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Mass spectrometry imaging (MSI) to investigate xenobiotics metabolism in Salix alba leaves.

--Manuscript Draft--

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Strasbourg, February 25th, 2020

Benjamin Werth, JoVE Editorial Board

Object: Submission of a revised manuscript.

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Dear Benjamin Werth,

We are pleased to submit the revised version of our manuscript entitled "Mass spectrometry imaging (MSI) to investigate xenobiotics metabolism in *Salix alba* leaves." in response to your kind invitation for publication in JoVE.

I think there was a bit of confusion concerning this manuscript, from my side and for the reviewers. You contacted me based on a manuscript published in Metabolomics (Villette et al, 2019) to propose a protocol which could be adapted to the JoVE video format. This protocol was expected to be short and not as detailed as the one previously published, otherwise it would be a duplicate, which is not a really attractive exercise.

From my point of view, the reviewers provided a review as in a classical journal reviewing process. I wonder if you carefully informed them and explained them the specific process to follow for JoVE ?
I don't think so, which explains the confusion and the fact that most of their questions can find an answer in the previously published manuscript. Consequently, I can only politely invite them to read the manuscript from Metabolomics.
When possible, we made the modifications proposed by the reviewers.

I hope you understand my discomfort.

On behalf of all co-authors, Dimitri Heintz

TITLE:

Investigation of Xenobiotic Metabolism in *Salix alba* Leaves via Mass Spectrometry Imaging

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KEYWORDS:

mass spectrometry imaging, MSI, *Salix alba*, xenobiotics, metabolism, metabolites prediction, MALDI

SUMMARY:

This method uses mass spectrometry imaging (MSI) to understand metabolic processes in *S. alba* leaves when exposed to xenobiotics. The method allows the spatial localization of compounds of interest and their predicted metabolites within specific, intact tissues.

ABSTRACT:

The method presented uses mass spectrometry imaging (MSI) to establish the metabolic profile of *S. alba* leaves when exposed to xenobiotics. Using a non-targeted approach, plant metabolites and xenobiotics of interest are identified and localized in plant tissues to identify specific distribution patterns. Then, in silico prediction of potential metabolites (i.e., catabolites and conjugates) from the identified xenobiotics is performed. When a xenobiotic metabolite is located in the tissue, the type of enzyme involved in its alteration by the plant is recorded. These results were used to describe different types of biological reactions occurring in *S. alba* leaves in response to xenobiotic accumulation in the leaves. The metabolites were predicted in two generations, allowing the documentation of successive biological reactions to transform xenobiotics in the leaf tissues.

INTRODUCTION:

Xenobiotics are widely distributed around the world due to human activities. Some of these compounds are water-soluble and absorbed by soil[1], and enter the food chain when they accumulate in plant tissues[2–4]. The plants are eaten by insects and herbivores, which are prey to other organisms. The intake of some xenobiotics and their impact on a plant's health have been described[5–8], but only recently at a tissue level[9]. Therefore, it is still unclear where or how the metabolism of xenobiotics occurs, or if specific plant metabolites are correlated to xenobiotic accumulation in specific tissues[10]. Moreover, most research has overlooked the metabolism of xenobiotics and their metabolites in plants, so little is known about these reactions in plant tissues.

Proposed here is a method to investigate enzymatic reactions in biological samples that can be associated to the tissue localization of substrates and products of the reactions. The method can draw the complete metabolic profile of a biological sample in one experiment, as the analysis is non-targeted and can be investigated using custom lists of analytes of interest. Provided is a list of candidates tracked in the original dataset. If one or several analytes of interest are noted in the sample, the specific tissue localization can provide important information on the related biological processes. The analytes of interest can then be modified in silico using relevant biological laws to search for possible products/metabolites. The list of metabolites obtained is then used to analyze the original data by identifying the enzymes involved and localizing the reactions in the tissues, thus helping to understand the occurring metabolic processes. No other method provides information on the types of reactions occurring in the biological samples, the localization of the compounds of interest, and their related metabolites. This method can be used on any type of biological material once fresh and intact tissues are available and the compounds of interest can be ionized. The proposed protocol was published in Villette et al.[12] and is detailed here for use by the scientific community.

PROTOCOL:

1. Sample preparation

1.1. Obtain the biological sample and either keep it fresh and intact (e.g., do not force it into a tube) or freeze it. The proposed protocol applies to any type of solid biological sample (i.e., plant, animal, or human tissues) to localize compounds in specific tissues.

1.2. Cool down a cryomicrotome to -20 °C. Keep the sample holder and the blade at the same temperature.

1.3. If necessary, embed the object in M1 embedding medium to preserve it during cutting.

1.3.1. Pour some matrix in a plastic mold placed in the cryomicrotome chamber. Rapidly add the sample and pour some more embedding medium to cover it. Maintain the sample in the center of the mold as the matrix solidifies while cooling down.

NOTE: Embedding is not necessary for all biological objects. Homogenous objects such as mouse brains do not need embedding medium and can be cut frozen.

1.4. Place the embedded or frozen sample on the cryomicrotome holder and cut it with a sharp blade. A thickness of 5–30 µm is good for plant samples, which are difficult to cut due to their heterogeneity. Adapt the cutting thickness and temperature to the sample, making several cuts to find the best conditions. In the example provided, the sample was cut at -20 °C. Consider keeping the sample under 0 °C to avoid sample degradation.

NOTE: The use of a binocular microscope placed next to the cryomicrotome can help determine the quality of the slices. The tissues must stay intact.

1.5. Carefully move the slice to an ITO-coated slide using forceps or a small paintbrush, then place a finger under the slide to warm up and dry the sample. Keep the slide in the cryomicrotome chamber until all samples are ready. Take the slide out of the chamber slowly to avoid heat shock.

NOTE: To check which side of the slide is ITO-coated, place a drop of water on the slide at room temperature (RT). The drop will stay round on the ITO-coated side or flatten on the uncoated side.

1.6. Draw marks on the slide using a thin marker pen or correction fluid and scan it with a high-resolution scanner before matrix deposition. The marks will be used to determine the sample's exact position on the slide when it is in the mass spectrometer. The best way to obtain precise points of reference is to draw a grid.

2. Matrix deposition

2.1. Prepare the MALDI matrix: weigh 70 mg of α-cyano-4-hydroxyconnamic acid (HCCA) matrix and dilute it in 10 mL of a water and methanol solution (50:50) with 0.2% TFA. Sonicate the matrix for 10 mins at RT. There may be extra solid matrix after sonication.

NOTE: Different types of MALDI matrices can be used depending on the analytes of interest.

2.2. Clean the matrix deposition robot with 100% methanol.

2.3. Using methanol and a precision wipe, clean the slide, holding the samples without touching them and without removing the marks. Add a clean coverslip over the slide in an area without sample. That part of the slide is then placed over the optical detector to track matrix deposition.

NOTE: To optically track matrix thickness, the slide and coverslip must be very clean.

2.4. Use the robot for matrix deposition: place only 6 mL of the matrix solution in the reservoir, and add 2 mL of 100% methanol for a total volume of 8 mL.

NOTE: Recording of matrix thickness along deposition is automatic and can be recovered using a USB stick. The development of a spraying method is not detailed here.

3. Data acquisition

3.1. Place 1 µL of the matrix on a MALDI plate to calibrate the mass spectrometer using the matrix peaks as references. The development of an acquisition method on the mass spectrometer is not detailed here.

3.1.1. Insert the MALDI plate in the source, click "**Load Target**" in ftmsControl software, and wait for the plate to be loaded.

3.1.2. Choose the position of the matrix spot by clicking on the spot in the image representing the MALDI plate.

3.1.3. Indicate the sample name and the folder in the "**Sample Info**" tab of the software.

3.1.4. Start acquisition by clicking "**Acquisition**".

3.1.5. Move the plate slightly during the acquisition using the mouse pointer on the "**MALDI video**" tab so that the laser points at different spots.

3.1.6. When the acquisition is finished, go to the "**Calibration**" tab, choose **HCCA calibration** list in **Quadratic Mode** and click **Automatic**. The global calibration result is indicated in the "**Calibration Plot**" window and should be less than 0.2 ppm for a SolariX XR 7T.

3.1.7. If the calibration is good, click "**Accept**" and save the method.

3.2. Once the matrix deposition on the samples is finished, place the slide in a slide adapter and retrieve the position of the marks using a plastic cover.

3.3. In flexImaging software, set up a new imaging run using the first window that opens with the software.

3.3.1. Name the imaging run, select the **Result Directory**, and click **Next**.

3.3.2. Indicate the raster size (i.e., user-defined measurement region), choose the method to be used (calibrated at section 3.1), and click **Next**.

3.3.3. Load the optical image of the slide obtained at step 1.6 after scanning the slide and click **Next**.

3.3.4. The image opens in a wider window. Use the marks on the slide to teach the position of the slides: in **ftmsControl**, place the target of the MALDI video window on the exact position of a mark, then come back to **flexImaging** and click on the exact same point on the optical image. Repeat this for three independent points. The plastic cover bearing the marks is placed on a MALDI plate to recover its position and facilitate teaching.

NOTE: If the marks were made with a marker, adding white correction fluid at the back of the slide can make them stand out during teaching.

3.4. Draw the regions of interest in the samples in the flexImaging software using the "**Add Measurement Regions**" tools.

3.5. Save the imaging run and an "**AutoXecute Sequence**" if several samples are to be analyzed.

3.6. Launch a sequence using "**AutoXecute Batch Runner**" if several samples are analyzed.

NOTE: Synchronize the data regularly to a secure location.

4. Data processing

4.1. Import the raw data to the visualization software (SCiLS Lab) and create a dataset. This is done in two separate steps in this software.

4.1.1. Use the "**Batch Importer**" tool and select the raw data, indicate the target directory, and click on "**Import**".

4.1.2. After import, several datasets can be combined for further analysis. To combine datasets, use the "**File|New|Dataset**" tool.

4.1.3. Select the type of instrument used for acquisition, add the imported datasets by clicking "**+**", and arrange the images by clicking and dragging the objects.

4.1.4. Check the mass range settings or modify if needed by indicating the range of interest. Click **Next** to see the import summary and launch the import.

4.2. Visualize the m/z of interest in the different tissues of a sample and/or in different samples. Simply click on the **spectra** to select the m/z of interest or type the value in the **m/z box**.

4.2.1. Perform statistical tests if needed to search for colocalized or discriminative values between different tissues and/or different samples to determine the m/z of interest. The tools are available in the "**Tool**" menu and will not be described in this protocol.

4.3. Export the m/z of interest (e.g., colocalized, discriminative compounds) as a .csv file from the **Object** tab. The whole dataset can also be exported if it is not too big (i.e., maximum 60,000 lines). Click on the "**Export**" icon on the right side of the region of interest indicated by a red arrow.

4.4. Import the .csv file to create a new dataset in the annotation software (Metaboscape). Click on "**Projects | Import CSV project**". Be aware that only the exact m/z will be considered, and the isotopic profile is lost.

NOTE: The 5.0 software version allows direct import of data from the visualization software, which enables a more accurate annotation, because the isotopic profile can be considered.

4.5. Annotate with custom-made analyte lists, which can be derived from publicly available databases. A template is given by the software to create analyte lists.

4.6. Use the prediction software (Metabolite Predict) to perform in silico prediction of the metabolites of the annotated compounds. The developed formula of the compound of interest is needed, either drawn by the user in the software or imported as a .mol file. Then the method is a simple step-by-step protocol.

4.7. Recover the list of metabolites, create an analyte list, and use it in the annotation software to annotate the raw data with predicted metabolites. Alternatively, if the list of metabolites is short, manually search for it in step 4.8.

4.8. Visualize the tissue distribution of the metabolites in the raw data in the visualization software.

4.9. Recover the names of the enzymes involved in the metabolic processes in the prediction software by right-clicking on the predicted metabolite of interest.

REPRESENTATIVE RESULTS:

This protocol was applied to fresh leaves sampled from a *S. alba* tree exposed to xenobiotics in the environment. The process is depicted in **Figure 1**. The first step is to prepare thin slices of the sample of interest. Plant samples are often more difficult to cut than animal samples, as the tissues are heterogeneous and can contain water and/or air. This difficulty is handled using embedding medium, which forms a homogeneous block around the sample. The matrix deposition is facilitated by the use of a robot, avoiding hand manipulation and assuring reproducible results. The MALDI matrix layer thickness is followed during the entire process and can be recorded. Data acquisition requires learning to handle a high-resolution mass spectrometer and to adapt the method to the type of samples and compounds being investigated. Raw data are imported into a visualization software to search for the compounds of interest and display their tissue localization. Discriminative or colocalized compounds can be found using statistical tools available in the software. At this step, only the exact m/z of the compounds are known. The compounds of interest are then exported to the annotation software, which can compare the exact m/z with a custom list of compounds of interest defined by the user. If the exact m/z matches the m/z of a compound of interest, it is annotated. In the context of a metabolic profile investigation, the annotated compounds are selected for in silico prediction of metabolites. The types of biological reaction rules used to generate metabolites are easily chosen by the user, as well as the number of generations over which the metabolites are predicted. The list of predicted metabolites can be used in the annotation software to search for matches between raw data exact m/z and predicted metabolites m/z (**Figure 1**). The annotated metabolites can be searched for in the visualization software to obtain their tissue localization (**Figure 2**). The enzymes involved in the metabolism of the original compounds of interest can be recovered to draw the metabolic reactions occurring in the biological sample (**Figure 3**).

In this example, the drug telmisartan was identified in the plant leaves; it was distributed throughout the tissues. Telmisartan's metabolites were predicted and searched for in the raw data. The annotations showed that one first-generation (I) metabolite was detected in the internal tissues of the leaves and further degraded into second-generation (II) metabolites, which

were localized in internal tissues or were more generally distributed in all leaf tissues (**Figure 2**). These results suggest an active metabolic reaction in the leaves to degrade telmisartan. The process was applied to several compounds of interest annotated in the leaves, and the enzymes involved in the reactions were recovered to investigate their role in the plant's response to xenobiotics accumulation. This gives an overview of the enzymes involved in xenobiotics metabolism in *S. alba* leaves (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1. General structure of the method. A fresh sample is cut and placed on an ITO-coated slide sprayed with the appropriate MALDI matrix. The MALDI acquisition provides raw data from which the localization within the tissue can be observed with the SCiLS Lab software. Metaboscape is used for annotation, and Metabolite Predict is used for metabolite (i.e., catabolites and conjugates) prediction.

Figure 2. Example of the results obtained on *S. alba* leaves exposed to xenobiotics. Telmisartan was identified in the plant leaves and visualized in all the tissues. Telmisartan metabolites were predicted and annotated on the raw data to visualize their tissue localization. The first-generation (I) metabolite $C_{33}H_{32}N_4O_3$ was localized mainly in the internal tissues, while second-generation (II) metabolites were sometimes more generally distributed. This figure was adapted with permission from Villette et al.[12].

Figure 3. Global enzymatic profile proposed for potential reactions occurring in *S. alba* leaves in response to xenobiotics exposure. The metabolite prediction and annotation on the object of interest suggested the potential enzymatic reactions responsible for the metabolism of the compound of interest. This figure was adapted with permission from Villette et al.[12].

DISCUSSION:

The critical part of this protocol is the sample preparation: the sample must be soft and intact. Cutting is the most difficult part, as the temperature and thickness of the sample can vary depending on the type of sample studied. Animal tissues are usually homogeneous and easier to cut. Plant samples often incorporate different structures and therefore are more difficult to keep intact as the blade encounters soft, hard, or empty vascular tissues. It is highly recommended to use fresh tissues when working with plant samples to avoid the formation of ice in the hydrophilic tissues and their destruction. The slices must be moved gently when deposited on the ITO-coated slide. The MALDI matrix was slightly diluted to avoid the clogging of the spray sheet with matrix crystals, which can happen if the 2 mL of 100% methanol are not added at step 2.4.

This method offers an easy one-day sample preparation protocol that provides reproducible results due to the use of a robot for MALDI matrix deposition. The proposed protocol necessitates competence in tissue cutting and mass spectrometry imaging and only applies to compounds that can be ionized. However, it provides molecular identification without the need for labelling as used in immunohistochemistry[11]. High sensitivity is achieved because the compounds are directly ionized in the tissues or cells, avoiding dilution effects produced by an extraction protocol[12]. The samples are analyzed in a non-targeted way, which allows for a large-scale

profiling of the endogenous or xenobiotics compounds in the samples. Therefore, biological responses to exogenous compounds can be followed. The in silico prediction of metabolites coupled to non-targeted analysis adds another dimension to classical mass spectrometry imaging, because metabolic reactions can be monitored without a priori knowledge of the exogenous compounds that will accumulate in the tissues. To date, only known compounds and a few metabolites have been followed with this method (e.g., drugs of interest fed to rats)[13]. With the proposed protocol, the original compounds and their metabolites can be localized within the tissues, and the biological responses to the accumulation of exogenous compounds and/or their metabolites can be followed.

This protocol does not only apply to the response of plants to xenobiotics, but can also be used to understand animal metabolism in response to drugs, to follow plant/fungi interactions, plant response to biotic or abiotic stresses, or to understand the evolution of diseases, revealing the metabolic processes in the tissues of interest.

ACKNOWLEDGMENTS:
We thank Charles Pineau, Mélanie Lagarrigue and Régis Lavigne for their tips and tricks regarding sample preparation for MALDI imaging of plant samples.

DISCLOSURES:
The authors have nothing to disclose.

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phenanthrene degradation, and active microbiome in an aged-contaminated industrial soil. *Frontiers in Microbiology*. **7** (February), 1–15 (2016).
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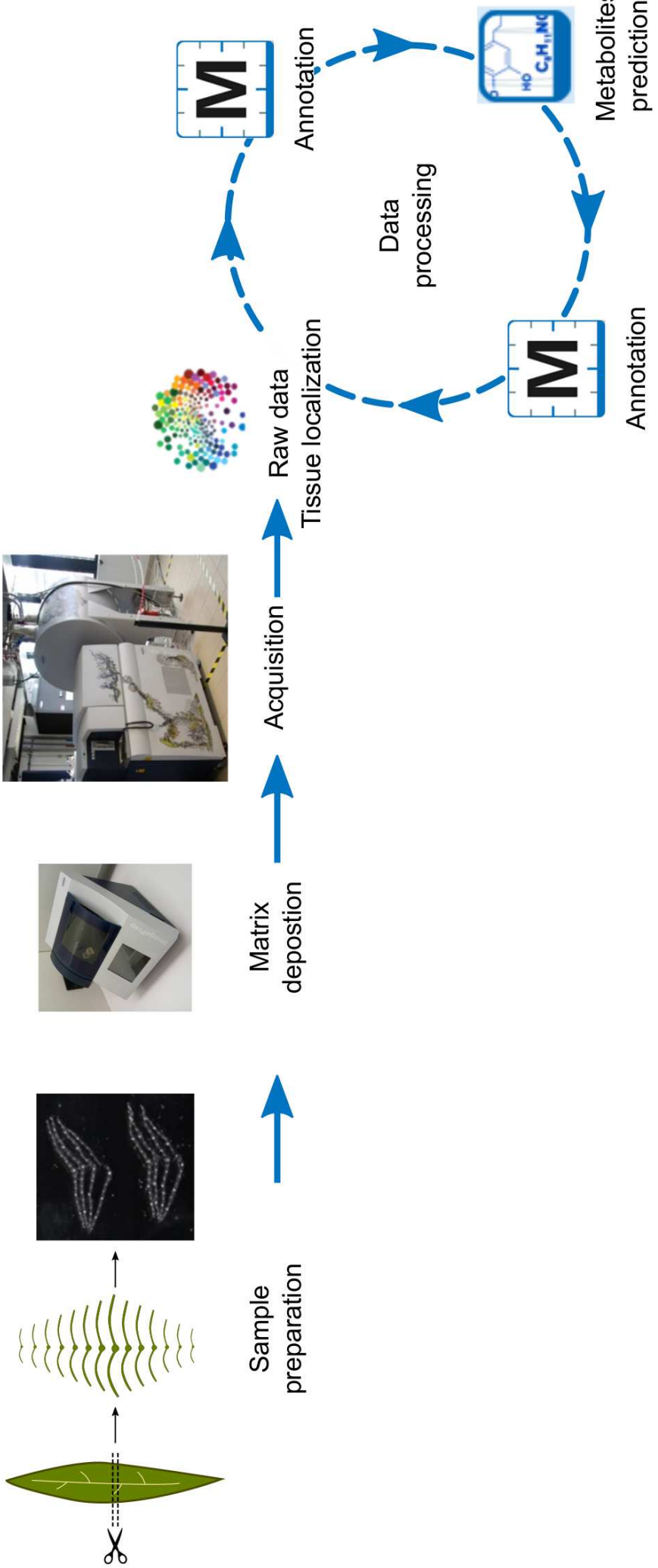
Figure 1



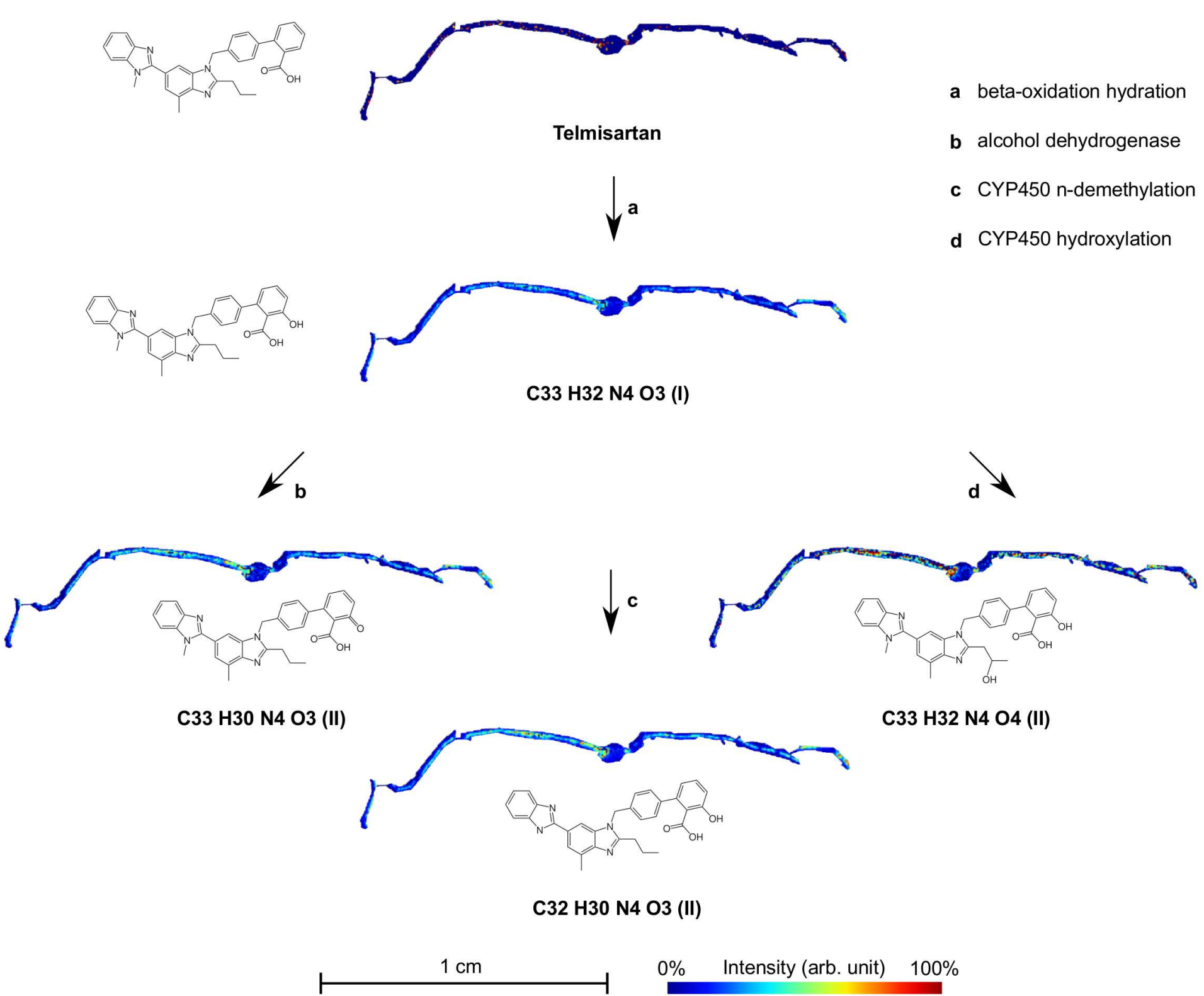
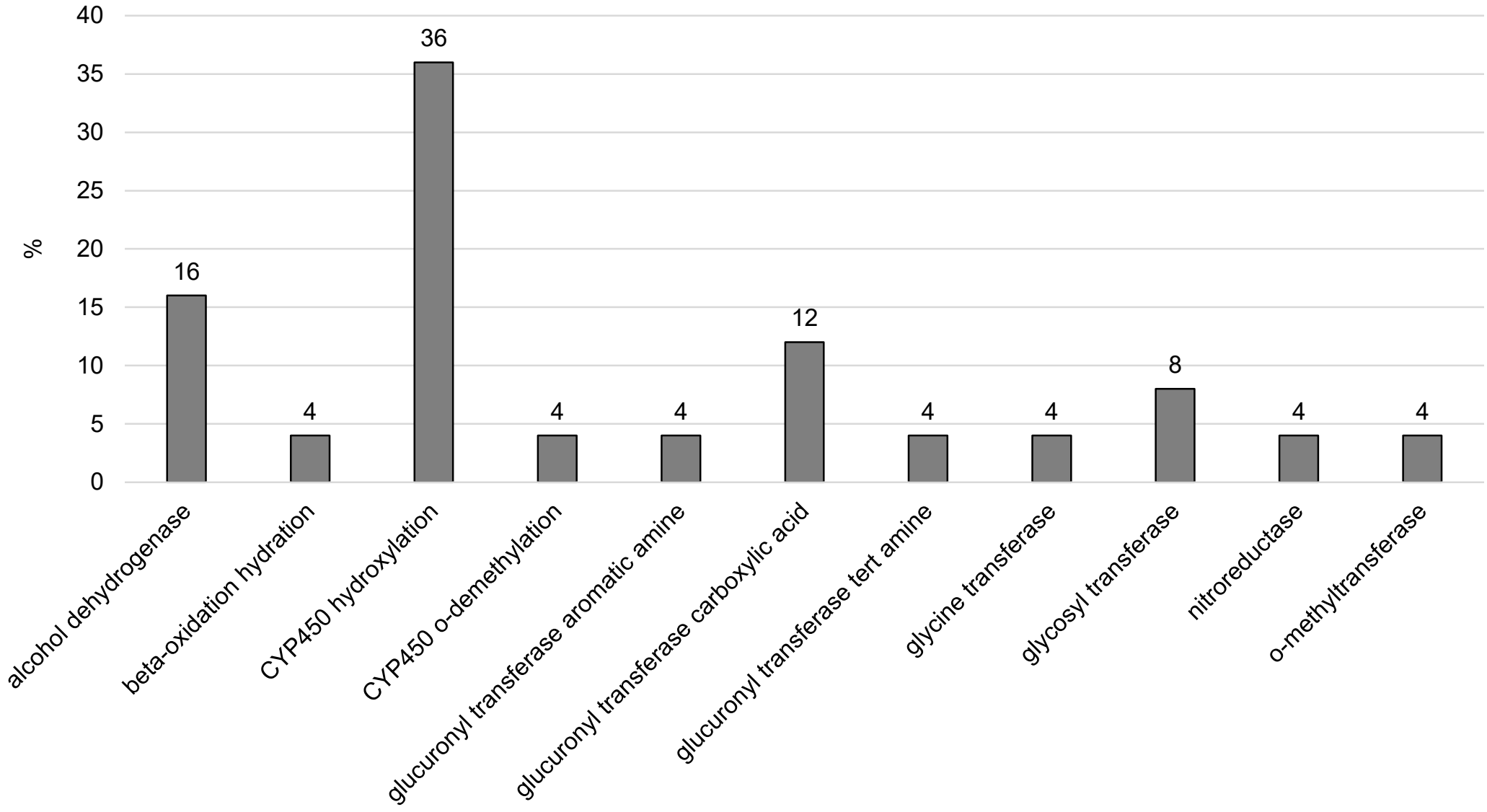


Figure 3

Click here to access/download;Figure;Figure 3.eps



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Cover slips	Bruker Daltonics	267942	
Cryomicrotome	Thermo Scientific		
Excel	Microsoft corporation		
flexImaging	Bruker Daltonics		
ftmsControl	Bruker Daltonics		
GTX primescan	GX Microscopes		
HCCA MALDI matrix	Bruker Daltonics	8201344	
ImagePrep	Bruker Daltonics		
ITO-coated slides	Bruker Daltonics	237001	
M1-embedding matrix	ThermoScientific	1310	
Metabolite Predict	Bruker Daltonics		
Metaboscape	Bruker Daltonics		
Methanol	Fisher Chemicals		No specific reference needed
MX 35 Ultra blades	Thermo Scientific	15835682	
Plastic molds			No specific reference needed
SCiLS Lab	Bruker Daltonics		
SolariX XR 7Tesla	Bruker Daltonics		The method proposed is not limited to this instrument
Spray sheets for ImagePrep	Bruker Daltonics	8261614	
TFA	Sigma Aldrich		No specific reference needed

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The whole manuscript was checked for spelling and grammar issues.

Protocol:
1. If applicable, please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care and/or human research ethics committee guidelines of your institution.

Not applicable

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

This point was checked.

Specific Protocol steps:
1. 1.1: Please clarify what types of samples are appropriate here.

A sentence was added to clarify what types of samples are appropriate:

« The proposed protocol applies to any type of solid biological sample (plant, animal, human tissues for example) for which there is an interest to localize compounds in specific tissues. »

2. 1.6: How exactly are marks made? What should they look like?

A sentence was added to clarify how the marks are made:

« The marks can be made using a thin marker pen or correction fluid. Drawing crosses is the best way to obtain precise points of reference for teaching. »

3. 2.3: How are slides cleaned?

The sentence was modified to explain how the slides are cleaned:

« Using methanol and a precision wipe, clean the slide… »

4. Sections 3 and 4: Please provide more details on software steps if they are to be filmed ('click, 'select', etc.).

Details were added in sections 3 and 4

Discussion:
1. Please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

The discussion already contains

a) critical steps lines 254-261

b) modifications and troubleshooting of the technique lines 261-264

c) limitations of the technique line 267

A sentence was added to remind the reader that skills are necessary in tissue cutting and mass spectrometry.

d) significance with respect to existing methods lines 269-272 and 278-281

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We checked and completed the table of materials

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

In this paper, the authors have used imaging mass spectrometry with a non-targeted approach to establish the metabolic profile of Salix alba leaves exposed to xenobiotics. From the identified xenobiotics, they have used in-silico prediction of potential catabolites and conjugates. In this study, authors highlight the biological responses to the accumulation of exogenous compounds and/or their metabolites. Metabolites were predicted on two generations, allowing the recording of successive biological reactions to transform xenobiotics in the leaves.

This manuscript is clearly among the top 10% technical papers I have read over tha past years in the field of environmental research. The study generally aims at understanding the role of plants in xenobiotics removal from the environment by following xenobiotics metabolism in leaf tissues, a topic of sufficient general interest to justify publication in JoVE.

Besides, the manuscript is well written. Experimental design is very elegant and the combination of techniques used does strengthen the hypotheses proposed by the authors. This reviewer has no comment but minor remarks and does congratulate the authors for such a nice piece of work.

Minor remarks:

Line 68 (Protocol 1.2): On certain equipment the temperature of sample holder and blade can be adjusted differently. Please provide information when relevant in the "Sample preparation" paragraph.

In our case the sample holder and blade were not adjusted differently, we added a sentence to provide the information

« The sample holder and the blade were kept at the same temperature in the described protocol. »

Line 77 (Protocol 1.4): Authors might want to provide more information about the temperature range.

In our example the sample temperature and cryomicrotome temperature were both set at -20°C, a sentence was added to provide the information and give an idea of a temperature range to be used.

« In the example provided here, the sample was cut at -20°C. Consider keeping the sample under 0°C to avoid sample degradation. »

As already written line 81, thickness and temperature are to be adapted depending on the sample type and several cuts are necessary for the user to decide which conditions are the best.

Line 81 (Protocol 1.5): Please correct the sentence - …a small paintbrush (plural : paintbrushes).

The sentence was corrected

Line 102 (Protocol 2.4): …6 mL of matrix solution in the reservoir…

The sentence was corrected

Line 125 (Protocol 4.2): In the sentence, please replace masses by m/z. (….Visualize the m/z of interest….)

The sentence was corrected

Line 129 (Protocol 4.3): In the sentence, please replace masses by m/z.

The sentence was corrected

Line 132 (Protocol 4.4): In the sentence, please replace masses by m/z.

The sentence was corrected

Chapters Representative results, Discussion and Legends to figures: Please check whether it is appropriate to replace mass by m/z.

The manuscript was checked to replace mass by m/z where needed.

Based on the above comments, I consider this manuscript granted for publication in JoVE.

Thank you for your time and for providing helpful suggestions.

Reviewer #2:

Manuscript Summary:
This protocol describes a work flow to understand metabolism of xenobiotics in plants, by analyzing cross sections of leaves by mass spectrometry imaging (MSI) and - using prediction software - propose metabolic pathways for the compounds of interest including enzymes and enzymatic products.

Major Concerns:

I have many concerns, most of which are related to the lack of discussion of common issues with (plant) MSI or lack of discussion of the robustness or relevance of the predictions. All of them are listed and explained below. In addition, it is never really explained anywhere why it is necessary to determine the localization of compounds to understand how they are metabolized when the prediction software itself does not require localization data to predict enzymatic conversions.

Specific points:

Protocol:

1. Sample preparation
This protocol for sample preparation includes several steps which are known to cause either dislocation of metabolites or false metabolic results. Sometimes it can be 'good enough' to perform the sample prep as described, but the authors do not point out what the drawbacks of their approaches are. Not here, and not in the discussion later on. E.g. if one wants a snapshot of the metabolic state of a sample, it is important to quench metabolism as soon as possible after perturbing the organism, for example by cutting off a leaf. It is standard practice to snap freeze directly into liquid nitrogen if possible, alternatively on dry ice, or as soon as possible upon returning from the field. Many metabolic reactions are triggered by wounding, and this is not mentioned by the authors when they state that freezing is optional. The same is the case for the option to embed samples by placing unfrozen samples in embedding medium and let it freeze slowly at -20 degrees. This can both allow metabolic reactions to happen in response to the cold and definitely cause dislocation of metabolites during the slow disintegration of the plant cells that happens during the slow freezing process. The authors do not seem to be aware

of this. Finally, the authors use thaw-mounting of the sample sections on the slides and do not mention that this is a well-known cause of metabolite dislocation and even degradation and loss of signal.

As plant biologist- biochemist I fully agree with the comment. Probably not enough or well explained here. This manuscript made for JoVE is a summary of a previously published manuscript in a peer reviewed journal Villette et al 2019 metabolomics. It is obviously well explained in the original paper. A protocol that has been applied to preserve plant tissue integrity by rapid deep freezing with embeeding is described in two of our recent papers, Villette et al Env International 2019 and Villette et al Metabolomics 2019. For further explanation I encourage reviewers to read that there.

2. Matrix deposition
Again, I find it odd that it is no where discussed that matrix deposition by liquid spraying is known to cause metabolite dislocation effects, although there are other advantages to spray coating over e.g. sublimation, that may make it a good choice depending on the scope of the analysis.
It is true that liquid matrix spraying can give rise to metabolite dislocation if it is not well done. Aware of that major point we optimized our spraying method, including adapted volume, adapted spraying time and drying time before each spray step. Those steps were repeated and optimized for each type of plant tissue as described in our previously published studies.

3. Data acquisition
l. 113-114: 'use the marks to teach the position of your slides'. Does this imply that the marks should have been made with something which can be detected by the MALDI-MS? This was not mentioned in the sample prep section.

Section 1.6. was completed to explain how the marks are to be made.

« The marks can be made using a thin marker pen or correction fluid. Drawing crosses is the best way to obtain precise points of reference for teaching. »

4. Data processing
This whole section uses vendor specific software (at least SCiLS and Metaboscape), which are not freeware and therefore mainly accessible if one has access to a specific instrument. I.e. the method is instrument specific. The section is difficult to understand when one does not know the software, but my guess is that these instructions will not be enough to fully understand the required operations - and hence this is not really a protocol allowing people to perform the experiments. Also, apparently the enzyme identification part is something that one of these pieces of software performs automatically, but nothing is stated about the reliability or curation of the results.
Yes SCiLS Lab and Metaboscape are not freeware and provided by Bruker. Yes we are happy with both softwares I recommend them for non targeted metabolomics analysis. I agree it is a pity that no equivalent freeware exists. Who is guilty ? I think we the academics people are guilty not to have yet provided something equivalent. Same question for proteomics why the community use mainly Mascot software ?
Parts 3 and 4 were completed to explain the steps to follow in the softwares.

Also here I am lacking a discussion about the limitations of the applied approaches and of the robustness of the predictions from the software. First of all, plants generally display much more complicated and diverse metabolism than animals, so it would be nice to know what the applied 'biological laws' are, and how they have been curated for plants - to understand why it is relevant to do these in silico analyses. Figure 3 is described as a 'Global enzymatic profile proposed for potential reactions occurring in S. alba leaves in response to xenobiotics exposure'. The most common conjugation reactions in plant detoxification pathways are glycosylations and glutathionylations, whereas glucuronylations are more important in animals. Nevertheless, Figure 3 suggests mainly glucuronylation enzymes to be involved in the S. alba xenobiotic conjugation reactions, and only few glycosylation enzymes and no glutathionylation enzymes, despite the fact that plants have 3-4 times as many of these enzymes as animals. This does not appear convincing when there is no metabolite data shown to support that this is indeed how the detoxification of the molecule in question happens.

I clearly do not agree with the reviewer comment regarding plant metabolism in the view of defense. Indeed the major enzymatic key player in plant responses to biotic and abiotic stressors is P450 cytochrome which leads mainly to hydroxylation but not only. It is well known and well documented that this enzyme family is the major actor for micropollutants degradation usually described as a first enzymatic step followed by several other steps where glycosylation is present that's true. Coming to the point of the predictive metabolic activity it means "predictive" and framed at which level it is coined as putative as described by Schimansky et al 2014.

Regarding Figure 2: It is unclear to me how the authors claim to know where on the molecule the specific reactions happen. Personally, I can think of several positions on the molecule that may be hydroxylated in plants. Also, a major flaw here is that there is no untreated control sample (or replicates). The Solarix of course has very high mass accuracy, so the sum formula is likely to be correct, but telmisartan is basically an alkaloid, and such compounds are produced by 25 % of all vascular plants. With the data presented, it is therefore completely possible that the detected compounds with sum formulae corresponding to telmisartan and its suspected metabolites correspond to different molecules from the plant endogenous metabolism.

The sites of hydroxylations used by P450 cytochromes cannot be predicted. The only criteria used by the software is the stereochemical information of the compound (which can also be found from pubchem), which determines whether the compound can be used by the enzyme or not.

Protocol:

1. Sample preparation
l. 88, what does it mean to 'scan' the sample? With a scanner? Also, I think, but I might be wrong, that it is only necessary to use ITO coated slides if one uses instruments from specific vendors, such as Bruker. However, the overview on p. 12 says that the protocol is not specific to the Bruker Solarix instrument used by the authors.

Details regarding the scanning of the slides were added in section 1.6..

« 1.6. Draw marks on the slide and scan it with a high resolution scanner before matrix deposition, ... A GTX primescan scanner was used to scan the slides. »

This procotol can be adapted to any type of instrument, of course we cannot describe into details how to adapt each step the any other instrument. Therefore the user can choose to use non-coated slides if they are not necessary on another instrument.

A small point: Considering that this protocol is for a Matrix Assisted Laser Desorption Ionization based method, where the matrix is something used to coat the sample to obtain desorption and ionization, it is rather confusing to refer to the embedding medium as an embedding 'matrix'. This becomes very confusing indeed when the two are mentioned in consecutive sentences on p. 5, l. 154-155.

Embedding matrix was replaced by embedding medium in all the manuscript to avoid confusion.

2. Matrix deposition
l. 99: This may make more sense if one also watches the video, but it seems counterintuitive to place a cover slide on the sample slide when we want to apply matrix to the sample. Where should the cover slide be placed exactly and why?

The sentence was modified to better explain how and why to add the cover slip

« Add a clean cover slip over the slide in an area where there is no sample. The part of the slide covered by the cover slip is then placed over the optical detector to follow matrix deposition. »

l. 102-103: One adds a matrix solution to the chamber, so what does it mean that one should 'avoid[ing] solid matrix in excess'?

The matrix solution is prepared with an excess of matrix powder, therefore it is important to avoid too much of this matrix powered which could clog the spotter. We added this information in section 2.1.

« 2.1. …Sonicate the matrix for 10 minutes at room temperature. It is normal to have solid matrix in excess after sonication. »

3. Data acquisition
l. 113-114: 'use the marks to teach the position of your slides'. Does this imply that the marks should have been made with something which can be detected by the MALDI-MS? This was not mentioned in the sample prep section.

Section 1.6. was completed to explain how the marks are to be made.

« The marks can be made using a thin marker pen or correction fluid. Drawing crosses is the best way to obtain precise points of reference for teaching. »

Reviewer #3:

Manuscript Summary:

Plant metabolism of xenobiotics is, without a doubt, a relevant and interesting research topic.

In this manuscript the authors provide a proposal for studying the local distribution of xenobiotics as well as their respective in-silico predicted catabolites and conjugates by using mass spectrometry in a non-targeted approach. The presented in-silico prediction of potential catabolites/conjugates from the identified xenobiotics in combination with together with the tissue localization has, indeed the highest news value. However, the major issue with the presented proposal concerns the implementation of this interesting strategy, - more specifically the lack of relevant information and confirmatory test.

Major Concerns:

(1) Information on the generation (plant contamination by xenobiotics) and handling of biological material needs to be added. Particularly, the number of technical and biological replicates used for MSI needs to be mentioned.
Biological replicates of the MSI images shown in Figure 2 should be provided, at least as supplementary images.

Maybe this was not clearly explained but this protocol is derived from a previously published manuscript (Villette et al 2019 metabolomics) where all the details about replicates are given. We do not consider that it is our duty in this protocol to tell scientists how many replicates they have to make for their own experiments.

(2) Information or a table showing relevant data on the detected/predicted analytes is missing (e.g. molecular formulas, adduct, theoretical mass, mass accuracy for metabolite identification).

Once again, these data were already published and cannot be given twice. Sufficient results are given here to show an example of what can be done using the proposed protocol.

(3) On-tissue MS/MS experiments need to be done to assist and confirm compound identification of MSI detected and in-silico predicted analytes.
These results were already published and therefor confirmed by the community. Moreover, most of the predicted analytes are not commercially available and their MS/MS spectra could not be used to further confirm their identification. As explained in the published manuscript, these annotations are are putative as described in Schimansky et al 2014.

Altogether, the requested information and data are essential to demonstrate the reliability and reproducibility of the described method

The described method was already published and proved reliable according to the reviewing process of the published manuscript. We were invited by JoVE to share the protocol only, as the method was already validated.

Minor Concerns:

The authors frequently use the term "at play". This is an unusual term which, moreover, is not adequate regarding the mapping of metabolite localizations, because it indicates live recording where, in fact, only a still image is shown.

“At play” was replaced by “occurring” or “involved” in all the manuscript.

In the final section Name of Material/Equipment, I can find the statement "The method proposed is not specific to this instrument". The phrase "is not limited to" may be more suitable than "not specific to".

The sentence was modified to “is not limited to”.

Generally, some passages would require some editing with regard to English grammar and style.

The whole manuscript was carefully checked again.

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