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APPLICATION NOTE

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Use of whole-body cryosectioning and desorption electrospray ionization mass spectrometry imaging to visualize alkaloid distribution in poison frogs

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Abstract

Ambient mass spectrometry is useful for analyzing compounds that would be affected by other chemical procedures. Poison frogs are known to sequester alkaloids from their diet, but the sequestration pathway is unknown. Here, we describe methods for whole-body cryosectioning of frogs and use desorption electrospray ionization mass spectrometry imaging (DESI-MSI) to map the orally administered alkaloid histrionicotoxin **235A** in a whole-body section of the poison frog *Dendrobates tinctorius*. Our results show that whole-body cryosectioning coupled with histochemical staining and DESI-MSI is an effective technique to visualize alkaloid distribution and help elucidate the mechanisms involved in alkaloid sequestration in poison frogs.

KEYWORDS

Ambient Analysis, Amphibia, chemical defense, Dendrobatidae, histology

1 | INTRODUCTION

Mass spectrometry imaging (MSI) is a technique whereby the analyte is desorbed, ionized, and detected directly from biological samples. A set of mass spectra is acquired, which represents the local molecular composition at known *x*,*y* coordinates of the tissue section and then assembled in an image by a specific software (for review in MSI see McDonnell and Heeren[1]). In ambient ionization mass spectrometry techniques, ionization occurs at ambient conditions with minimal or no preparation of samples.[2] Among ambient ionization techniques, desorption electrospray ionization (DESI) has been applied extensively in pharmaceutical and natural products research to detect both large and small molecules.[3–6] In DESI, a fine spray of charged droplets is directed at the sample surface, generating ions that are transferred to the spectrometer.[2] The lack of sample preparation and the noninvasive ionization in DESI-MSI allows histological analysis of the same tissue after MSI, providing correlation and confirmation of the morphological structures in the sample.[7] These analytic characteristics

allow this technique to be used in a wide variety of research, and, in particular, where mapping of molecules is critical to understand the location, movement, and/or metabolism of specific compounds.

An example is the study of defensive chemicals in amphibians. The production, storage, and mechanism of secretions have been studied extensively, and the biochemical pathways and distribution of these compounds are known for some amphibian species. Barbosa et al.[8] and Brunetti et al.[9] used matrix-assisted laser desorption ionization (MALDI)-MSI in studies of peptide distribution in the skin of two different frog species. Although MALDI-MSI is one of the most commonly used methods of mass spectrometry for *ex vivo* molecular viewing of targeted organs or whole-body sections from an animal,[10,11] its reliance on a ultraviolet (UV)-absorbing matrix to ionize molecules can hinder the detection of small molecules. More specifically, any matrix-related peaks that fall within similar mass ranges to the compounds of interest could prevent their detection,[12] which could be the case of smaller molecules found in some species of frogs, such as the lipophilic alkaloids of poison frogs (150-500 Da).[13] High resolution mass spectrometers like Orbitraps and Fourier-transform ion cyclotron resonances (FT-ICRs) can circumvent this problem due to the very narrow peaks. However, the high price of these systems makes them inaccessible to many research labs.

Poison frogs are a polyphyletic group of anurans that independently evolved the capacity to sequester lipophilic alkaloids from their diet.[14–17] These compounds are found in dietary arthropods like ants, mites, and beetles,[18] and laboratory-controlled experiments have shown that these defensive chemicals are stored in the frog's skin glands with little or no chemical modification.[15–17] Although many ecological and evolutionary aspects of the causes and consequences for the presence of lipophilic alkaloids in poison frogs have been studied,[19] little is known about the sequestration mechanism or the organs and tissues involved in the process.

Visualization and mapping of alkaloids in the body of a poison frog could elucidate the pathway alkaloids travel through different organs and tissues. Standard histological procedures have been used to study the morphology and ultra-structure of the skin glands in which frog alkaloids are stored after uptake from dietary sources[20,21]; however, no histochemical procedures are available for staining all alkaloids, which is largely due to the considerable diversity of alkaloids present in poison frogs (more than 1400 alkaloids of 24 classes have been described to date).[19,22,23] For this reason, techniques that allow for visualization of small molecules and metabolites on a surface of interest without specific chemical targets are interesting strategies to analyze poison frog alkaloids.

In this study, we present the use of DESI-MSI to map alkaloids in whole-body sections of poison frogs. To do this, we used an alkaloid from the histrionicotoxin (HTX) class (HTX **235A**) in an experimental procedure that included oral administration of the alkaloid, DESI-MSI, and histology. HTXs are a class of spiropiperidine alkaloids that have highly selective inhibition of nicotinic acetylcholine receptors and are commonly found in dendrobatid poison frogs.[13,24] Here, we describe the method for the whole-body sectioning of frogs and the detection and mapping of synthetic HTX **235A**.

2 | MATERIALS AND METHODS

2.1 | Poison frog experimental procedures

Because wild poison frogs sequester alkaloids from their natural diet, captive-bred frogs lack alkaloids.[25] For our study, we used two juveniles of the poison frog species *Dendrobates tinctorius* (Dendrobatidae). All experimental procedures with live animals were performed at John Carroll University, Ohio, USA (IACUC protocol #1700). The juvenile frogs (approximately 1.5 cm snout-vent length) were purchased in the pet trade (Josh's Frogs, MI, USA), maintained in terraria under controlled temperature (20°C-25°C) and humidity (70%-100%) and a 12-hour light cycle[26] and fed *Drosophila melanogaster* dusted with multivitamin powder *ad libitum*. Based on the estimated daily consumption of alkaloid-containing arthropods, we administered 3.2 μg of HTX **235A** to one of the frogs by pipetting 5 μL of a 0.64 μg/μL solution of HTX **235A** suspended in 50% ethanol directly into the posterior portion of the buccal cavity. After 1 hour, the frog was euthanized by cooling followed by flash freezing in liquid nitrogen. The other frog was used as a negative control for alkaloid presence. The frogs were stored at −18°C until being transported to York University, ON, Canada (CITES permit #18US17079D/9) and prepared for cryosectioning. The racemic HTX **235A** used in this study was synthesized via Hg $(OTf)_2$-catalyzed cycloisomerization and SmI_2-mediated ring expansion reactions.[27]

2.2 | Whole-body cryosectioning

We tested three different embedding media: 2% carboxymethyl cellulose (CMC), 5% CMC, and 5% CMC + 10% gelatin based on procedures described for zebrafish by Nelson et al.[28] and Perez et al.[29] We removed the arms and legs of the frogs[7], placed the whole body in a flexible, peel-away mold with embedding media, and oriented it to sagittal sectioning. To visualize simultaneously as many internal organs as possible, we analyzed three regions of the body through mid-sagittal, left, and right parasagittal sections. We prepared 15-μm tissue sections using C.L. Sturkey Diamond Disposable Blades (Lebanon, PA, USA; dimensions: 76 × 8 × 0.25 mm) at −18°C on a Shandon E cryotome, Thermo Fisher (Nepean, ON, Canada) and thaw-mounted each section on a glass microscope slide. Serial tissue sections for DESI-MS and histology were collected alternated in order to allow correlation and organ identification. The slides were stored at −18°C until DESI analysis. The tissue sections for DESI-MS were air-dried for 30 minutes before the procedure. The sections for histological analysis were stained with hematoxylin and eosin (H&E). We pipetted all staining reagents on slides until sections were immersed. In brief, we immersed the sections in hematoxylin for 1 minute, washed thoroughly with tap water, immersed sections for a few seconds (approximately 3 seconds) in eosin, washed thoroughly with ethanol 95%, and mounted slides with Cytoseal 280 (Thermo Scientific). We took digital photographs of the histological sections using a Zeiss Discovery.V12 stereoscope and a Zeiss AxioCam ICc5 camera.

2.3 | DESI-MS of HTX 235A

We analyzed a 0.5 μg/μL HTX **235A** solution using DESI-MS and DESI-MS/MS in a linear ion trap mass spectrometer (LTQ; Thermo Scientific, USA). We analyzed a 0.5 μL drop of the HTX **235A** solution in a silica plate and a 0.5-μL drop on the tissue section of the control frog. For optimal desorption and ionization, we used the following DESI ion source parameters: capillary temperature of 180°C, capillary voltage of 14 V, solvent spray voltage of 5 kV, incident angle of 52°, 2 mm capillary tip to surface, 3 to 5 mm distance from mass inlet to capillary tip, nitrogen gas pressure of 100 psi, and flow rate of 4 μL/min of 95% methanol/5% water solvent. We obtained full scan DESI-MS spectra in the mass range of *m/z* 100 to 1000 and conducted MS/MS on HTX **235A** peaks using collision energies between 25 and 30 arbitrary units (manufacturer's unit). We used Qual Browser Xcalibur 2.0 to process the mass spectral data.

2.4 | DESI-MSI

The DESI ion source was mounted on a 2D moving stage that automatically moves in the *x*- and *y*-directions for imaging acquisition. The poison frog whole-body sections were 1.6 cm long and 0.6 and 0.7 cm wide. We performed DESI-MSI in positive ion mode with the same DESI ion source parameters described above. Ion injection time was set to 250 milliseconds, summing three microscans. Full scan mass spectra were in the mass range of *m/z* 100 to 1000. Imaging acquisition times varied between 3.0 and 3.5 hours with a spatial resolution of 100 μm for all images. We used ImageCreator ver. 3.0 software to convert the Xcalibur 2.0 mass spectra files (.raw) into a format compatible with BioMap (freeware, http://www.maldi-msi.org/).

3 | RESULTS AND DISCUSSION

Our study is the first to use DESI-MSI to map the distribution of defensive chemicals in frogs. In Figure 1, we present the summarized method developed to detect HTX **235A** in the sagittal sections of the experimental specimen and to determine the tissues in which the alkaloid was detected by comparison with the H&E-stained section. We determined HTX **235A** peaks through DESI-MS in the silica plate and on the control section (base peak *m/z* 236, Figure 2A). Figure 2B shows the MS/MS profile of the alkaloid. A fragmentation pathway is proposed on Figure S3. Experiments using stable isotopically labeled standards, MS^n and theoretical calculations are necessary to confirm this pathway. The same MS and MS/MS profile was detected in the experimental poison frog. On the basis of these data, we were able to determine that 1 hour after oral administration HTX **235A** was present in the mucous membrane of the mouth, stomach, intestine and, with less intensity, liver (Figure 3). We cannot affirm, however, if the alkaloid has already been absorbed by the guts tissue or is still located extracellularly. No peak at *m/z* 236 was detected in the control frog, confirming the absence of HTX **235A** (Figure S2I).

We also observed several chemical species in the full scan spectra and their different localizations (Figures S1 and S2). These species can be grouped in (a) small metabolites (e.g., *m/z* 134), (b) surface/solvent contaminants (e.g., *m/z* 551, present only outside the tissue), and (c) phospholipids (e.g., *m/z* 768, 797, and 813). Phospholipids are the most abundant class of chemicals observed by DESI in biological tissues in the *m/z* 700 to 900 range.[30] These chemical species will be identified in the future by high-resolution mass spectrometry and chemical standards.

One of the difficulties of mapping the distribution of target molecules in vertebrates is whole-body cryosectioning because it involves sectioning both soft (e.g., skin, muscle) and hard (e.g., bone) tissues

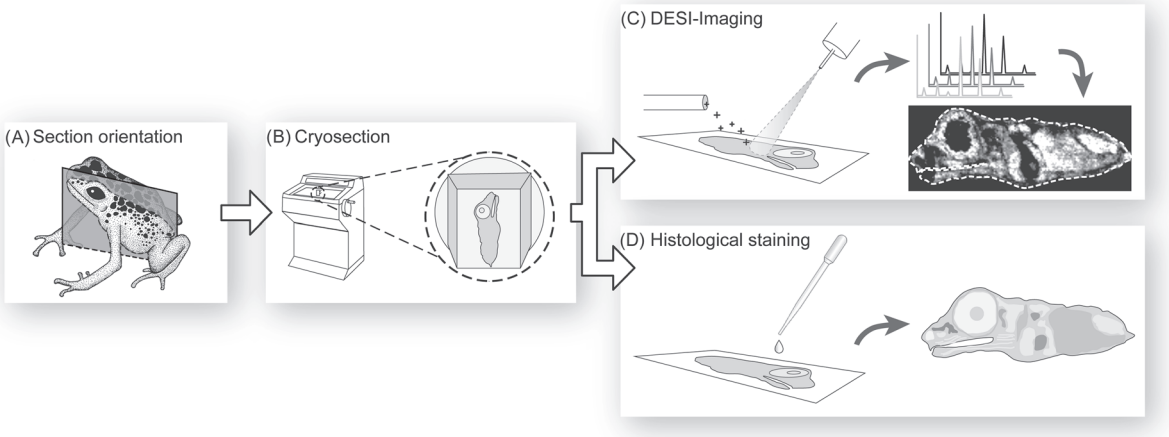

FIGURE 1 General overview of the major steps in desorption electrospray ionization mass spectrometry imaging (DESI-MSI) of whole frog specimens. A, Determine optimal orientation for sectioning. Sagittal sectioning allows simultaneous visualization of as many organs as possible. B, Cryosection whole frog from posterior to anterior (−18°C, 15 μm sections), thaw-mount on glass microscopy slides. C, Perform DESI-MSI, including generation of mass spectra and conversion to image. D, Stain with hematoxylin and eosin for comparison with MSI

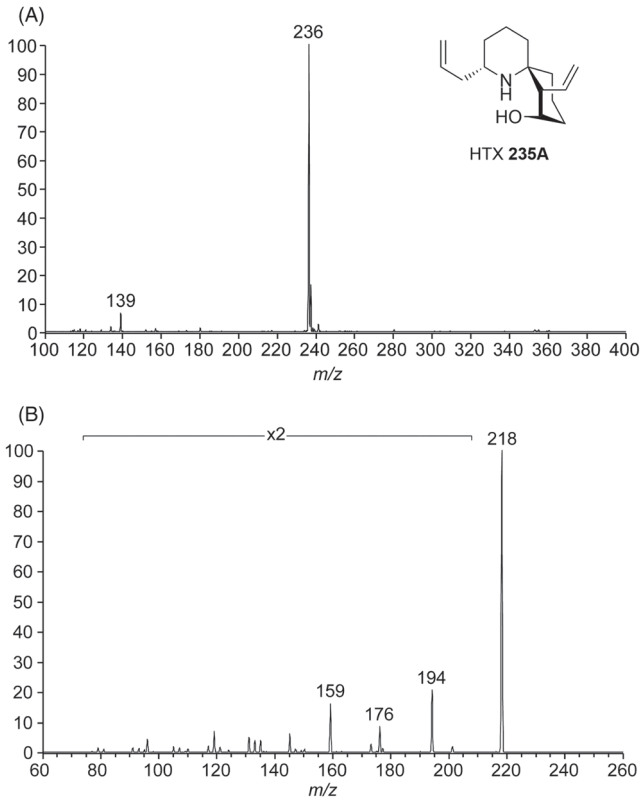

FIGURE 2 A, Full desorption electrospray ionization mass spectrometry imaging (DESI-MS) of histrionicotoxin (HTX) **235A** spotted on silica plate. B, MS/MS spectra of HTX **235A**

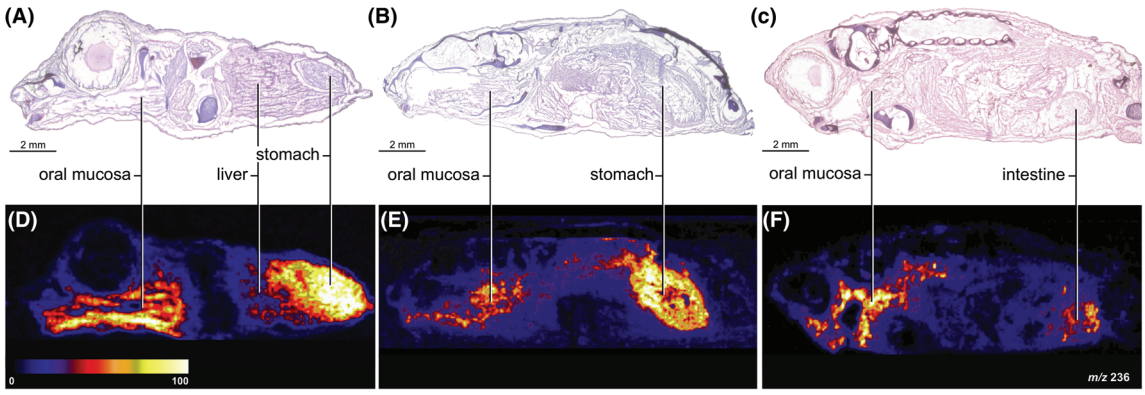

FIGURE 3 A to C, Histological sections and D to F, desorption electrospray ionization mass spectrometry imaging (DESI-MSI) of *Dendrobates tinctorius* after 1 h of oral administration of histrionicotoxin (HTX) **235A**. A and D are images of the left parasagittal section of the frog, B and E represent the mid sagittal sections, and C and F represent the right parasagittal sections. Organs and areas of the body where HTX **235A** was detected are identified

simultaneously, which requires careful selection of embedding media and specific attention to other practical techniques during sectioning. The optimal medium for whole-body cryosectioning used in the present study was 5% CMC + 10% gelatin. This was the only medium that was not too hard to tear the section, disintegrating the tissues and hindering the histological analysis, but hard enough to maintain rigidity at a proper temperature. The solution was also the most pliable and stable medium for frog cross-sectioning, in agreement with similar results found for zebrafish cryosectioning.[28] The minimum section thickness we achieved was 15 μm. Also, given that whole-body sectioning involves different tissue densities, the best orientation of the body relative to the blade was found to be posterior to anterior (Figure 1B), which resulted in soft tissues being cut before most bony structures (e.g., skull, vertebral column, and pectoral girdle).

Even though we experimented and optimized whole body sectioning, the sections obtained in our histological analysis are not suitable for cellular level analysis; nevertheless, they permitted identification of major tissues and organs, which was our main goal.

Our results show that whole-body cryosecting and DESI-MSI are effective tools to study alkaloid sequestration in poison frogs. Whole-body mapping of alkaloids in poison frogs using DESI-MSI is a first step toward further studies aiming to understand the pharmacokinetics and biochemical mechanisms of alkaloid sequestration. For example, some poison frogs are known to metabolize certain alkaloids,[15] but the physiological role of metabolism and the organs involved in these modifications are still unknown. In a controlled oral administration experiment with the specific alkaloid that is metabolized, DESI-MSI could show the tissues involved in biomodification. Further, the use of stable labeled alkaloids would allow a metabolomic study of these compounds and structural elucidation of metabolites. Also, information is lacking on the detailed location of alkaloids in specific parts of the body (e.g., different types of skin glands). Given the challenges involved in whole-body cryosectioning and consequent lack of resolution, after initially mapping the molecule in whole-body sections and targeting the regions or organs of interest in one experimental specimen, one could remove the target organs from another experimental specimen and section them separately. This approach would allow for cellular level analysis, as well as higher resolution during DESI-MSI acquisition.

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SUPPORTING INFORMATION

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