

In Situ Analysis of *Asimina triloba* (Paw Paw) Plant Tissues for Acetogenins via the Droplet-Liquid Microjunction-Surface Sampling Probe Coupled to UHPLC-PDA-HRMS/MS

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Abstract:

Asimina triloba, commonly known as paw paw, is one of approximately 2100 species in the Annonaceae family, scores of which are known to biosynthesize bioactive secondary metabolites, termed Annonaceous acetogenins. Even with over 400 acetogenins identified, a high-throughput screening protocol for these compounds does not exist. Advances in direct ambient ionization mass spectrometry have opened the door to many metabolite profiling methodologies, but for acetogenins, this is often complicated by the abundance of isomers that are present. A droplet-liquid microjunction-surface sampling probe coupled to UPLC-PDA-HRMS/MS system was employed to detect acetogenins *in situ* from *A. triloba*. The seeds, fruit pulp, twigs, leaves, and flowers of *A. triloba* were all examined for acetogenins. Additionally, lithium was infused post-column to increase the sensitivity of the fragments, thus allowing for characterization of the structural classes, and mass defect filtering was used to mine the data for the various acetogenin analogues. This surface sampling system allowed for the rapid identification and differentiation of Annonaceous acetogenins directly from the various organs of *A. triloba*, including the never before studied flowers.

Keywords: *Asimina triloba* | paw paw | mass spectrometry | acetogenins | *in situ* analysis

Article:

Introduction

Asimina triloba (L.) Dunal, Annonaceae, commonly known as paw paw (Fig. 1A), has been studied for decades, notably by McLaughlin and colleagues,¹⁻³ due to the presence of Annonaceous acetogenins, which are polyketide-derived fatty acid derivatives. These compounds contain at least one tetrahydrofuran (THF) ring, a methylated γ -lactone, and a

number of hydroxy groups along the hydrocarbon chain (Fig. 1B). Over 400 acetogenins have been isolated from various plants in the Annonaceae family²⁻⁴ with more discovered every year.⁵⁻⁷ *A. triloba* is of particular interest because out of the roughly 120 genera and 2100 species from Annonaceae, it is the prominent temperate species; the rest are predominantly tropical or subtropical.^{2,8,9}

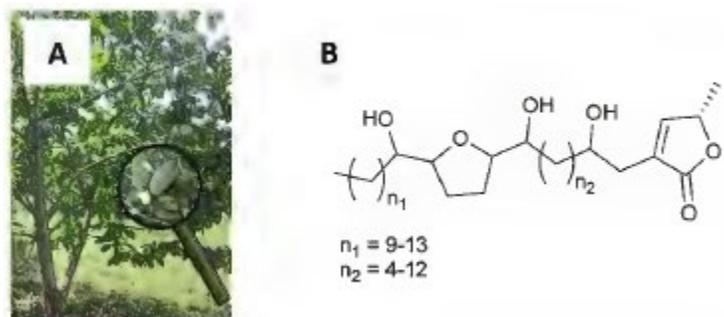


Fig. 1. (A) Photograph of *Asimina triloba* and magnification of the fruit. (B) General backbone for the mono-tetrahydrofuran structural class of Annonaceous acetogenins.

Annonaceous acetogenins have shown activity in a number of assays, including antimalarial, anthelmintic, pesticidal,¹⁰ piscicidal, antimicrobial, antiviral and antitumor.^{11,12} Currently, they are used in commercial products such as pesticidal shampoos¹³ and sprays, ointment for oral herpes (HSV-1), an anthelmintic pill, and as a botanical supplement for certain cancer patients.^{2,7}

Annonaceous acetogenins can be subdivided into three structural classes: mono-THF, adjacent bis-THF, and nonadjacent bis-THF.¹⁻³ Full characterization of each acetogenin typically involves a compilation of techniques, including LC, NMR, MS, and CD.¹⁴⁻¹⁶ However, a majority of the structure can be determined solely by HRMS/MS, specifically the positions of the THF ring(s) and the hydroxy groups along the hydrocarbon backbone. This is important because by identifying the structural class, number of hydroxy groups, and the distance between the lactone and the THF ring(s), one can infer the relative bioactivity of an acetogenin.^{11,17} However, to utilize HRMS/MS, chromatography is vital due to the numerous isomers that exist in the plant.¹⁸⁻²¹ Thus, while direct ambient ionization techniques have provided in situ MS screening protocols for many classes of secondary metabolites,²²⁻²⁴ none currently exist for acetogenins. Moreover, only a few direct ambient ionization studies have been performed on entire plant materials.²⁵⁻²⁸ For this reason, *A. triloba* provided a great test case to explore direct ambient ionization techniques in situ for plants that contain a complex mixture of isomers.

Direct ambient ionization mass spectrometry techniques, such as desorption electrospray ionization (DESI) and nanoDESI, have shown to be efficient at detecting trace amounts of secondary metabolites directly on an organism,^{26,28-31} but can struggle with the determination of isomers due to the lack of chromatographic separation.³² Detecting the presence of acetogenins using accurate mass without the ability to differentiate between isomers is insufficient when trying to rapidly screen for particular constituents.

The recently developed droplet-liquid microjunction-surface sampling probe (droplet-LMJ-SSP) has the ability to perform microextractions directly on a sample while affording chromatographic separation.³²⁻³⁴ This technique reintroduced this key element to direct ambient ionization mass

spectrometry techniques, thus making the differentiation of isomers possible.³² Here, we demonstrate the application of droplet-LMJ-SSP to elucidating the structures of the acetogenins observed in the seeds and pulp of the fruit, twigs and leaves of the branches, and the petals and ovaries from the flowers of *A. triloba*. Interestingly, the flowers of plants are rarely studied, especially from the Annonaceae family,³⁵ therefore this was the first comparison of the acetogenins in the flower tissues to that of the rest of the well-studied tissues (i.e. seeds and twigs) for any plant in the Annonaceae family.³⁶

Historically, electron impact (EI) and fast atom bombardment (FAB) ionization techniques were used to acquire MS/MS data to elucidate the structures of acetogenins.^{18,37} However, when using the more modern approach of electrospray ionization (ESI), acetogenins do not form prominent product ions, confounding the use of MS/MS for structure elucidation. Thus, a clever development for gaining discernible fragmentation patterns from acetogenins using ESI was the post-column infusion of lithium.¹⁴ Lithium addition greatly increased the sensitivity for HRMS/MS by forming prominent lithiated adducts ($[M + Li]^+$) to both the precursor and product ions.¹⁴ Structures of acetogenins were then elucidated by obtaining the molecular formula from HRMS, and then determining the placement of the THF ring(s) and the hydroxy groups by deciphering the MS/MS fragmentation patterns.¹⁴

Finally, mass defect filtering (MDF) was applied to deconvolute the chromatograms afforded by the analysis of these complex mixtures.³⁸ MDF capitalized on the fact that related analogues will be within an easily isolated mass range (i.e. ± 100 Da), but perhaps more importantly, similar in mass defect (i.e. ± 25 mDa).³⁸ Additions and losses of carbons, hydrogens, and/or oxygens to an acetogenin only adds or subtracts 0.00 mDa, 7.82 mDa, or -5.09 mDa, respectively, to the overall mass defect.³⁸ This process rapidly generates a list of possible analogues to be explored further. This project aimed to combine LC-MS surface sampling with MDF to quickly profile the acetogenins observed in *A. triloba*.

Results and discussion

Identification of acetogenins from complex mixtures

Annonaceous acetogenins are produced in abundance from *Asimina triloba*.^{10,18,21,39-49} Not only are a variety of analogues present, but each analogue is coupled with multiple isomers, resulting in a complex mixture of acetogenins (Fig. 2A). Therefore, the identification of acetogenins was impossible by direct ambient ionization techniques without some form of separation. The droplet-LMJ-SSP provided in situ analysis of *A. triloba*, and by coupling it to UPLC-HRMS/MS, the separation of isomers was achieved. Furthermore, post-column lithium infusion increased the sensitivity for tandem mass spectrometry, thus providing discernible fragmentation patterns (Fig. 2B).

When sampling the various tissues from *A. triloba* (seeds, pulp, twigs, leaves and flowers), acetogenins were readily identified by their characteristic MS/MS spectra. This allowed for the rapid characterization of the acetogenins present in each of the *A. triloba* tissues. For instance, a prominent accurate mass (m/z 603.4807) that was detected corresponded to an acetogenin with the formula of $C_{35}H_{64}O_7$. When searching the Dictionary of Natural Products,⁵⁰

there were 46 matches to that formula. Pairing the results with the MS/MS fragmentation patterns, three matches remained, all of which only differed by chirality: asitrilobin B,¹⁸ annonacin,⁵¹ and cis-annonacin.⁵² Using an alternate way to search the data, a Dictionary of Natural Products⁵⁰ search for *A. triloba* yielded 36 matches that were acetogenins. Narrowing this search by adding a filter for the formula of the prominently detected mass ($C_{35}H_{64}O_7$), resulted in 6 matches. Based on the MS/MS fragmentation pattern, only asitrilobin B remained a match for the mass.

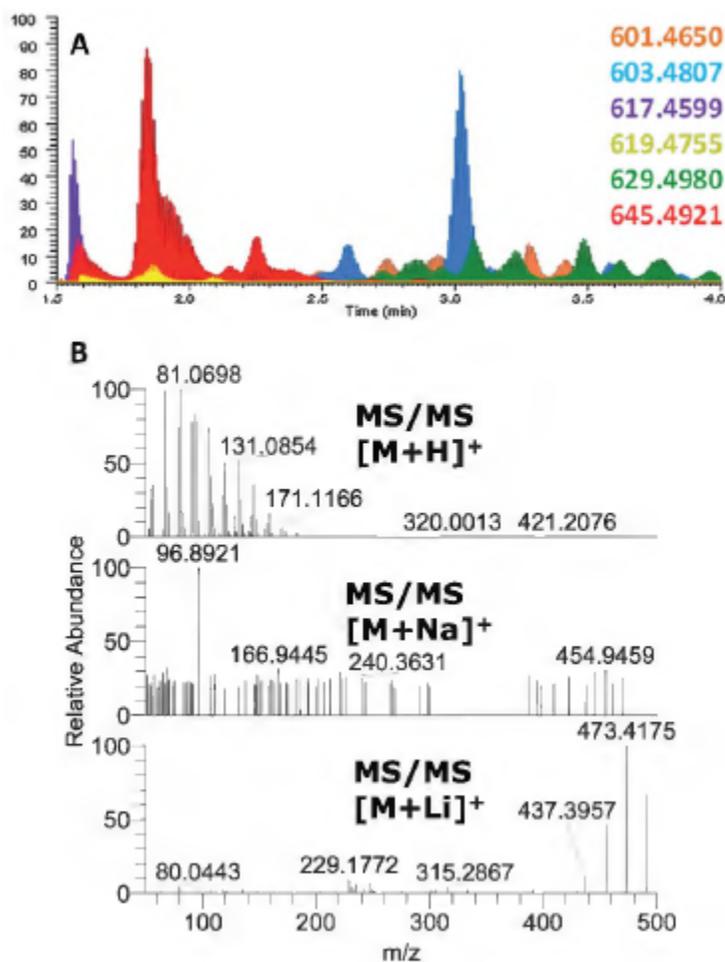


Fig. 2. (A) Overlay of the exact mass chromatograms for various acetogenin analogues and isomers detected in the pulp of a paw paw fruit by the droplet-LMJ-SSP-UPLC-HRMS system. (B) Comparison of the MS/MS fragmentation patterns with ESI after an HCD of 60 was applied to the molecular ions of $[M + H]^+$, $[M + Na]^+$, and $[M + Li]^+$ for annonacin (m/z 603.4807).

Screening *A. triloba* for acetogenins in situ with a more direct ambient ionization technique, DESI-MS, had less conclusive results. Reactive DESI⁵³⁻⁵⁵ was performed to increase the detection of acetogenins by having a solvent spray system with lithium fluoride in it. While the HRMS signals for the acetogenins were observed, the MS/MS signals were indiscernible due to the complexity of the mixture (data not shown). This did nothing to alleviate the overwhelming issue of differentiating between isomers. Those same searches in the Dictionary of Natural Products⁵⁰ resulted in 46 matches for the formula $C_{35}H_{64}O_7$, but the lack of chromatography

made differentiating between them difficult. Even when the search was further limited to *A. triloba*, it was difficult to rule out any of the remaining 6 matches.

While the droplet-LMJ-SSP system provided the differentiation of isomers, it was not without its challenges. The chromatograms obtained by the droplet microextraction were difficult to navigate due to the complexity of the mixture and the small extraction volume (5 μL) of the droplet. With such a complex mixture and low extraction volume, the chromatographic peaks for the acetogenins were very low in intensity, such as with the flower petal (Fig. 3A). This was especially true when analysing the pulp considering the amount of sugars extracted from the fruit, further suppressing the signal.

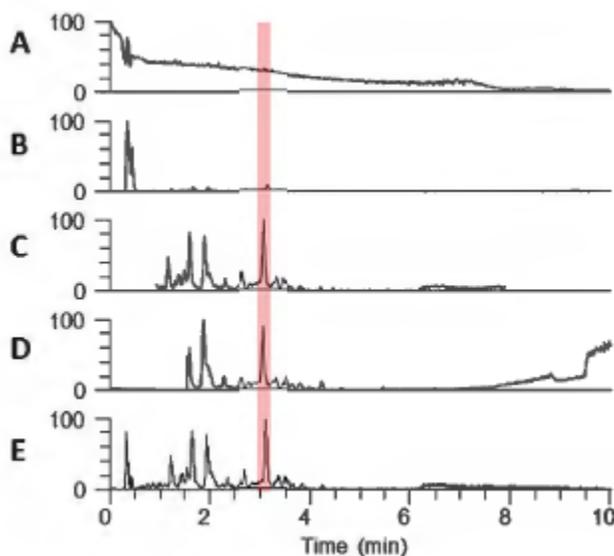


Fig. 3. (A) The unfiltered base peak, (B) the extracted ion chromatogram (m/z 550–700), and (C) the retention time filtered data (1.0–8.0 min) chromatograms for the direct analysis of a paw paw flower petal. (D) A subsequent injection with a narrow mass range (m/z 550–700) and 1.5–8.0 min. (E) The original sample (A) after applying a mass defect filter of ± 25 mDa to 603.4807 ± 100 Da. The highlighted region (red) indicates the location of annonacin (m/z 603.4807).

Consequently, scan filters were applied to make the data easier to navigate. Extracted ion chromatograms of the m/z range of 550–700 displayed some minor peaks associated with acetogenins, but were still insignificant (Fig. 3B). Furthermore, narrowing the retention time range to 1.0–8.0 min, where the acetogenins typically eluted (Fig. 3C), gave rise to several discernible peaks that were attributed to acetogenins. Alternatively, rather than filtering the existing data, a new microextraction was performed with a narrow mass window (550–700) and with the first 90 s diverted from the mass spectrometer (Fig. 3D). This too resulted in the rise of several peaks identified as acetogenins. However, while these methods helped generate lists of the acetogenins present, the lists were not necessarily comprehensive. The questions of how many and which acetogenins were present were left inconclusive and imprecise when compiled manually.

To obtain a more comprehensive list when answering how many and which acetogenins were present, mass defect filtering (MDF) was performed.³⁸ The most prominent acetogenin in most

of the samples was attributed to annonacin based on the HRMS and MS/MS data. This assignment was confirmed by isolation and structure elucidation (Fig. S1†).^{20,51} A mass range of ± 100 Da with a mass defect of ± 25 mDa was applied around the accurate mass of annonacin (m/z 603.4807 $[M + Li]^+$), and the newly created chromatogram (Fig. 3E) displayed an abundance of prominent peaks that were acetogenins.

Although the mass defect filtered chromatogram was relatively similar to the manually filtered chromatograms, the more important information obtained was the population of a comprehensive list of acetogenin peaks (Table S1 and Fig. S2†). This provided a comprehensive target set to perform data dependent fragmentation on subsequent samples. Furthermore, MDF did not limit the amount of data collected (i.e. m/z ranges, diverted flows, etc.), therefore the data could always be re-examined in search for other substituents that may become of interest in the future.

Elucidation of acetogenins

Like peptides, acetogenins fragment in predictable patterns. Acetogenins fragment around the hydroxy groups, which help determine the placement of the THF ring(s), the length of hydrocarbon chains, and the positions of each hydroxy group (Fig. 4).¹⁻³ The loss of m/z 112 Da (red) indicated the removal of the lactone moiety. The subsequent losses of water (18 Da) denoted the number of hydroxy groups throughout the molecule (red).

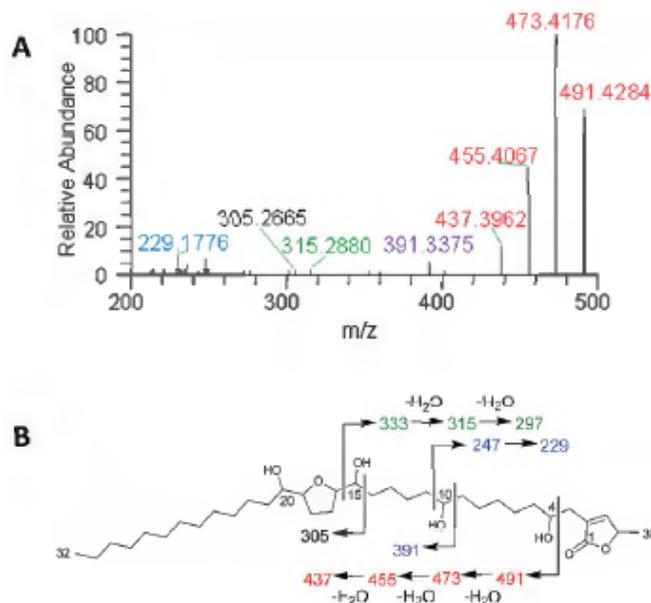


Fig. 4. (A) Fragmentation pattern of annonacin from direct analysis of a paw paw seed. (B) Elucidation of annonacin. The accuracy of annonacin between the observed and calculated was 0.8 ppm (603.4802 observed vs. 603.4807 calculated for $[C_{35}H_{64}O_7 + Li]^+$).

The remaining fragmentations indicated breaks at each hydroxy group, including those that flank the THF rings. In the case of annonacin, the fragments on both sides of C-10 were observed by m/z 247 & 229 (blue) and m/z 391 (purple) and similarly with C-15 at m/z 333 & 315 (green) and m/z 305 (black). These fragments complete the portion of the molecule to the right of the THF ring (Fig. 4). The only remaining pieces must be the THF ring, its flanking hydroxy group, and

the other hydrocarbon chain, which finalized the structure to the left of the THF ring. The ability to separate the acetogenins and then elucidate the structures via MS/MS reopens the possibility of high-throughput screening for particular acetogenins of interest.

Spatial distribution of acetogenins

Annonaceous acetogenins from *A. triloba* have been primarily isolated from the seeds^{18,44} and twigs.^{39,49} While there is some research investigating the leaves,³⁶ the flowers have not yet been explored for the presence of acetogenins, even for other members of the Annonaceae family. A comparison of the fruits (seed and pulp), twigs, leaves, and flowers (petal and ovary) was performed using the droplet-LMJ-SSP coupled to a UPLCHRMS/MS system with post-column lithium infusion (Fig. 5). The samples were analyzed using MDF to build chromatograms of the acetogenins that were present in each sample (Fig. 5B). Ultimately, the secondary metabolite profiles of each plant tissue were similar, with some shifts in the relative abundances of distinct isomers (Fig. 5B). Interestingly, MDF revealed that the ovaries contained the most extensive list of Annonaceous acetogenin analogues (Table S1 and Fig. S2†), however, they are an underexplored organ from *A. triloba* due to the short life cycle, thus low availability. This data suggested that the flowers may be an untapped resource when searching for new acetogenins. Furthermore, this technique may prove beneficial for other plant species, where the flowers are underexplored for bioactive secondary metabolites.

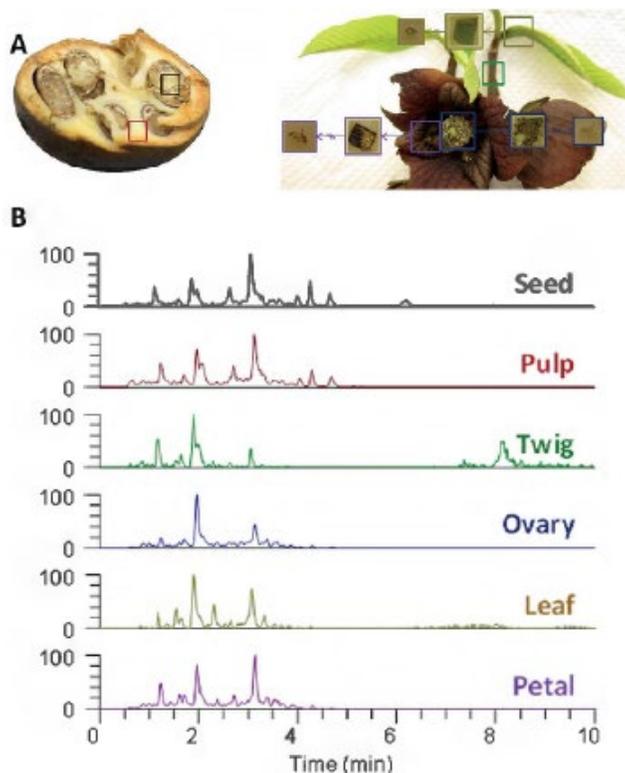


Fig. 5. (A) Locations of paw paw where the droplet-LMJ-SSP directly sampled seed (black), pulp (red), and twig (green) and the portions that were cross-sectioned: ovary (blue), leaf (yellow), and petal (purple). (B) The mass defect filtered chromatograms around annonacin; 603.4807 ± 100 Da with a mass defect of ± 25 mDa.

The seeds, pulp, and twigs were all directly sampled with the droplet-LMJ-SSP, and signals for the lithiated acetogenin adducts were readily detected via the mass spectrometer. The droplet-LMJ-SSP protocol on a suite of plant parts provided a detailed profile of secondary metabolites in situ, including the ability to differentiate between isomers. However, when the leaves and flowers were sampled directly, the metabolites were not detected. This is a common issue when performing direct ambient mass spectrometry experiments on leaves due to their waxy, hydrophobic surfaces. There are three common methods to overcome this challenge: imprint the metabolites on Teflon,⁵⁶ remove the waxy surface with chloroform,²⁷ or create cross-sections using a cryotome.⁵⁷

Initially, chloroform was used to remove the waxy, hydrophobic surface of the leaves and flower petals (Fig. 6A and B). This procedure worked for the leaves, albeit with low signal (Fig. 6C), but acetogenins were still not detected on the petals (Fig. 6D). Instead, the leaves, petals, and ovaries were subjected to cross-sectioning using a cryotome (Fig. 6E and F). After sectioning, the sliced plant materials were subjected to surface analysis, and acetogenins were readily detected on all three plant tissues (leaves, petals, and ovaries). Since this project aimed to perform direct sample surface analysis, the imprint methodology was not examined.

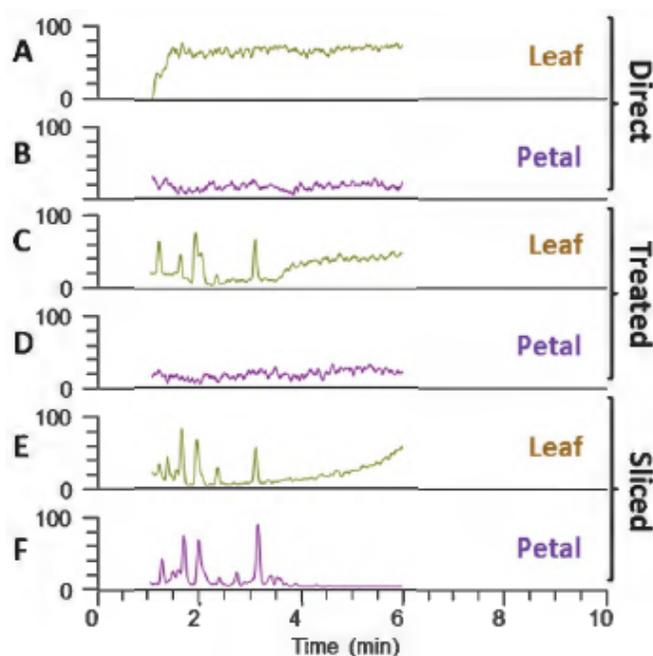


Fig. 6. The droplet-LMJ-SSP directly sampled a (A) leaf (yellow) and a (B) petal (purple). CHCl_3 was used to remove the waxy surface and the (C) leaf and (D) petal were resampled with only minimal success on the leaf. Finally, the cryotome sliced tissues of the (E) leaf and (F) petal were sampled with the droplet-LMJ-SSP. Extracted ion chromatograms of the m/z range of 550–700 over the retention times of 1.0–6.0 min were compared.

Experimental

Instrumentation methods

The data were collected on a QExactive Plus mass spectrometer (ThermoFisher, San Jose, CA) with a spray voltage of 3.7 kV, a nitrogen sheath gas set to 25 arb, and an auxiliary gas set to 5 arb. The HCD fragmentation used a normalized collision energy of 60.0 for all compounds. An Acquity ultra-performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA) was used with a flow rate set to 300 $\mu\text{L min}^{-1}$ using a BEH C₁₈ column (2.1 \times 50 mm \times 1.7 μm) equilibrated at 40 °C with a 2 mM solution of LiF in MeOH infused post-column at a rate of 5 $\mu\text{L min}^{-1}$. The mobile phase consisted of Fisher optima LC-MS grade CH₃CN–H₂O (acidified with 0.1% formic acid), starting at 70% CH₃CN and increasing linearly to 100% CH₃CN over 8 min. It was held at 100% CH₃CN for 1.5 min before returning to starting conditions for re-equilibration. Microextractions of 5 μL were performed using a CTC/LEAP HTC PAL auto-sampler (LEAP Technologies Inc., Carrboro, NC) converted into an automated droplet-LMJ-SSP system by using the dropletProbe Premium software.^{32–34} The 5 μL droplet was composed of 50 : 50 MeOH–H₂O. Compound Discoverer (ThermoFisher, San Jose, CA) was used to perform the mass defect filtering. The filter was set to ± 100 Da and ± 25 mDa around m/z 603.4807.

Preparation of leaves and flowers

A voucher specimen of the plant material was deposited in the Herbarium of the University of North Carolina at Chapel Hill (NCU602030). The leaves and flowers from a paw paw tree were removed and cut into small (0.5 \times 0.5 cm) pieces (Fig. S3†). A customized tray (Fig. S3†) was designed using SketchUp Make (Trimble Navigation Limited), sliced using Simplify3D (Simplify3D LLC), and printed out of poly(lactic acid) using an F306 3D printer (Fusion3 Design LLC). The design contained four removable cells, and a piece of each material was placed in an individual cell. Each cell was filled with Tissue-Tek optimum cutting temperature (O.C.T.) embedding medium and the tray was placed in a –80 °C freezer. Once frozen, the material was removed from the cell and cut to 15 μm cross-sections using the Leica CM1100 cryostat (Leica Biosystems Inc., Buffalo Grove, IL). The cross-sections were thaw mounted to a microscope slide.

Conclusions

The droplet-LMJ-SSP provided a method for characterizing acetogenins directly from the seeds, pulp, and twigs of *A. triloba* with no sample preparation. Furthermore, it allowed for the comparison of these well-studied plant tissues to those that are less studied (e.g. leaves) or have never been studied (e.g. the petals and ovaries of the flower). While other direct ionization technique, such as DESI, nanoDESI, and MALDI, can profile some of the plant's substituents, the lack of chromatographic separation greatly limits their abilities to discern isomers. By coupling the droplet-LMJ-SSP with LC-MS and applying it towards *A. triloba* analysis in situ, the power of chromatographic separation in conjunction with mass spectrometry fragmentation was revealed. Acetogenins were rapidly screened, characterized, and compared directly from the various organ tissues. The use of post-column lithium infusion and MDF provided increased sensitivity and comprehensive data analysis, respectively. Furthermore, the flowers from *A. triloba* were analysed for acetogenins for the first time, revealing an abundance of analogues that warrant further exploration. In particular, the ovaries were a rich source of acetogenins previously unreported using traditional natural products protocols.

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