



Application of multiple isotope pattern searches for in-depth plant metabolism determination

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ABSTRACT

Here, we illustrate the utility of high-resolution LC-MS workflows for trace-level detection of metabolites in complex sample matrix based on characteristic isotope patterns, and the utility of the collision-induced dissociation (CID) and electron-activated dissociation (EAD) fragmentation to propose and confirm structures of these compounds.

INTRODUCTION

Identification of major xenobiotic metabolites is required for registration of agrochemicals around the world. Mass spectrometry (MS) has been instrumental for understanding the environmental impact of new product candidates by enabling the detection and structural characterization of metabolites and impurities of the active ingredients in complex matrices.

Halauxifen-methyl (Arylex™), a broad-spectrum herbicide effective for controlling broadleaf and sedge weeds and some grass weed species, is an agrochemical that generates a series of metabolites that can conjugate with various moieties in plant and animal species. In this study, we investigated metabolism products of a mixture of native and isotopically enriched Arylex, with the aim to characterize trace amounts of its phase II and phase III metabolites.

MATERIALS AND METHODS

A simulated metabolite sample was generated by dosing an isotopically labeled mixture of Arylex into wheat cell cultures. These were allowed to metabolize the active ingredient for 0, 24, 48 and 120 hours. After the incubation, cell cultures were harvested and lysed with acetonitrile.

LC was performed using an Exion LC system and compounds were separated on a Zorbax Eclipse Plus C18 (2.1x50mm, 1.8u) column using a 20-minute gradient from 0%-65% organic (water / acetonitrile). The extracts were analyzed in positive ion mode on a ZenoTOF 7600 system with both data-dependent acquisition (DDA) and SWATH data-independent acquisition (DIA) workflows using CID and EAD fragmentation modes (see Table 1 for a summary of the MS settings). The data were interrogated with Molecule Profiler software applying isotope pattern-derived peak finding strategies and a customized set of biotransformation reactions.

	MS/MS collection strategy	DDA	SWATH
Source and Gas parameters	Spray voltage (V)	5500	5500
	Spray temperature (°C)	550	550
	Ion source gas 1(psi)	25	25
	Ion source gas 2(psi)	70	70
	Curtain gas (psi)	35	35
	CAD gas	7	7
	Declustering potential	80	80
MS range	MS	100 to 1000	100 to 1000
	MS/MS	80 to 1000	100 to 1000
	SWATH Q1 range	NA	300 to 1030
MS/MS parameters	MS/MS accumulation time(ms)	50	20
	CE (V)	40	40
	CES (V)	15	0
	KE (V)	10	10
	Zeno trap	on demand	on
	Q1 window	1	39
	Q1 window overlap	NA	9

Table 1. Mass spectrometer settings for DDA and SWATH DIA acquisitions.

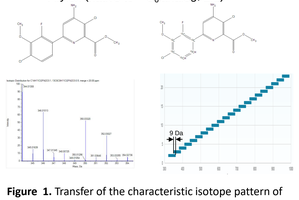


Figure 1. Transfer of the characteristic isotope pattern of the Arylex native and labeled mixture into a variable window SWATH DIA method design.

RESULTS

Major metabolic pathways of Arylex have been established[1]. Two processes transform the active metabolite, halauxifen acid, into inactive metabolites: o-demethylation of the aryl ring and glucose conjugation.

With a dedicated set of typical plant phase I, II and III metabolism reactions, we could find 50 metabolites covering 3 orders of magnitude (see Table 2 for the list of metabolites that contribute by >0.1% to the total metabolic profile at 48 hours). The metabolites were mainly glucoside conjugates combined with O-demethylation, loss of water and the addition of malonic acid to the sugar conjugate.

In our study we focussed on minor metabolites and the utility of isotope patterns in both MS and MS/MS measurements, to uncover these metabolites in matrices of different complexities. Halauxifen-methyl molecule has 2 chlorine atoms and therefore the mixture of native and $^{13}C_6$-enriched molecules gives a comprehensive, unique isotopic fingerprint that holds across a wide range of concentrations. With SWATH DIA, we set up the Q1 windows to transmit the full isotopic pattern within a single SWATH DIA experiment (Figure 1), maintaining the full isotope fingerprint of MS/MS fragments.

For the isotope pattern-based data mining the conditions were set to accommodate for the chemical changes due to polysaccharide conjugations. The relative signal of peaks at M+2, M+6 and M+8 had the smallest spread over the series of Arylex conjugates (M from 300 to 1000), and these peaks were considered in the pattern-driven MS data mining (Figure 2A).

While using just the 2-peak chlorine-derived isotope pattern to extract LC/MS peaks from the cell culture matrix sample was sufficient, the 4-peak isotope-enriched pattern was needed to get comprehensive metabolite coverage in the typical environmental matrix.

For the fragment isotope patterns in the SWATH DIA data, we found that Q1 window overlaps of 9 Da covered the full isotope pattern of the isotopically enriched molecule and gave the best metabolite coverage. The 4-peak fragment isotope patterns did not vary much across the wide molecule size range of the detected metabolites (Figure 3). This approach also allowed to detect additional singly and multiply charged metabolites (Figure 4).

EAD MS/MS data pinpointed the location of glucose conjugations. The CID data helped to confirm phase III metabolites (Figure 5).

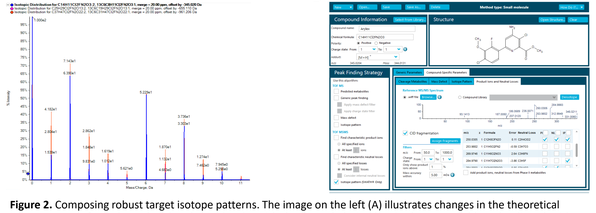
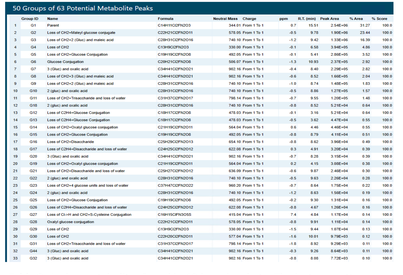


Figure 2. Composing robust target isotope patterns. The image on the left (A) illustrates changes in the theoretical isotope pattern for a glycosylated Arylex molecule, with the demethylation (M=344) pattern in blue, the disaccharide (M=654) pattern in pink and the tetra saccharide (M=960) pattern in orange. The image on the right (B) shows fragment isotope patterns reflecting 1 and 2 chlorine atoms selected from the annotated Arylex MS/MS spectrum.



	Group ID	Name	Formula	Neutral Mass	Charge	ppm	R.T. (min)	Peak Area	% Area	% Score
1		G4	Parent		C14H10Cl2FN2O3		9.7	10.41	2.54E+06	34.37	100.0
2		G2	Loss of CHO+Malonyl glucose conjugate		C23H21Cl2FN2O11		-0.5	9.78	1.90E+06	25.44	100.0
3		G3	Loss of CHO+2 (Glac) and malonic acid		C19H13Cl2FN2O10		-1.2	9.42	1.53E+06	19.59	100.0
4		G4	Loss of CHO		C13H9Cl2FN2O3		-0.1	9.58	3.94E+05	4.86	100.0
5		G5	Loss of CHO+Glucose Conjugation		C19H19Cl2FN2O8		-0.1	9.41	2.56E+05	3.52	100.0
6		G6	Glucose Conjugation		C20H21Cl2FN2O8		-1.3	10.93	2.33E+05	2.92	100.0
7		G7	2 (Glac) and acetic acid		C18H17Cl2FN2O7		-0.4	9.49	2.26E+05	2.82	100.0
8		G8	Loss of CHO+2 (Glac) and malonic acid		C14H11Cl2FN2O5		-0.6	8.52	1.66E+05	2.04	100.0
9		G9	Loss of CHO+2 (Glac) and malonic acid		C20H17Cl2FN2O10		-1.6	8.74	1.48E+05	1.83	100.0
10		G10	2 (glac) and oxalic acid		C20H19Cl2FN2O10		-0.5	8.96	1.27E+05	1.57	100.0
11		G11	Loss of CHO+Triacetylamide and loss of water		C31H31Cl2FN2O17		-0.7	9.55	1.09E+05	1.46	100.0
12		G12	2 (glac) and acetic acid		C26H25Cl2FN2O16		-0.8	8.52	9.23E+04	0.64	100.0
13		G13	Loss of C2H4+Glucose Conjugation		C18H11Cl2FN2O8		-0.1	3.16	9.21E+04	0.64	100.0
14		G13	Loss of C2H4+Glucose Conjugation		C18H13Cl2FN2O8		-0.5	3.62	6.47E+04	0.55	100.0
15		G14	Loss of CHO+Oxalyl glucose conjugation		C21H15Cl2FN2O11		0.6	4.46	4.48E+04	0.55	100.0
16		G15	Loss of CHO+Glucose Conjugation		C19H19Cl2FN2O8		-0.6	8.79	4.18E+04	0.51	100.0
17		G16	Loss of CHO+Disaccharide		C25H27Cl2FN2O13		-0.6	8.62	3.96E+04	0.48	100.0
18		G17	Loss of C2H4+Disaccharide and loss of water		C24H23Cl2FN2O12		0.3	9.91	3.39E+04	0.38	100.0
19		G20	3 (Glac) and acetic acid		C24H21Cl2FN2O13		-0.7	8.28	3.15E+04	0.39	100.0
20		G19	Loss of CHO+Oxalyl glucose conjugation		C21H15Cl2FN2O11		-0.3	4.15	3.08E+04	0.38	100.0
21		G21	Loss of CHO+Disaccharide and loss of water		C25H27Cl2FN2O12		-0.6	9.87	2.46E+04	0.30	100.0
22		G22	2 (glac) and acetic acid		C26H25Cl2FN2O16		-0.5	9.63	2.06E+04	0.28	100.0
23		G23	Loss of CHO+4 glucose units and loss of water		C37H47Cl2FN2O22		-0.7	8.64	1.75E+04	0.22	100.0
24		G24	2 (glac) and acetic acid		C26H25Cl2FN2O16		-1.2	8.63	1.58E+04	0.19	100.0
25		G25	Loss of CHO+Glucose Conjugation		C19H13Cl2FN2O8		-0.2	9.39	1.31E+04	0.16	100.0
26		G26	Loss of C2H4+Disaccharide and loss of water		C24H21Cl2FN2O12		-0.3	4.87	1.28E+04	0.16	100.0
27		G27	Loss of Cl+H and CHO+S-Cysteine Conjugation		C18H15Cl2FN3O6S		7.4	4.84	1.17E+04	0.14	100.0
28		G28	Oxalyl glucose conjugation		C22H21Cl2FN2O11		-0.8	9.91	1.15E+04	0.14	100.0
29		G29	Loss of CH2		C13H9Cl2FN2O3		-1.5	9.44	1.07E+04	0.13	100.0
30		G30	Loss of CHO		C20H19Cl2FN2O10		-1.6	10.81	9.79E+03	0.12	100.0
31		G31	Loss of CHO+Triacetylamide and loss of water		C31H31Cl2FN2O17		-1.6	8.92	9.29E+03	0.11	100.0
32		G44	3 (Glac) and acetic acid		C32H29Cl2FN2O19		-0.3	9.39	8.64E+03	0.11	100.0
33		G32	3 (Glac) and acetic acid		C26H25Cl2FN2O16		-0.6	9.68	7.72E+03	0.10	100.0

Table 2. Arylex metabolites found in 48-hour incubate. The isotope pattern agreement is indicated in the "% Score" column.

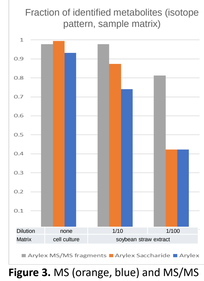


Figure 3. MS (orange, blue) and MS/MS (grey) isotope pattern efficiencies to detect Arylex metabolites in varying matrices.

sample	metabolites inspected	average isotope signal accuracy	average isotope signal precision	log 10 linear dynamic range inspected
t48	12	-0.9%	4.6%	2.2
t120 1/10 in matrix	10	1.8%	9.4%	1.5
t120 1/100 in matrix	4	6.4%	6.4%	0.6
t120 1/10 in matrix SWATH	14	2.4%	15.0%	1.6
t120 1/100 in matrix SWATH	9	3.6%	24.0%	1.9

sample	MS/MS patterns inspected	m/z offset accuracy (mDa)	m/z offset precision (mDa)	log 10 linear dynamic range inspected
t120 1/10 in matrix SWATH	14	1.4	1.10	1.6
t120 1/100 in matrix SWATH	9	0.80	2.9	1.9

Table 3. Characteristics of the 4-peak isotope pattern across a dynamic range of metabolites detected with the Molecule Profiler software. The m/z tolerance for the pattern was set to 3mDa and the intensity tolerance to 15%.

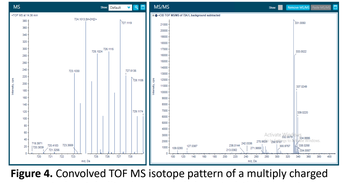


Figure 4. Convoled TOF MS isotope pattern of a multiply charged unexpected metabolite gives several characteristic fragment pattern signatures in the SWATH DIA experiment.

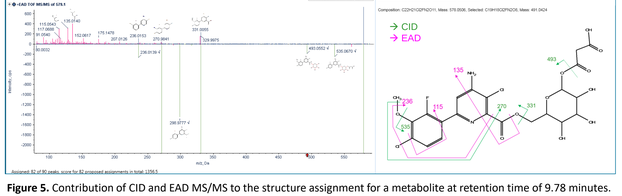


Figure 5. Contribution of CID and EAD MS/MS to the structure assignment for a metabolite at retention time of 9.78 minutes.

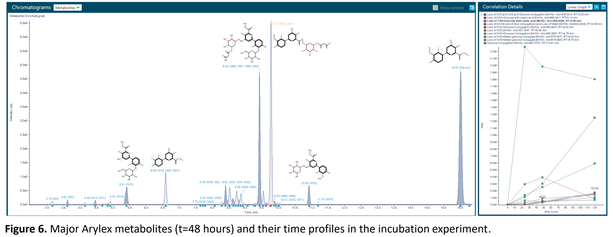


Figure 6. Major Arylex metabolites (t=48 hours) and their time profiles in the incubation experiment.

CONCLUSIONS

- Characteristic isotope pattern-driven HRMS data mining offers unprecedented specificity and allows for the detection of impurities and metabolites in complex sample matrices across a wide dynamic range
- SWATH DIA Q1 window positioning that preserves the full fragment isotope pattern is instrumental in the search for unexpected low-level material since:
  - Multiple fragments could carry complementary isotope pattern signatures
  - Pattern reproducibility and accuracy across metabolites is superior due to a lesser variation in target fragment sizes
- Multiple mass pattern template-based data extraction and filtering enabled in Molecule Profiler software is suited to studies of the agrochemical metabolism pathways in complex environmental matrices
- Deploying 2 independent fragmentation mechanisms, DDA and SWATH DIA, in the ZenoTOF 7600 system generated complementary diagnostic fragments leading to an in-depth understanding of Arylex metabolism in plants

REFERENCES

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