edu.au
Fax: +61 2 9385-6141

Abstract

The metabolic fate of a compound can often determine the success of a new drug lead. Thus, significant effort is directed to identifying the metabolites formed from a given molecule. Here, an automated and non-targeted procedure is introduced for identifying drug metabolites without authentic metabolite standards via the use of stable isotope labelling, liquid chromatography mass spectrometry (LCMS), and high performance computing. LCMS of blood plasma extracts from rats that were administered a 1:1 mixture of acetaminophen (APAP) and $^{13}\text{C}_6$ -APAP resulted in mass spectra that contained “twin” ions for drug metabolites that were not detected in control spectra (*i.e.*, no APAP administered). By developing a program (High-resolution Twin Ion Metabolite Extraction; HiTIME) that can identify twin ions in high resolution mass spectra without centroiding (*i.e.*, reduction of mass spectral peaks to single data points), 9 doublets corresponding to APAP metabolites were identified, which is nearly twice that obtained by use of existing programs that make use of centroiding to reduce computational cost. By manually searching for all reported APAP metabolite ions, no additional twin ion signals were identified. These data indicate that all the major metabolites of APAP and multiple low abundance metabolites (*e.g.*, acetaminophen hydroxy- and methoxy- sulphate) that are rarely reported were detected. This methodology can be used to identify drug metabolites without prior knowledge of their identity. HiTIME is freely available from <https://github.com/bjpop/HiTIME>.

Keywords: mass spectrometry, stable isotope labelling, non-targeted metabolite identification, twin ion method

Running title: High-resolution Twin-Ion Metabolite Extraction (HiTIME) Mass Spectrometry

Introduction

Liquid chromatography mass spectrometry (LCMS) is widely used for detecting and identifying drug metabolites at trace levels in extremely complicated biological fluids that can contain thousands of endogenous compounds.¹ For metabolites that are unknown and unexpected, it can be challenging to determine which of the thousands of ions detected in an LCMS experiment correspond to the downstream metabolites of precursor compounds. This “needle-in-a-haystack” problem requires the development of methods to rapidly identify the products of compounds in complex mixtures, at trace levels, and without prior knowledge about the identity of the metabolites.

An elegant approach to the rapid non-targeted identification of drug metabolites was demonstrated in the early 1970s by Knapp *et al.* who simultaneously administered a 1:1 mixture of nortriptyline and [²H₃]-nortriptyline to human subjects and then analysed urine samples using gas chromatography mass spectrometry (GCMS).² They observed that metabolites of nortriptyline which retained the isotopic label eluted from the GC column at the same time and with the same abundance as those derived from the unlabelled drug, but the masses of the labelled and unlabelled metabolite ions differed in the mass spectra by a precisely known amount due to the isotopic label. These ‘twin-ions’ provide a signature in the GCMS or LCMS data that is unique to drug metabolites so that they can be distinguished from the multitude of endogenous compounds in a non-targeted manner; that is, without authentic standards or any information about the identity of the metabolites. Despite the obvious practical advantages of non-targeted twin-ion metabolite detection, the use of this method has been limited³ partly because (i) the resolution of many types of common mass spectrometers were insufficient to detect low abundance metabolite ions, and (ii) it was challenging to identify twin-ion metabolite signatures in large data sets.⁴

Given the significant advances in mass spectrometer performance, there has been renewed interest in using the twin ion method for a range of biological applications including the identification of trace level metabolites of insecticides in susceptible insects *in vivo*,⁵ the determination of pharmacokinetic parameters such as bioavailability⁶ and the elucidation of metabolic pathways and mechanisms.^{3a} Due to the number of ions that can be detected in high resolution LCMS experiments, it is important that twin-ion metabolite identification is automated to alleviate the laborious and inefficient task of manually searching through such datasets. Several computer algorithms have been developed to identify twin-ion metabolite doublets in LCMS data.⁷ For example, MetExtract developed by Schumacher and co-workers^{7f} initially considers each data point in each mass spectrum to be the unlabelled metabolite and the algorithm then searches for a theoretical peak at a higher m/z containing a defined number of isotopic labels. A twin-ion “hit” is assigned if this peak is found at a comparable intensity to the unlabelled peak. However, all existing programs (to our knowledge) for automated twin ion detection of unknown isotope labelled metabolite ions are optimized for datasets that have been “centroided,” in which the number of data points corresponding to a given ion is reduced to a single point. While this significantly reduces the computational cost for identifying the twin-ion peaks, centroiding can increase the signal-to-noise (S/N) that is required to confidently detect a given ion. Ideally, all data reduction steps should be eliminated to enable trace level metabolites to be reliably detected.

Here, we demonstrate a non-targeted approach to detecting drug metabolites that combines the twin ion method with automated data-mining software for non-targeted identification of drug metabolites. We have selected acetaminophen (APAP), a clinically relevant and widely used analgesic, to test this approach because it has a metabolic profile that is well established. By eliminating centroiding, the number of APAP metabolites that can

be detected in plasma extracts from rats (therapeutic dose) by a custom program entitled High-resolution Twin Ion Metabolite Extraction (HiTIME) increased by nearly a factor of 2.

Experiments

A 1:1 mixture of APAP:¹³C₆-APAP (5 mg kg⁻¹ of each isotopomer; *ca.* therapeutic dose) was administered intravenously to adult male Sprague-Dawley rats (327-366 g; isoflurane anaesthetized). Blood samples were drawn after 30 min. Blood plasma was prepared by addition of methanol to precipitate proteins and analysed using an Agilent 1100 LC coupled to an Agilent 6520-B Quadrupole-Time-of-Flight MS over a 67 min water/acetonitrile solvent gradient in both positive and negative ion polarities. Full details of the experiments and the HiTIME algorithm are in supporting information.

Results and Discussion

Results and Discussion

Positive ion mode LCMS analysis of extracts of blood plasma obtained from rats exposed to a saline solution that contained 10 mg kg⁻¹ of 1:1 ratio of APAP and [¹³C₆]-APAP resulted in the detection of 4957 and 2128 peaks in the respective positive and negative ionization modes (mass-spectral intensity threshold of 500 ion counts; average of $n = 3$ separate rats) that can be identified by standard algorithms using the native Agilent software. Unknown/unexpected metabolite ions can be identified by use of a mass defect filter⁸ to identify metabolites based on well-defined shifts in the differences between the accurate and integer m/z values. By use of a 50 mDa filter, which is sufficiently wide to cover many phase I and II metabolic transformations,⁸ an average of 1567 and 1460 peaks for positive and

negative ionization modes were respectively identified, which presents a challenge for metabolite identification.

To identify twin-ion signatures, a custom computer program was designed to automatically search through LCMS data and highlight data segments wherein the “twin-ion” metabolite ions are observed. For example, in **Figure 1a**, an example of a twin ion signature for the unlabelled ($[\text{APAP}+\text{H}]^+$, m/z 152) and labelled ($[\text{}^{13}\text{C}_6\text{-APAP}+\text{H}]^+$, m/z 158) is shown. HiTIME searches for twin-ion pairs that are defined as having: (i) identical retention times for the heavy and light isotopomers (*i.e.*, for the precursor ions in **Figure 1a**, the retention times of each ion is 13.1 min); (ii) identical abundances for the heavy and light isotopomers; and (iii) a difference in m/z values that corresponds to the known mass difference between heavy and light isotopomers (the labelled and unlabelled APAP ions differ by 6.0201 m/z , theoretical value: 6.0201).

For HiTIME, each LCMS data point is weighted based on the goodness-of-fit to an idealised twin ion shape. Such a shape is approximated by two 3-dimensional Gaussian surfaces (retention time, m/z value, and ion abundance are the x , y and z coordinates, respectively) that are centered at the retention time and m/z value of the data point of interest, spaced by the mass difference between heavy and light isotopes (6.0201 Da), and have the same widths and heights (**Figures 1b and S1**). To test for an ideal twin ion ‘hit,’ the absolute heights and widths of both Gaussian distributions are optimised (and keeping the ratios between the widths and heights of each distribution constant). Each LCMS data point is assigned a confidence score that indicates how well the twin-ion model centered at that point fits the surrounding data (**Figure 1c**). The results can then be visualised as a heat map that depicts both mass spectral and chromatographic domains with confidence scores that are indicated by a colour gradient (**Figure 1d**). To facilitate the identification of ions detected in

trace abundances, the HiTIME algorithm does not consider absolute peak intensity when assigning confidence scores to a data point; *i.e.* a low-abundance twin-ion should be assigned the same score as that for a high abundance twin ion (in the absence of other factors) .

Applying this algorithm to the negative ion LCMS data from the blood plasma of acetaminophen treated rats generates the heat map shown in **Figure S2a**. Numerous ‘bright spots’ are seen that are assigned weighted intensities of >5 indicating a strong correlation between the data in these regions and the model twin-ion signature. These signals likely arise from the elution of isotopically labelled drug metabolites. However, endogenous compounds unrelated to one another may by chance give an LCMS profile similar to that of the twin-ion signature. Cumulative intensity histograms depicting the distribution of weighted values are shown in **Figure S3**. 80% of the data in the raw files (representing tens of thousands of data points) are assigned a very low confidence value (<2). On this basis, data points assigned a weighted intensity value of <2 are considered to have negligible correlation to the twin-ion model and are excluded from the plots shown here. Endogenous compounds can be discounted by comparing the treatment sample against the control sample shown in **Figure S2b**. Corresponding bright spots are absent in the heat map generated from control LCMS experiments on blood drawn prior to APAP dosing. To remove these interferences, the control data were subtracted from those produced following APAP treatment and the results are shown in **Figure 2**. White space in the plots indicates a score of 2 (or less) meaning that essentially no correlation is observed between the hypothetical twin ion model and the raw data ($>99.7\%$ of all data points). This indicates that the vast majority of the data does not

match the expected APAP metabolite twin ion profile and has been removed by the HiTIME algorithm.

For some of the highly scoring regions in **Figure 2**, multiple high scoring data points are identified at the same retention time but at different m/z values. These are not observed in the control data and, based on high-resolution mass measurements and isotope distributions, arise from simple non-covalent adducts between twin-ion metabolites and inorganic salts (*e.g.*, $[M+Na]^+$ and $[M+H+NaHCO_2]^+$) or other endogenous products that are commonly formed in the ESI process (**Table S1**). As it is highly unlikely that unrelated endogenous compounds would produce this phenomenon simply by chance (based on the control data; **FigureS2b**), such adduct ions can provide additional confirmation that a metabolite was detected.

Analysing the subtracted heat maps, a total of 9 unique highly scoring regions were identified that had a unique retention time and a weighted intensity greater than 5 in either positive or negative background subtracted data sets (**Figure 2**). The 9 regions were manually investigated by plotting extracted ion chromatograms (EICs for both the heavy and light isotopes of potential metabolites using m/z values obtained for ions that were assigned a high confidence value in the **Figure 2 data**. In this way, the presence of APAP metabolites could be readily verified by searching for regions where the EIC traces for heavy and light isotopes of suspected metabolites overlap.

To demonstrate for a particularly low abundance metabolite, the twin ion signature for the rarely reported APAP metabolite, 3-methoxyacetaminophen-4-sulphate (APAP-OMe- SO_3), is detected at m/z 260 and m/z 266 in the negative ion mode (**Figure 3**). The EIC traces of each ion closely overlap for $t = 12.86$ min (**Figure 3 inset**), which is indicative of a twin-ion hit. For the vehicle control data, the analogous twin ions were not observed (Figure 3,

inset). The accurate m/z difference between $^{13}\text{C}_6$ -APAP-OMe-SO₃ and APAP-OMe-SO₃ is 6.0230 (within 11 ppm of the ideal value of 6.0201), and the abundances of these ions are essentially identical. In this case the mass spectral peak for the unlabelled isotope of APAP-OMe-SO₃ (m/z 260.0238, marked in **Figure 3** with a red arrow) is immediately adjacent to an endogenous compound at m/z 260.1318.

In total, 9 acetaminophen metabolites were identified by use of HiTIME and confirmed by plotting the EICs of the heavy and light isotopomers. The APAP metabolites that were identified are listed in **Table 1** and their structures and nomenclature are shown in **Figure 4**. The metabolites identified are: APAP-SO₃ and APAP-GLC (the two predominant metabolites of APAP), APAP-GSH, APAP-CYS, APAP-NAC (derived from APAP bioactivation to the putative toxic reactive metabolite N-acetyl p-quinone imine, NAPQI), as well as APAP-OH-SO₃ and APAP-OMe-SO₃, which are downstream products of the (unobserved) intermediated APAP-OH. Both N- and O- PAP-SO₃ are also observed which are likely to arise from deacetylation of APAP forming *p*-aminophenol followed by sulfation which can occur at either the amine or the phenolic oxygen atom.

The twin ion signature that corresponds to APAP-OMe-SO₃ (Figure 3) is representative of a low abundance metabolite ion that was identified by use of HiTIME. Of the 9 acetaminophen metabolites identified in this study, 5 were detected at *ca.* 10³ ion counts, which is close to the mass-spectral intensity threshold of 500 ion counts. If the identity of these metabolites were not known in advance, it would be challenging to identify and assign these low abundance twin-ion signals. Overall, these data indicate that HiTIME can identify twin-ion signatures even at relatively low *S/N* values (> *ca.* 10).

To rule out the possibility of “false-negatives” being assigned by the HiTIME algorithm (*i.e.*, low confidence scores assigned to actual acetaminophen metabolites showing

the twin-ion signature), a list of all reported metabolites of APAP and the related compound (and known APAP metabolite) 4-aminophenol was assembled from literature sources (31 metabolites in total, see Supporting Information for references) and their corresponding m/z values for the heavy and light isotopes.⁹ EIC traces were plotted for each potential twin ion that was not already assigned by the HiTIME algorithm. In no case was a region of clear overlap between the EIC traces of heavy and light isotopomers observed. These data provide confidence that HiTIME has accurately assigned all known metabolites of APAP that were present in the plasma samples, ionized, resolved and detected.

While the abundances of the 9 metabolites detected here vary considerably in the raw LCMS data, they are assigned similar confidence scores after weighting with the HiTIME algorithm. For example, APAP-CYS is detected with only a very low abundance (integrated peak area = 63186, **Table 1**) and is assigned a weighted intensity of 9.4 by the HiTIME algorithm. By contrast the precursor compound, APAP, was detected in high abundance (peak area = 13942546) that is *ca.* 200 fold greater than that for APAP-CYS and is assigned the same weighted intensity value of 9.4. These data demonstrate that the HiTIME algorithm can provide a non-targeted and unbiased assessment of twin-ion signatures present in a sample for a wide range of ion abundances.

To benchmark HiTIME, we compared our results to those generated by processing the same dataset with the freely available MetExtract software, which is also designed to identify twin-ion signatures in LCMS data.^{7f} As MetExtract is optimized for the interpretation of LCMS data in the ‘centroided’ format, our raw, profile data were centroided with the commonly used application MSConvert¹⁰ and these data were manually inspected to ensure that the signals for these metabolites were preserved throughout the data formatting process. MetExtract correctly identified 5 of the 9 twin-ion signatures known to be present in the data

set, however there were 4 metabolites that were not identified (APAP-GSH, APAP-CYS, APAP-OH-SO₃ and APAP-OMe-SO₃). By use of an m/z tolerance of 99 ppm (the maximum allowed value by MetExtract) and the ion doublet intensity ratio threshold was set to 0.5, one additional metabolite (APAP-CYS) was identified.

The discrepancy between the numbers of twin ion signatures that were identified may result from a detrimental impact of centroiding on (i) signal-to-noise ratio, and/or (ii) mass accuracy. **Figure S4** shows a plot of the ratio between profile S/N and centroid S/N as a function of signal intensity. Profile S/N is routinely 2-3 times higher than that for centroided data and is largely independent of signal intensity. In addition, the four metabolites that were not identified in centroid mode were detected in the lowest abundance. The absolute m/z accuracy values (in ppm) between the labelled and unlabelled twin ions (ideal: 6.0201 Da) tend to be slightly higher for profile (average of 4.2 ppm) than for centroid data (average of 6.4 ppm; **Figure S5**), which indicates that centroiding can have a detrimental effect on mass accuracy. Both effects could result in the identification of fewer twin ion signatures in the centroided data than the profile data. Overall, these results indicate that by using HiTIME, in which high-resolution mass spectral data are directly processed without data reduction (*i.e.*, peak centroiding), a significantly larger number of metabolites can be identified in these experiments.

Conclusion

An automated process for the non-targeted detection of drug metabolites was demonstrated for the detection of APAP metabolites in blood plasma taken from rats that were administered a 1:1 mixture of APAP and [¹³C₆]-APAP. The HiTIME algorithm successfully identified a series of doublet signals in unprocessed LCMS data that correspond to 9 metabolites of acetaminophen, including 3 which are important indicators of

acetaminophen bioactivation and 4 that are rarely reported. The combination of isotopic labelling, LCMS, and the direct high performance computational processing of high resolution mass spectral data files without data reduction enabled all APAP metabolites to be rapidly and comprehensively identified in complex biological mixtures in a non-targeted and unbiased approach. By eliminating peak centroiding, which can lower S/N values and m/z accuracy, lower abundance ions can be identified more readily in complex mixtures. Although this can increase computational cost, the use of HiTIME should benefit many LCMS measurements for the identification and detection of the downstream metabolites of analytes in complex mixtures. HiTIME has been made freely available on the World Wide Web (<https://github.com/bjpop/HiTIME>).

Acknowledgements

We thank the University of Melbourne Interdisciplinary Seed Grant program for funding. This research was further supported by the Victorian Life Sciences Computation Initiative (VLSCI), an initiative of the Victorian Government hosted by The University of Melbourne, Australia. MGL thanks the Elizabeth and Vernon Puzey foundation for the award of a PhD research scholarship. WAD thanks the Australian Research Council for a Discovery Early Career Researcher Award and the Faculty of Science at the University of Melbourne for a Centenary Research Fellowship. We gratefully acknowledge Ms. Heather Daykin for performing rat surgeries, drug administration and blood sampling and Mr. Kin Kuan Hoi for evaluating early versions of the HiTIME algorithm. We also thank Prof. Michael Small for useful discussions on mathematical approaches to identifying twin-ion peaks and Prof. Peter Taylor for facilitating access to VLSCI.

Supporting Information

Detailed descriptions of drug administration, blood sampling and LCMS sample preparation and parameters are provided. Figures S1-S5: a schematic of the parameters involved in the twin-ion fitting procedure, weighted data intensity histograms, positive ion HiTIME output heatmaps and comparisons of profile and centroid data signal-to-noise ratio and mass accuracy, respectively. Table S1-S4 Q-TOF ionisation and acquisition parameters, MsConvert data export parameters, HiTIME fitting parameters and non-covalent adduct ion assignments respectively.

Figures and Table

Table 1. List of APAP metabolites identified in LCMS datasets by the HiTIME algorithm.

Compound	RT (min)	Ion Polarity	Peak Area ^a	Measured m/z (¹² C ₆ - labelled) ^b	$\Delta m/z$ ^{b,c}	HiTIME confidence
APAP	13.15	+	13942546	152.0718	6.0201	9.4
PAP-SO ₃ ^d	10.60	-	1288950	188.0027	6.0199	11.1
APAP-SO ₃	12.70	-	94083840	230.0130	6.0202	17.7
APAP-OH-SO ₃	12.67	-	53731	246.0081	6.0167	7.7
APAP-OMe-SO ₃	12.86	-	53850	260.0238	6.0230	7.8
APAP-CYS	11.97	+	63186	271.0690	6.0206	9.4
APAP-NAC	13.08	+	74847	313.0890	6.0199	11.8
APAP-GLC	11.60	-	2776708	326.0886	6.0202	18.3
APAP-GSH ^e	12.34	+	42206	457.1388	6.0242	3.6

^a Values are the average of three replicates. ^b Exemplary values from a single analysis.

^c Difference in m/z values of ¹³C₆ and ¹²C₆ labelled ions. ^d Combined ion abundances for both N- and O- sulphate aminophenol metabolites. ^e m/z values from baseline subtracted mass spectra.

Figure 1. A) Profile LCMS mass spectrometry data in which doublet peaks assigned to APAP $[M+H]^+$ are shown. B) Representative 'ideal' twin-ion peaks that were used to weight the data. C) Output data after weighting centring on the light isotope. D) Visualisation of the results from HiTIME weighting as a two-dimensional heat map. RA and RT correspond to relative abundance and retention time, respectively.

Figure 2. Negative ion LCMS data from rats treated with 1:1 APAP:[¹³C₆]-APAP 10 mg kg⁻¹ following subtraction of vehicle control. To aid in visualisation of the results, only a small segment that contains the majority of the twin-ion ‘hits’ is displayed.

Figure 3. Mass spectrum showing co-elution of the heavy and light isotopes of the low level metabolite [APAP-OMe-SO₃-H]⁻. Inset shows the EICs for heavy (red) and light (blue) APAP-OMe-SO₃ where solid and dashed lines correspond to the treatment and vehicle control data.

Figure 4. Structures and nomenclature of APAP metabolites that were identified in this study.

References

1. (a) Zhu, M.; Zhang, H.; Humphreys, W. G., *J. Biol. Chem.* **2011**, *286*, 25419-25425; (b) Prasad, B.; Garg, A.; Takwani, H.; Singh, S., *TrAC, Trends Anal. Chem.* **2011**, *30*, 360-387; (c) Holčapek, M.; Kolářová, L.; Nobilis, M., *Anal. Bioanal. Chem.* **2008**, *391*, 59-78; (d) Kostianinen, R.; Kotiaho, T.; Kuuranne, T.; Auriola, S., *J. Mass Spectrom.* **2003**, *38*, 357-372.
2. Knapp, D. R.; Gaffney, T. E.; McMahon, R. E.; Kiplinger, G., *J. Pharmacol. Exp. Ther.* **1972**, *180*, 784-790.
3. (a) Mutlib, A. E., *Chem. Res. Toxicol.* **2008**, *21*, 1672-1689; (b) Richards, D. P.; Sojo, L. E.; Keller, B. O., *J. Labelled Compd. Radiopharm.* **2007**, *50*, 1124-1136.
4. Wright, P., *Xenobiotica* **2011**, *41*, 670-686.
5. Hoi, K. K.; Daborn, P. J.; Battlay, P.; Robin, C.; Batterham, P.; O'Hair, R. A. J.; Donald, W. A., *Anal. Chem.* **2014**, *86*, 3525-3532.
6. Schellekens, R. C. A.; Stellaard, F.; Woerdenbag, H. J.; Frijlink, H. W.; Kosterink, J. G. W., *Br. J. Clin. Pharmacol.* **2011**, *72*, 879-897.
7. (a) Lin, L.-C.; Wu, H.-Y.; Tseng, V.-M.; Chen, L.-C.; Chang, Y.-C.; Liao, P.-C., *J. Am. Soc. Mass. Spectrom.* **2010**, *21*, 232-241; (b) Feldberg, L.; Venger, I.; Malitsky, S.; Rogachev, I.; Aharoni, A., *Anal. Chem.* **2009**, *81*, 9257-9266; (c) Kielmas, M.; Kijewska, M.; Stefanowicz, P.; Szewczuk, Z., *Anal. Biochem.* **2012**, *431*, 57-65; (d) Zhang, J.; Zhang, T.; Jiang, L.; Hewitt, D.; Huang, Y.; Kao, Y.-H.; Katta, V., *Anal. Chem.* **2012**, *84*, 2313-2320; (e) Zhu, P.; Tong, W.; Alton, K.; Chowdhury, S., *Anal. Chem.* **2009**, *81*, 5910-5917; (f) Bueschl, C.; Kluger, B.; Berthiller, F.; Lirk, G.; Winkler, S.; Krska, R.; Schuhmacher, R., *Bioinformatics* **2012**, *28*, 736-738.
8. Zhang, H.; Zhang, D.; Ray, K.; Zhu, M., *J. Mass Spectrom.* **2009**, *44*, 999-1016.
9. Many of the 31 Apap Metabolites Found in Our Literature Search Were Observed in Studies Examining the Metabolism of Apap at Hepatotoxic Doses of >200 Mg Kg⁻¹ Rather Than the Therapeutic Doses That Were Employed in Our Experiments
10. Chambers, M. C.; Maclean, B.; Burke, R.; Amodei, D.; Ruderman, D. L.; Neumann, S.; Gatto, L.; Fischer, B.; Pratt, B.; Egertson, J., *Nat. Biotechnol.* **2012**, *30*, 918-920.

Table of Contents Graphic