

Identification of new metabolites of a pesticide in an anaerobic aquatic metabolism study

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Abstract

In the course of an Anaerobic Aquatic Transformation study, four known metabolites of the compound under study were proposed for confirmation by LC-MS/MS. The proposed metabolites were based on close retention time matches between reference standards and the metabolites found during HPLC-RAM analyses of the sample extracts. This study used sediments from two rivers. Each of the river sediments was divided into two "compartments" (called "aqueous" and "sediment extract") resulting in a total of four compartments analyzed. In the HPLC-RAM portion of the study, four metabolites were found at different levels in all the compartments. However, when LC-MS/MS analysis was done, two of the four proposed metabolites were found in some but not in all compartments. Further data analysis resulted in the discovery of new metabolites.

Methods

- The HPLC-RAM data were collected using the Agilent Series 1200 HPLC system and a Perkin Elmer radiochemical detector (Model 625TR) under the control of the Laura Radiochromatography Data/Collection software version 5.0.1.13. All HPLC-RAM data were processed using Laura software version 5.0.1.13.
- The LC-MS/MS data were collected using a Sciex TripleTOF[®] system and a Shimadzu Prominence HPLC under the control of Analyst[®] TF software alongside a Berthold FlowStar model LB513 radiochemical detector controlled by FlowStar software with that data also being captured by Analyst software through the use of an ADC converter. The data were processed using PeakView[®] software, version 1.2. Analysis of all samples was done in both positive and negative ion polarities.
- The LC-MS/MS data were acquired using IDA (information-dependent acquisition) methods which automatically generate product ion spectra for chromatographic peaks meeting specified criteria. In most cases the use of an IDA method avoids the need to re-inject the sample. The method used ion intensity, background subtraction, and real-time mass defect filtering which places ions with mass defects matching those of the test material and its predictable transformation products at the top of the list for automated product ion spectra collection, even if they're not the most abundant ions in the spectrum.

Data

HPLC-RAM Results

HPLC-RAM analysis of the aqueous and sediment extracts from samples of both sediment types was used to assign the metabolites found in the study to known reference standards based on retention time (RT) matching. However, since these structure assignments are based solely on RT matching, they usually require confirmation by LC-MS/MS analysis using reference standards. This LC-MS/MS analysis provides accurate mass information (that leads to a formula assignment that's consistent with the proposed metabolite based on the m/z and isotope pattern) and shows an MS/MS spectrum match between the metabolite and the reference standard along with a second RT match on a separate system. This additional information typically provides sufficient confidence for the structure assignments. If a metabolite cannot be confirmed, it must be identified and a structure proposed.

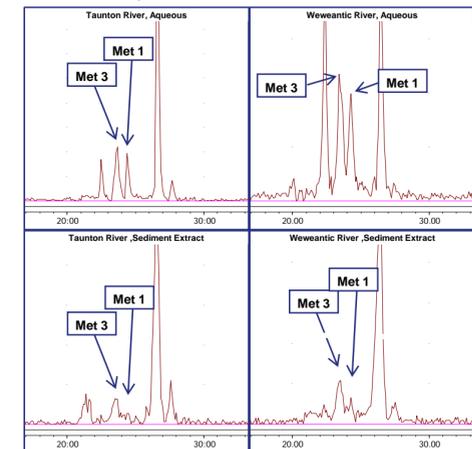


Figure 1: HPLC RAM Chromatograms. Radio Chromatograms with Met 1 and Met 3 labeled from the aqueous (top two panels) and sediment extract (bottom two) compartments are shown at the left. The two left-hand panels are from Taunton River sediments and the two right-hand panels are from Weweantic River sediments.

LC-MS/MS Results

Met 1 was found in all four compartments during the HPLC-RAM analysis. LC-MS/MS analysis of the aqueous and sediment extracts for the proposed Met 1 (3,5-dichloro-4-methylbenzoic acid) showed that it was found in both aqueous compartments and in one of the sediment compartments but practically not at all in the second sediment compartment (**Figure 2a**). This prompted a search for a compound that was found in all four compartments (**Figure 2b**), resulting in a new proposed metabolite which is a known metabolite that results from the replacement of a chlorine with an oxygen. The retention time differences for the originally and the newly proposed compounds are very small. **Figure 3** shows similar data for Met 3.

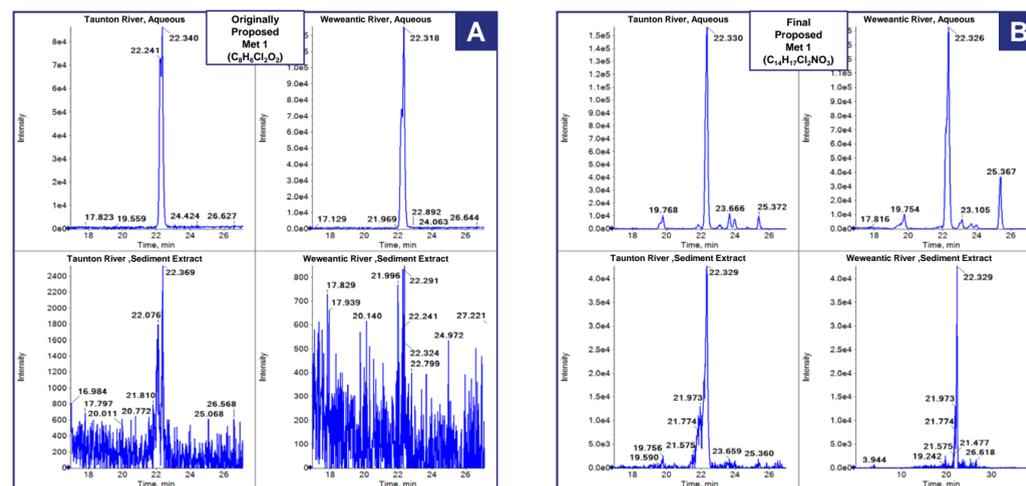


Figure 2: LC-MS/MS Analysis of Met 1. Panel A shows the extracted ion chromatograms (XICs) for the originally proposed metabolite (chemical formula: $C_8H_6Cl_2O_2$) in the four compartments and found only in three of the four. Panel B shows the extracted ion chromatograms for the newly proposed metabolite (chemical formula: $C_{14}H_{17}Cl_2NO_3$). This compound is found in all four compartments as was seen in the HPLC-RAM analysis. Notice the small difference in RT between the two metabolites.

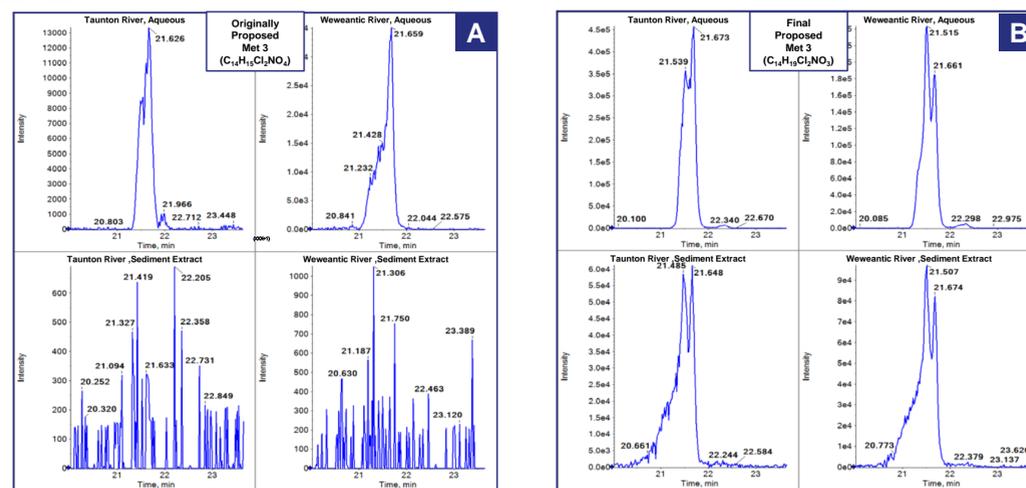
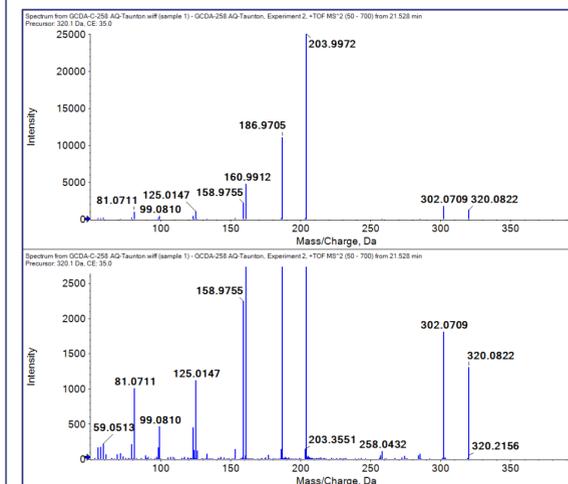


Figure 3: LC-MS/MS Analysis of Met 3. Panel A shows the extracted ion chromatograms (XICs) for the originally proposed metabolite (chemical formula: $C_{14}H_{15}Cl_2NO_4$) in the four compartments and found only in two of the four. Panel B shows the extracted ion chromatograms for the newly proposed metabolite (chemical formula: $C_{14}H_{19}Cl_2NO_3$). This compound is found in all four compartments as was seen in the HPLC-RAM analysis. This compound is not one of the known metabolites of the pesticide, but can be derived from dechlorination of the parent or by addition of H_2O to the structure of a known metabolite. The latter is believed to be more likely based on the observed MS/MS fragmentation.



Exptl	m/z	Calc.	mass error (ppm)	mass error (mDa)	Neutral Loss	Ionic Formula
81.0712	81.0699	15.7	1.3	$C_8H_{11}Cl_2NO_3$	$C_6H_7^+$	
122.9989	122.9996	-5.8	-0.7	$C_7H_6ClNO_3$	$C_7H_4Cl^+$	
125.0147	125.0153	-4.4	-0.5	$C_7H_4ClNO_3$	$C_7H_6Cl^+$	
158.9755	158.9763	-4.7	-0.8	$C_7H_5NO_3$	$C_7H_5Cl^+$	
186.9705	186.9712	-4.0	-0.7	$C_6H_5NO_2$	$C_8H_5Cl_2O^+$	
203.9972	203.9978	-2.9	-0.6	$C_6H_{12}O_2$	$C_8H_8Cl_2NO^+$	
302.0709	302.0709	0.1	0.0	H_2O	$C_{14}H_{18}Cl_2NO_2^+$	
Neutral Formula for the Precursor: Proposed Met 3					—	$C_{14}H_{19}Cl_2NO_3$

Figure 4 – Product Ion spectrum match to structure to substantiate the proposed structure. The two spectra on the left (un-zoomed y-axis on top) and zoomed on the bottom) are from Unknown 3. Matching the ions to a proposed structure lends credence to the assigned structure. The table on the right shows the proposed formulae for the ions and their associated neutral losses. The compound under study contains three chlorines per molecule two of which are at positions 3 and 5 on a phenyl ring the other is at the other end of the structure. This metabolite only has two chlorines and could form by addition of water to a known metabolite that is formed by replacement of the lone chlorine with a hydroxyl group or could form by dechlorination of the phenyl ring and then oxidation. The ions at m/z 158.9755, 186.9705, 203.9972 and 302.0709 are most readily explained by structures including the 3,5-dichlorophenyl ring arguing in favor of the addition of water to the known metabolite resulting from dechlorination outside the ring over dechlorination on the ring.

Conclusions

- The complete absence of a proposed compound from one of the compartments could be evidence that the proposed metabolite is only a partial answer to the identity of the metabolite found by HPLC-RAM analysis. This conclusion should hold despite the fact that there may be some variations in observable intensities due to different co-eluting compounds found in the several matrices which can lead to different levels of signal suppression in the ion source,
- LC-MS/MS is a much more reliable technique for metabolite confirmation when compared to co-elution or co-chromatography of a reference standard using HPLC-RAM analysis when using only one column and gradient combination.

Acknowledgments

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