

## Dereplicating and Spatial Mapping of Secondary Metabolites from Fungal Cultures *in Situ*

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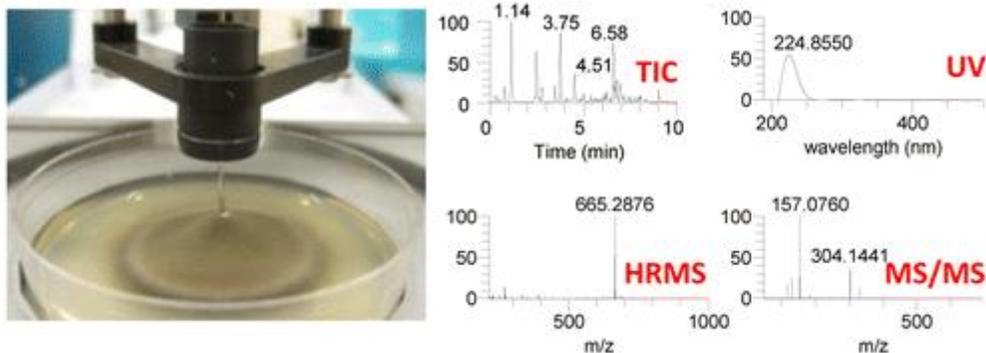
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### **Abstract:**

Ambient ionization mass spectrometry techniques have recently become prevalent in natural product research due to their ability to examine secondary metabolites *in situ*. These techniques retain invaluable spatial and temporal details that are lost through traditional extraction processes. However, most ambient ionization techniques do not collect mutually supportive data, such as chromatographic retention times and/or UV/vis spectra, and this can limit the ability to identify certain metabolites, such as differentiating isomers. To overcome this, the droplet–liquid microjunction–surface sampling probe (droplet–LMJ–SSP) was coupled with UPLC–PDA–HRMS–MS/MS, thus providing separation, retention times, MS data, and UV/vis data used in traditional dereplication protocols. By capturing these mutually supportive data, the identity of secondary metabolites can be confidently and rapidly assigned *in situ*. Using the droplet–LMJ–SSP, a protocol was constructed to analyze the secondary metabolite profile of fungal cultures without any sample preparation. The results demonstrate that fungal cultures can be dereplicated from the Petri dish, thus identifying secondary metabolites, including isomers, and confirming them against reference standards. Furthermore, heat maps, similar to mass spectrometry imaging, can be used to ascertain the location and relative concentration of secondary metabolites directly on the surface and/or surroundings of a fungal culture.



**Keywords:** dereplication | secondary metabolites

### Article:

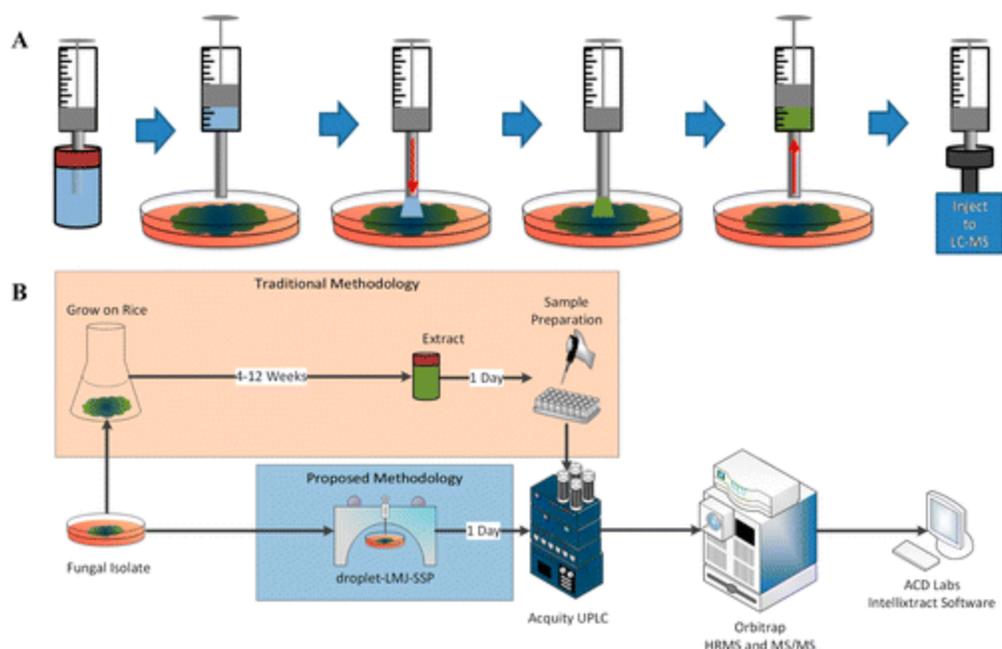
In the drug discovery realm of natural products, strategies to increase the output of new, bioactive compounds, while decreasing the isolation of previously known structures, are continually evolving. To achieve these goals, researchers strive to profile samples as early as possible in the isolation/identification procedures by utilizing LC–UV,(1) LC–MS,(2) LC–NMR,(3) or a combination of these techniques.(4) This identification of known compounds, which was coined “dereplication”,(5) allows for the early detection of known metabolites, thus saving time, effort, and cost.(6) Our methodology for the dereplication of fungal metabolites involves analyzing mutually supportive data by screening fungal culture extracts via an ultraperformance liquid chromatography–photodiode array–high-resolution tandem mass spectrometry (UPLC–PDA–HRMS–MS/MS) protocol and comparing the retention time, UV/vis data, and HRMS and MS/MS fragmentation patterns with a database.(7, 8) Presently, the method is used routinely to dereplicate fungal extracts from a growing library of over 300 secondary metabolites, particularly mycotoxins, in approximately 30 min. However, that time frame starts once the extract has been generated and does not take into consideration the 4–12 weeks that the culture was growing in solid-phase culture prior to extraction. The main goal of this study was to dereplicate fungal cultures with four stipulations: (1) eliminate the need to extract the fungal sample, (2) conduct the analysis directly from the culture dish, (3) avoid optimizing growth conditions to facilitate ambient ionization, and (4) include the acquisition of mutually supportive data.

Ambient ionization techniques for MS allow for direct culture analysis without the need for extracting the sample or extended fungal growth times.(9-13) Our team has explored desorption electrospray ionization–mass spectrometry (DESI–MS)(14) as a method for examining fungal cultures directly from Petri dishes.(13) DESI–MS has many advantages over other direct ionization techniques, such as MALDI, due to its ambient, minimally destructive nature. For instance, the ability to analyze and identify known metabolites using DESI–HRMS and MS/MS without destroying the culture was beneficial, as repeat analyses were possible. However, to optimize the DESI setup for fungal cultures, a few challenges must be overcome, particularly due to the dynamic topography of a fungal culture, which is not flat.(15) In a scenario that draws from the analysis of biological tissues,(16) a cryotome was used to afford thin, flat cross-sections of the desired culture.(17) Alternatively, an imprint of the culture could be made and then

scanned by DESI-MS.(17) Moreover, we developed methods to grow a fungal culture on an insert, so as to afford a firm, flat surface that was desired for DESI-MS analysis.(13) While these techniques worked for MS imaging experiments, they were not universally applicable to the goals of dereplication. They each required optimization of the individual fungal culture, which would be impractical for the analysis of hundreds of cultures annually. Moreover, none of them permitted the collection of mutually supportive chromatographic or UV/vis data, all being based solely on MS measurements.

Innovative MS techniques, such as molecular networking, have emerged that utilize ambient ionization sources to dereplicate cultures directly.(12) This technique involves creating a web of connectivity between compounds by plotting the HRMS and fragmentation patterns. The spectra are converted into unit vectors, and the cosine of the angle between vectors creates a similarity score.(12) This method works well as a complement to the traditional dereplication protocols, but the loss of chromatographic separation prevents the differentiation of isomers and limits the amount of mutually supportive data that can be generated concomitantly. The importance of these ancillary data should not be overlooked. For example, even in 2015, the UV/vis spectrum of a fungal metabolite can be valuable data for rapid dereplication, especially with compounds that were discovered prior to the common use of NMR and MS in structure elucidation.(18)

Recently, a droplet-liquid microjunction-surface sampling probe (droplet-LMJ-SSP) system was reported for analysis of drug-dosed animal thin tissue sections.(19-22) With this system, a droplet of 2-4  $\mu\text{L}$  is employed to perform a microextraction on the surface of a sample, which can then be injected directly into LC and any additional inline detectors, such as PDA, ELSD, and/or MS (Scheme 1).(23) By coupling the droplet-LMJ-SSP with UPLC-PDA-HRMS-MS/MS, dereplication(7) can take place by sampling a culture directly, thereby gaining chromatographic separation and obtaining retention time and UV/vis data. The retention time alone acts as a key identifier for specific metabolites, such as isomers (or isobars), which are often indistinguishable without chromatography. For the purpose of dereplication, the acquisition of mutually supportive data highlights a major advantage of the droplet-LMJ-SSP over other ambient ionization techniques (Table 1), while saving much time over the traditional protocol of performing dereplication on extracts (Scheme 1).



Scheme 1. (A) The Droplet-LMJ-SSP Can Extract Secondary Metabolites Directly off the Surface of a Culture, Including Compounds Exuded into the Agar, and Inject the Extract into the LC-MS Instrument for Analysis; (B) Comparison of the Current Dereplication Protocol(7) (Orange) and the Streamlined Methodology (Blue)

**Table 1. Comparison of Direct Ionization Sources (DESI, nanoDESI, MALDI, LAESI, and LESA) to Droplet-LMJ-SSP Functionalities for Direct Sample Analysis**

| Applications                       | DESI <sup>24</sup> | nanoDESI <sup>25</sup> | MALDI <sup>26</sup> | LAESI <sup>27</sup> | LESA <sup>28</sup> | droplet-LMJ-SSP <sup>19</sup> |
|------------------------------------|--------------------|------------------------|---------------------|---------------------|--------------------|-------------------------------|
| Direct Culture Analysis            | ✓                  | ✓                      | ✓                   | ✓                   | ✓                  | ✓                             |
| Repeat analysis                    | ✓                  | ✓                      | ✗                   | ✗                   | ✓                  | ✓                             |
| Accurate Mass                      | ✓                  | ✓                      | ✓                   | ✓                   | ✓                  | ✓                             |
| Tandem MS                          | ✓                  | ✓                      | ✓                   | ✓                   | ✓                  | ✓                             |
| Imaging/Heat Mapping               | Imaging            | Imaging                | Imaging             | Imaging             | Heat Mapping       | Heat Mapping                  |
| Sample Preparation                 | Minimal            | None                   | Matrix              | None                | None               | None                          |
| Separation of Isomers <sup>o</sup> | ✗                  | ✗                      | ✗                   | ✗                   | ✗                  | ✓                             |
| Points of Identification           |                    |                        |                     |                     |                    |                               |
| HRMS                               | ✓                  | ✓                      | ✓                   | ✓                   | ✓                  | ✓                             |
| MS/MS                              | ✓                  | ✓                      | ✓                   | ✓                   | ✓                  | ✓                             |
| Retention time                     | ✗                  | ✗                      | ✗                   | ✗                   | ✗                  | ✓                             |
| UV/VIS absorbance                  | ✗                  | ✗                      | ✗                   | ✗                   | ✗                  | ✓                             |

Table aGas-phase separation techniques (i.e., ion mobility) lack sensitivity and resolution compared to liquid-phase separation techniques (i.e., LC). Hence this table is comparing only solution-based processes.

Mass spectrometry imaging (MSI), or mapping, has become increasingly popular in the natural products community.(9) Mapping the locations of metabolites to the sample's surface has created opportunities to analyze the chemical interactions that take place between cultures.(29) Like many other ionization techniques, droplet-LMJ-SSP has the ability to map the location of compounds on a sample; however, it does so in a different format, termed heat mapping. Heat mapping shows the relative intensity of a compound at specific locations, rather than as a continuous image (Figure 1).(20) Heat mapping with the current system configuration has some limitations, compared to imaging, due to the low spatial resolution (0.7–1.0 mm).(23) However, it does have benefits, such as the ability to obtain LC separation, analyze by more than one detection type, and use various ionization sources (i.e., ESI, APCI, and APPI). Furthermore, high spatial resolution is not necessarily required for the analysis of fungal cultures, as the general trends are often just as informative as the minute differences obtained with high spatially resolved locations.

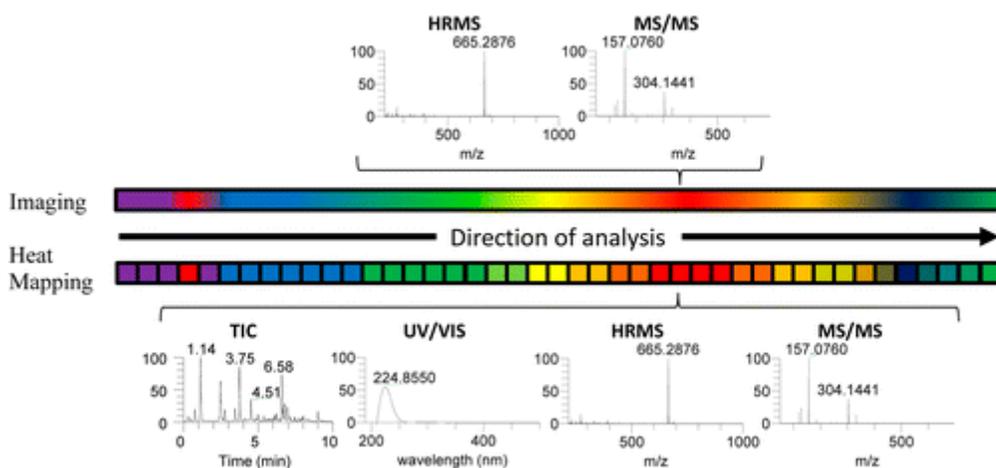


Figure 1. Conceptual comparison of MSI (top) and heat mapping (bottom) experiments as they scan a sample from left to right. Imaging experiments have a continuous flow of data, while heat maps are of specified locations. However, for each specific location in the heat map, there is chromatographic separation and UV/vis data associated with it. In this hypothetical example, the color scale indicates the relative amount of signal detected for the given analytes.(13)

## Results and Discussion

### Dereplication of Fungal Cultures

An in-house database of over 300 fungal secondary metabolites, encompassing a diverse range of structural classes such as polyketides, terpenoids, and peptides,(30) had been assembled, recording the chromatographic retention times, UV/vis data, full-scan HRMS, and MS/MS spectra in both positive and negative electrospray ionization (ESI) modes.(7) Once extracts were analyzed, these data were processed utilizing the ACD IntelliXtract software, which scans the data and reports molecular ions that match the database. The matches were investigated further by comparing the fragmentation pattern of the sample to that of the standard. However, for this study, there were two slight differences. First, the original database was built using collision-

induced dissociation (CID) with a normalized collision energy (NCE) of 30 on an LTQ Orbitrap XL, thereby generating low-resolution fragmentation data; at the time, that presented a more efficient way to process the data. In contrast, this study used a QExactive Plus and therefore used high-energy collision dissociation (HCD) with an NCE of 35, which had the benefit of having high-resolution fragmentation. Although the resulting fragments were mostly similar between HCD and CID, there were some differences, making it important to rerun the standards using HCD. Second, the QExactive Plus has the ability to perform polarity switching, thus allowing for the collection of both positive and negative ionization modes in a single run. The use of the QExactive Plus also provided increased sensitivity and the option of higher resolution (140 000 vs 100 000) as compared to the LTQ Orbitrap XL.

Initially, a representative 10% of the compounds from the fungal library(7) were spotted on Teflon-coated slides and sampled via the droplet-LMJ-SSP (Table S1, Supporting Information). Although our traditional drug discovery projects focus on metabolites soluble in organic solvents (such as CHCl<sub>3</sub>-MeOH), a droplet comprising 50:50 MeOH-H<sub>2</sub>O was used for two reasons. First, MeOH was chosen because of its compatibility with LC-MS systems and had the added benefit of mimicking the typical extraction process; CH<sub>3</sub>CN worked in an equivalent manner. Also, an equal volume of H<sub>2</sub>O was added to maintain droplet formation and integrity, as reported previously.(21) Polyketides, cyclic peptides, terpenoids, and peptides, as well as commonly dereplicated compounds, such as equisetin (20), aerofusarin (23), and alternariol analogues (Table S1), were all readily detected. For each standard, the HRMS, MS/MS, UV/vis data, and retention times were all reacquired and recorded to account for any changes to the retention times and fragmentation patterns due to the droplet-LMJ-SSP setup. Over a mass range of 225 to 1963 amu, complications of extraction and ionization were not observed with any of the compounds (Table S1, Supporting Information), suggesting that it would work for the entirety of the library. Subsequently, 12 fungi(13, 31-36) (Table S1) were selected that were known to biosynthesize those standards from traditional natural product studies, and each was dereplicated readily from cultures on Petri dishes using the droplet-LMJ-SSP (Table S1).

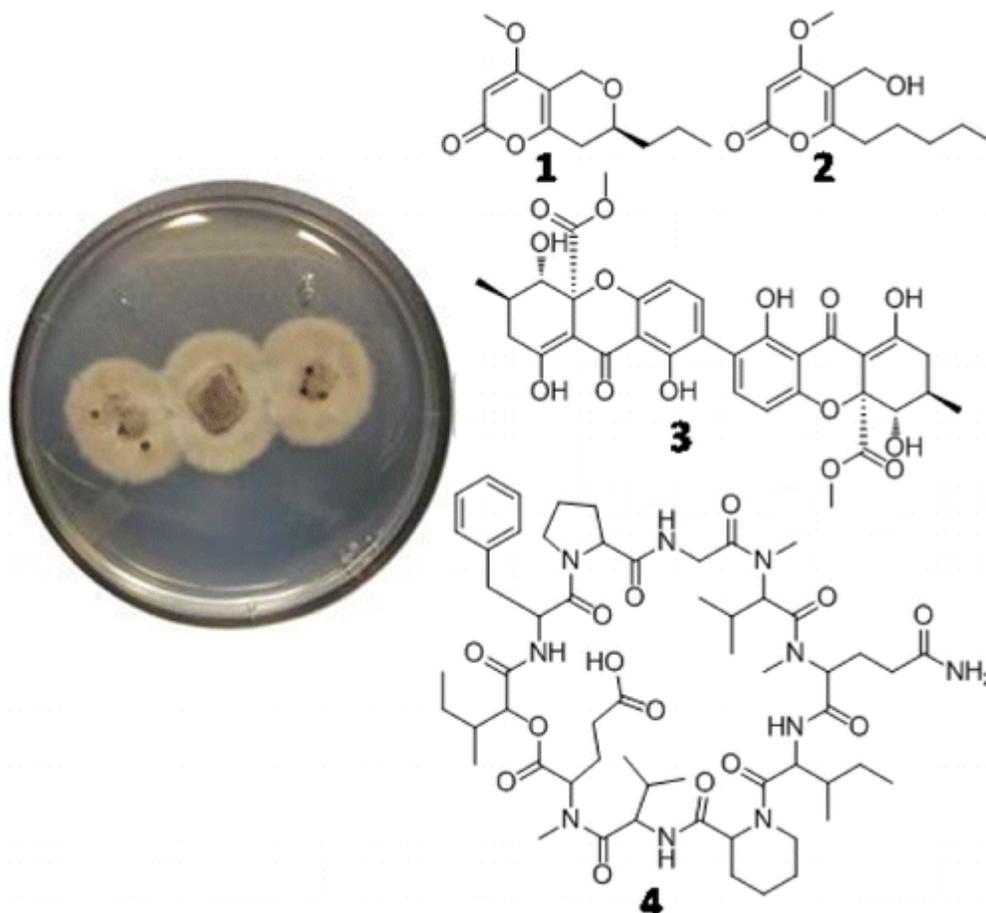


Figure 2. Fungal culture G100 and the structures of the identified metabolites using the droplet-LMJ-SSP.

Briefly, the first fungus selected for dereplication via the droplet-LMJ-SSP was a culture identified as *Clohesyomyces aquaticus* (Pleosporales, Dothideomycetes, Ascomycota) and coded G100 (Figure 2). Phomopsinone A (1) and three other metabolites (compounds 2–4) were all detected and identified by their retention time, UV/vis data, HRMS, and MS/MS data (Figure 3).

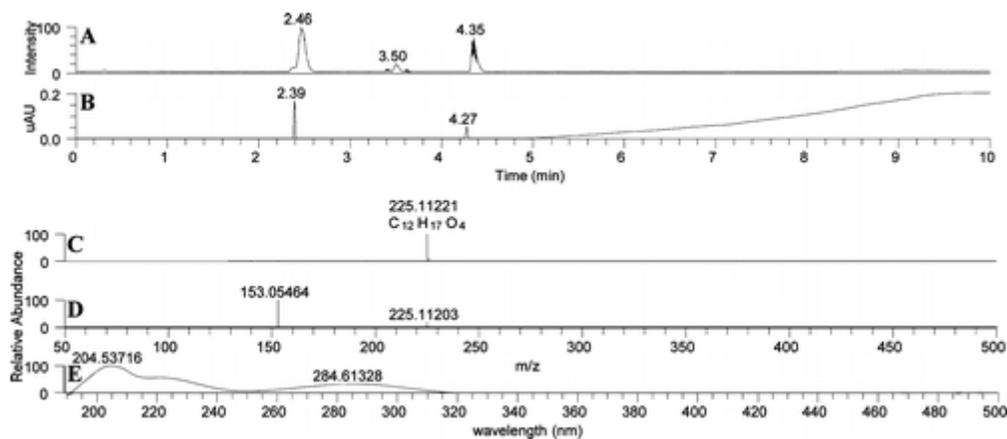


Figure 3. Droplet–LMJ–SSP coupled to UPLC–PDA–HRMS–MS/MS was used to sample fungus G100, thereby generating (A) the total ion chromatogram and (B) the UV/vis (190–500 nm) chromatogram (0.08 min delay between PDA and MS). At 4.35 min, (C) the HRMS spectrum, (D) the HCD fragmentation pattern, and (E) the UV/vis spectrum can be observed, all corresponding to 1. The mass accuracy of 1 was –0.6 ppm (225.1120 observed vs 225.1121 calculated for [C<sub>12</sub>H<sub>16</sub>O<sub>4</sub> + H]<sup>+</sup>).

An important aspect of the droplet–LMJ–SSP was its tolerance of diverse fungal topographies and its ability to analyze specific features of a fungal culture, such as guttates (i.e., liquid droplets).<sup>(17)</sup> While many fungi produce guttates, ambient techniques, such as DESI and nanoDESI, have reported the difficulty of analyzing such liquids on a culture’s surface.<sup>(13, 37)</sup> Previously, guttates on the surface of a fungus were explored using DESI–MS.<sup>(17)</sup> This required imprinting the culture onto Teflon-coated slides and analyzing with DESI–MS, rather than directly sampling the culture’s surface.<sup>(17)</sup> However, the droplet–LMJ–SSP was able to extract liquid droplets off the surface of a fungal culture without any sample preparation. To showcase this ability, the droplet–LMJ–SSP sampled both a guttate and the outer mycelium on a fungal culture of G100. The antifungal compound 1 was observed in significantly larger amounts (over two magnitudes) on the guttate than on the outer edge of the fungus (Figure S1). This interesting observation would be impossible with a standard natural products protocol that extracts the entire sample. Moreover, it allows us to now postulate and test questions about where, when, and why this fungus concentrates an antifungal compound in guttates.

### Separation of Isomers

Isomers are often encountered in natural products research.<sup>(31)</sup> One of the most powerful advantages of the droplet–LMJ–SSP over other ambient ionization techniques is the ability to differentiate between isomers using LC. Ion mobility has tried to alleviate this issue but has several limitations, such as decreased sensitivity, low ion mobility resolution, and requiring a mass spectrometer that has ion mobility capabilities.<sup>(38)</sup> Currently, ion mobility works best as a complementary technique with LC, rather than as the sole source of separation.<sup>(39)</sup> The separation that LC provides has a greater ability to resolve compounds due to the abundance of chemically diverse columns and chromatographic conditions available. Moreover, chromatography is likely more familiar to most specialists in natural products chemistry.

To display the separating ability of the droplet–LMJ–SSP coupled with LC–MS, a fungus, identified as *Halenospora* sp. and coded G87 (Figure 4), was reported previously to biosynthesize two sets of isomeric resorcylic acid lactones (compounds 5/6 and 7/8).<sup>(31)</sup> To apply the droplet–LMJ–SSP, the fungus was sampled in a Petri dish and had three peaks with an accurate match ( $\pm 5$  ppm) for  $m/z$  381.1099 (Figure 5). Standards were analyzed for compounds 5 and 6, and these displayed matching retention times (4.03 and 4.21 min, respectively), HRMS, and MS/MS fragmentation patterns (Figure S2) for two of the peaks.

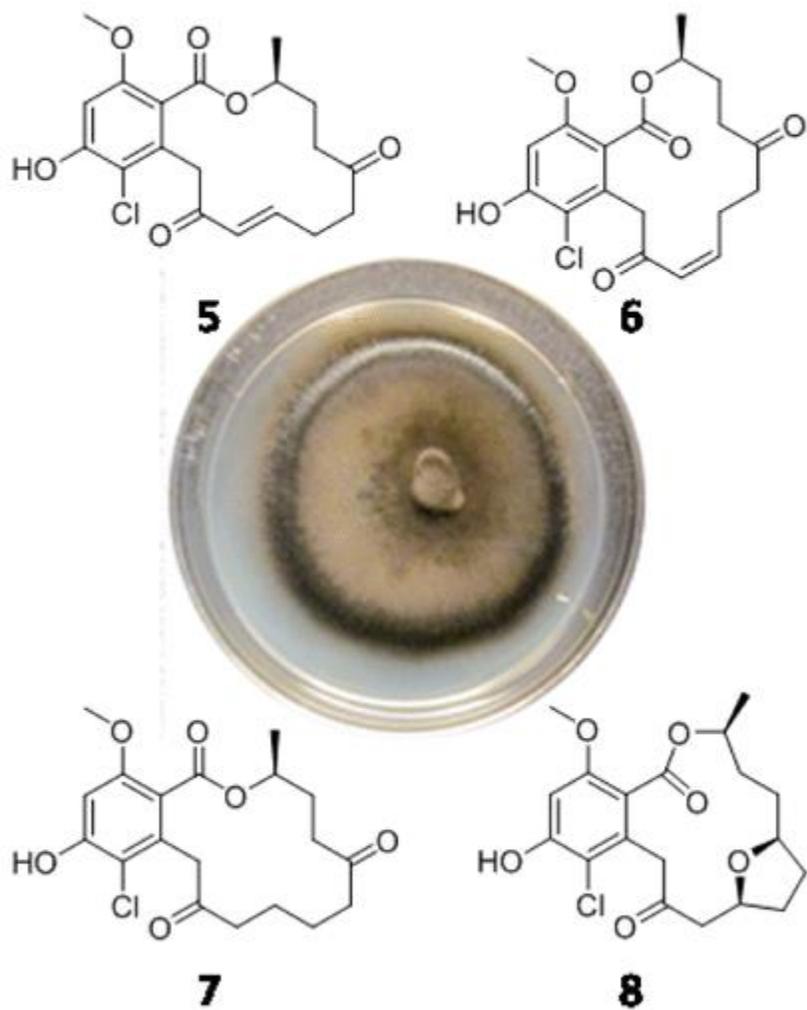


Figure 4. Fungal culture G87 and the structures of the identified metabolites using the droplet-LMJ-SSP.

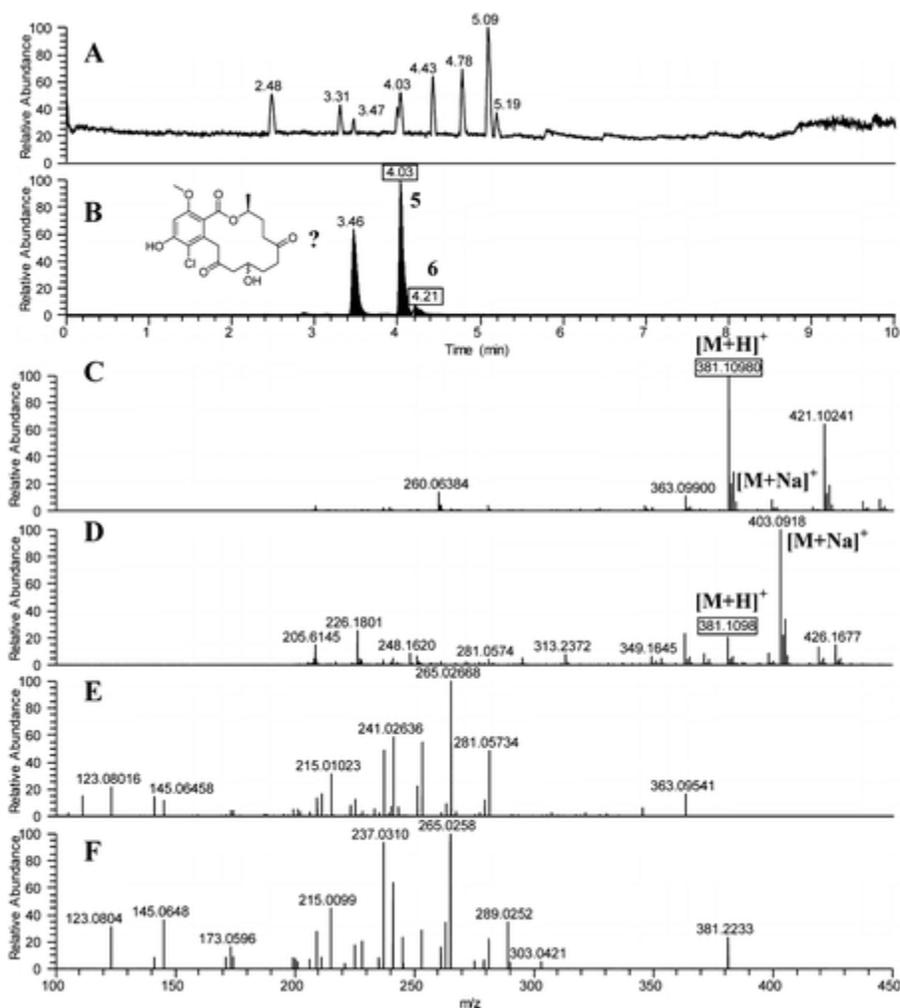


Figure 5. (A) Base peak chromatogram for fungus G87 sampled by the droplet-LMJ-SSP. (B) The XIC of  $m/z$  381.1099 ( $\pm 5$  ppm) displays matching retention times (boxed) for compounds 5, and 6 and a potential analogue. The full-scan MS at (C) 4.03 and (D) 4.21 min and the tandem MS of 381.11 at (E) 4.03 and (F) 4.21 min match the standards for compounds 5 and 6, respectively.

Interestingly, none of the previously isolated compounds from G87 matched the peak on the extracted ion chromatogram (XIC) at 3.46 min.<sup>(31)</sup> This was perplexing at first given its relative abundance, but upon further inspection, the mass was identified as a loss of water on the precursor ion at 399.1204 (Figure S3). This observation further highlights two important benefits to sampling a fungal culture directly with the droplet-LMJ-SSP. First, the chromatographic separation allows for the assignment of multiple adducts, such as assigning  $[M + H]^+$ ,  $[M - H_2O + H]^+$ , and  $[M + Na]^+$  to a compound. With ambient ionization techniques that directly infuse into the mass spectrometer, it is difficult to differentiate whether observed masses (i.e., 399.1204 and 381.1098) are analogues that differ by 18 amu or if one of them is a loss of water from the other (i.e.,  $[M + H]^+$  and  $[M - H_2O + H]^+$ ). Without chromatography, the two isomers (5 and 6) may have been presumed to be  $[M - H_2O + H]^+$  to the mass at 399.1204, rather than the presence of three unique metabolites. By gaining chromatographic separation, adducts can be differentiated from analogues, eliminating this concern. Second, because the proposed metabolite

that eluted at 3.46 min (Figure S3) was not encountered in the original study, which identified 14 new resorcylic acid lactones,(31) its detection with the droplet–LMJ–SSP indicated a suite of interesting possibilities. For example, the fungus might not have biosynthesized this compound when grown on rice in solid-phase culture, the fungus might have biosynthesized it only early in the growth of the fungus, or the compound decomposed during the initial extraction/isolation processes. Performing a microextraction of the culture directly from the Petri dish, such as afforded by the droplet–LMJ–SSP, could be used to probe these and related questions.

### Identification of Fungal Culture

An interesting circumstance arose while testing the ability of the droplet–LMJ–SSP as a dereplication tool. Four secondary metabolites (9–12) from a fungal strain, coded MSX19583 (Figure 6), had been isolated, characterized, and added to the dereplication database.(32) However, when a regrowth of this culture was requested in order to identify its genus and species via ITS sequencing,(40) it was discovered that there was a contaminant in fungal strain MSX19583 (Figure S4). To identify which fungus was the contaminant and which one biosynthesized the isolated metabolites, both fungi were isolated and subjected to analysis via the droplet–LMJ–SSP. The XIC of masses for the four previously isolated metabolites were compared for each fungus against the pure standards. Matches of retention time, accurate  $m/z$  match ( $\pm 5$  ppm), and tandem MS for compounds 9–11 were present in the green-colored fungus (Figures 7, S5, and S6), while the matches for these compounds were not observed in the purple-colored fungus. Initially, compound 12 appeared to be detected in the green-colored fungus. However, the retention time and MS/MS data demonstrated that this was a spurious observation and not the same compound (Figure 8), further exemplifying the benefits of mutually supportive data afforded by the droplet–LMJ–SSP. From this, culture MSX19583 and the contaminant were later identified as *Aspergillus sydowii* (green) and a *Chaetomium* sp. (purple), respectively.(32)

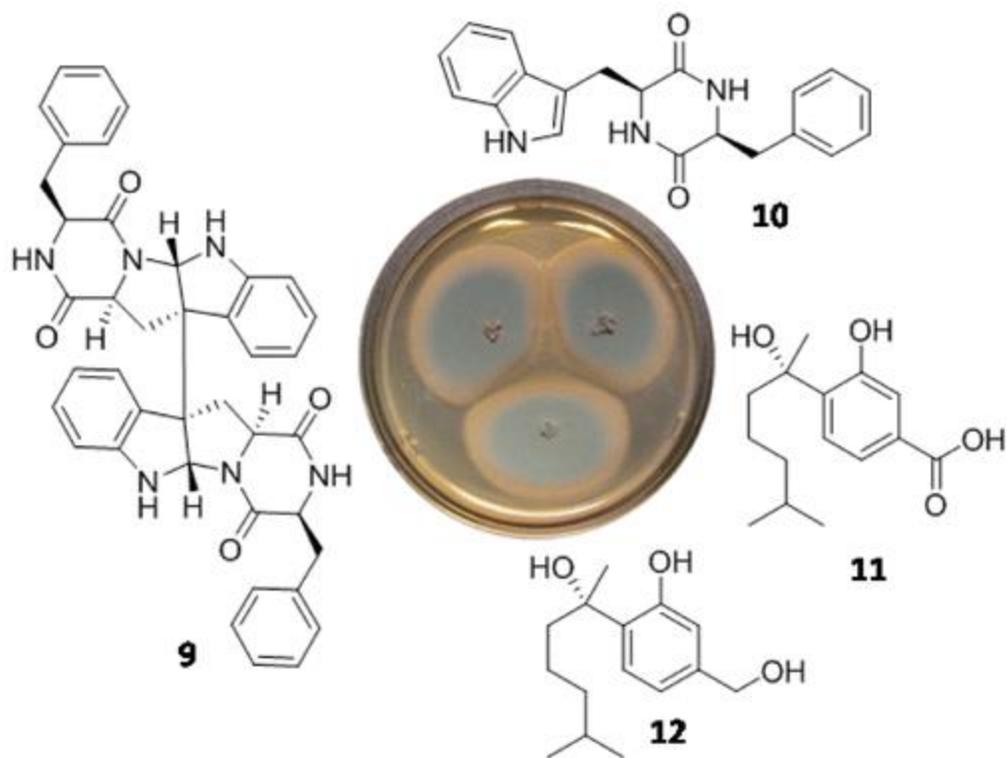


Figure 6. Fungal culture MSX15983 and the structures of the isolated metabolites.

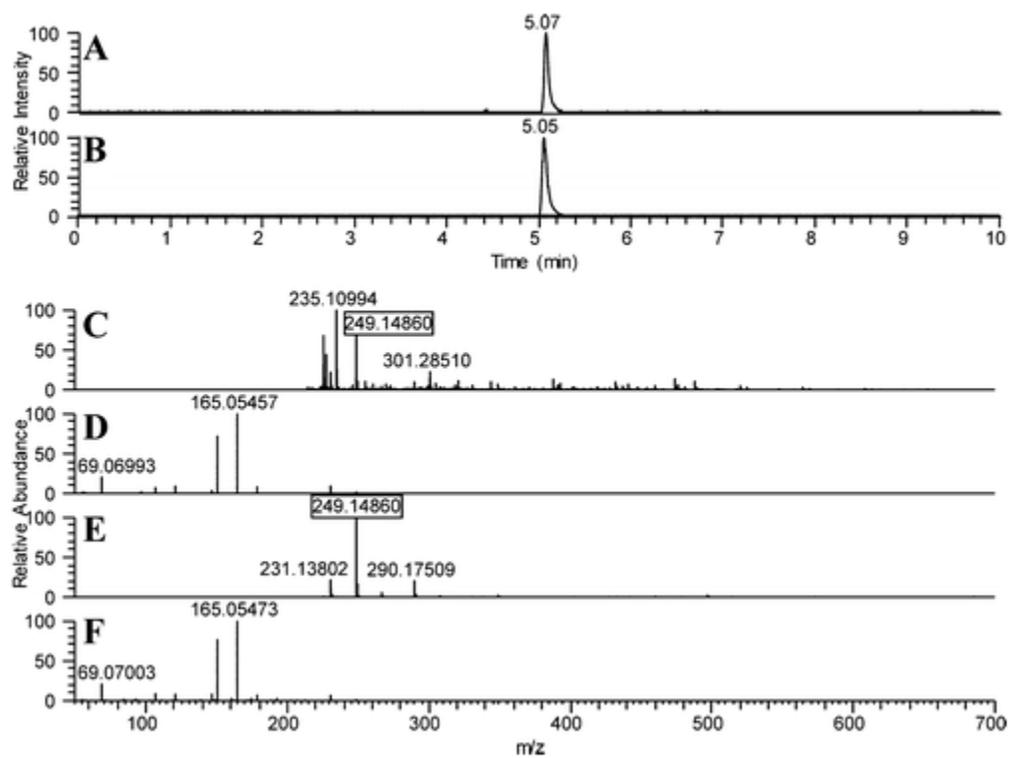


Figure 7. Compound 11 was previously isolated from fungal culture coded MSX19583. Matches in retention time can be observed by comparing the XIC of  $249.1485 \pm 5$  ppm from (A) the fungal culture and (B) the standard. Furthermore, the HRMS data for (C) the fungus and (E) the standard both matched, and the MS/MS spectra for (D) the fungus and (F) the standard have matching fragmentation patterns.

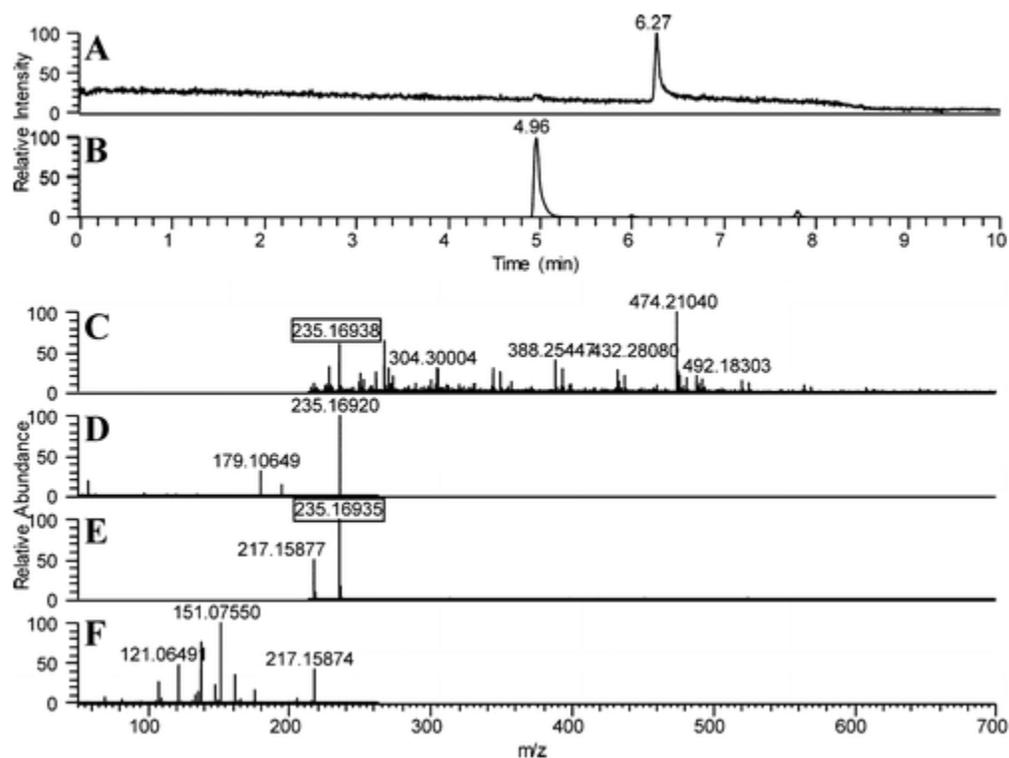


Figure 8. Compound 12 was previously isolated from fungal culture coded MSX19583. A difference in retention time can be observed by comparing the XIC of  $235.1693 \pm 5$  ppm from (A) the green-colored fungal culture and (B) the standard. The MS spectra display the difficulties of solely using MS for this metabolite identification. The HRMS spectrum for (C) the fungus and (E) the standard both matched  $[C_{15}H_{23}O_2 - H_2O + H]^+$  within a 5 ppm mass tolerance, but the MS/MS spectra showed differences between (D) the fungus and (F) the standard in fragmentation patterns.

### Mapping of Secondary Metabolites

Similar to MSI, the droplet-LMJ-SSP also has the ability to map the relative intensities of selected molecular ion peaks. The fungus coded MSX19583 was used to map the location of two of the key metabolites, compounds 9 and 10. As mentioned earlier, this culture was originally contaminated. Initially, the impure culture was analyzed, mapping a straight line from the contaminant (purple) to the desired fungus (green) and back to the contaminant (Figure 9). It was observed that compound 9 was detected primarily on the body of the fungus, while compound 10 was predominantly exuded into the surrounding media. This experiment was repeated on another culture of MSX19583, once the contaminant (purple) was removed. The results were the same, showing that compound 9 remained on the body of the fungus while compound 10 was exuded

into the surrounding media (Figure S7, Supporting Information). This was an important observation, as we initially pondered if the purple fungus had either been responsible for the biosynthesis of 10 or stimulated its biosynthesis by the green-colored culture. These sorts of measurements are impossible with extracted cultures, as all spatial information would be lost in the context of the entire extract. Moreover, sampling the agar region could be challenging in other MSI experiments, as we observed the formation of divots from the gas and spray pressure when attempting a similar experiment with a DESI source.(13)

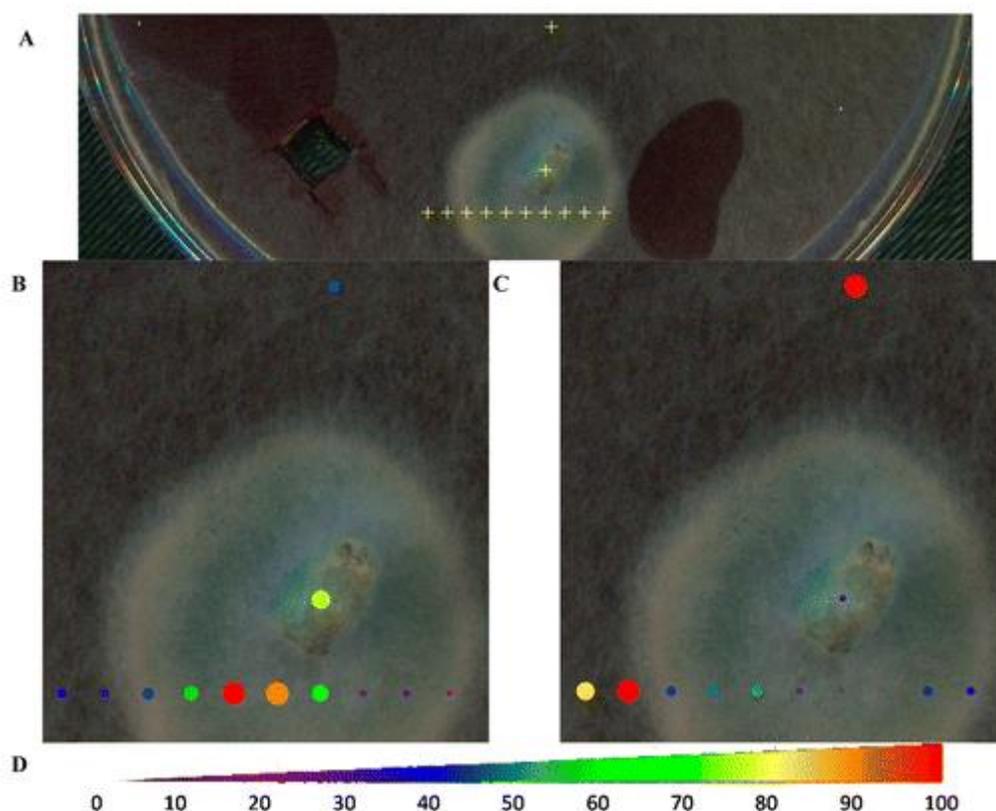


Figure 9. (A) Culture MSX19583 (greenish-gray) with the contaminant (purple); crosshairs illustrate location of sampling points. (B) Heat map of compound 9 as sampled from the contaminant to the culture. (C) Heat map of compound 10 as sampled from the contaminant to the culture. (D) The color scale and diameter of the spot indicate the relative amount of signal detected for the given analytes.

## Challenges and Conclusions

There are many questions and challenges that one could envision for the utilization of the droplet-LMJ-SSP for fungal culture analysis. First, there were concerns about whether the fungal culture would absorb the droplet rather than forming a liquid microjunction between the fungus and the syringe. In working with 12 cultures, the amount of droplet loss was considered negligible for most fungi. However, significant droplet loss was an issue for two fungi, coded G87 and MSX59553 (Figure 10). Interestingly, visual inspection of the fungus was not an adequate predictor of droplet loss. For example, G100 and G87 (Figures 2 and 4) were similar

looking and covered in mycelia (i.e., a hair-like surface), yet G100 permitted droplet–liquid microjunction formation readily, while G87 would absorb the droplet. A possible explanation of this could be due to the presence of hydrophobins that are often contained in the conidia/spores. Strains that produce conidia/spores on the surface of aerial mycelium tend to be hydrophobic (i.e., G100), while aerial mycelium that lack conidia/spores are more hydrophilic (i.e., G87).(41) Additionally, MSX59553 and MSX57715 (Table S1) resembled each other with flat, spore-covered surfaces, yet MSX59553 would absorb most of the droplet and MSX57715 would not. For the challenge concerning MSX59553, the droplet–LMJ–SSP still recovered some of the solvent from the sampled area. In this case, it was enough to generate data for dereplication, but would result in poor spatial resolution (3–5 mm) for mapping. Fortunately, these challenges for G87 and MSX59553 could be alleviated by resampling the same position several hours later. After the suboptimal sampling area dried, a hardened surface was created that was amenable to liquid microjunction formation. By resampling this area, the droplet was recoverable and multiple extractions could take place with the single droplet (i.e., sampling three or more times before injecting into the UPLC–PDA–HRMS–MS/MS).



Figure 10. Comparison of the topography of fungal cultures that resembled each other superficially, but either had challenges (i.e., G87 and MSX59553) or were amenable (G100 and MSX57715) with the droplet–LMJ–SSP.

Besides absorbency issues, occasionally a droplet was simply unrecovered. This typically happened in one of the following instances: (1) when sampling cultures with steep topography or (2) if repeated sampling eventually resulted in considerable droplet loss. Of the two scenarios, and given our previous studies with DESI–MS,(13) the steep topography was surprisingly not a prevalent issue, but could occur in a few instances, particularly with fungi covered in mycelia. It was also not a consistent issue, as immediately resampling the same steep location would often result in a successful microextraction. For repeated sampling with the droplet–LMJ–SSP, droplet loss could be minimized by utilizing three or less microextraction cycles, with the exception of the fungal cultures with absorbent surfaces, as noted above.

When working with solid-phase cultures for isolation studies, our typical protocol involves an overnight extraction, largely out of convenience.(17, 31, 36) Thus, another concern that arose was the amount of material a microextraction could absorb via a 4  $\mu$ L droplet during the two seconds of contact with the culture. This was addressed by repeating the microextraction three times with the same droplet before injecting the sample into the UPLC–PDA–HRMS–MS/MS system. This gave the droplet a higher concentration of fungal metabolites, while minimizing the risk of losing the droplet to the fungal surface. As previously reported,(21) several short microextractions were more effective for this application than a single long extraction. In fact,

when designing the experiments, a QExactive Plus (with enhanced resolution) was used with this concern in mind. However, after experimenting, a less sensitive instrument should also suffice. With the various types of structural classes tested (Table S1), limitations were not encountered with this method for secondary metabolite detection for both pure standards and direct fungal culture analysis.

A question that arose during peer review was how this process compared to rapid plug extractions, which were first described in the late 1990s.(42, 43) In this, a 6 mm plug is excised by hand from the fungal culture (including agar) in the Petri dish. The entire plug is extracted, and the effluent can then be analyzed by HPLC. While that method has corollaries with some of the droplet-LMJ-SSP benefits (such as sampling across a culture, the space between cocultures, etc.), it requires more human power for the sample manipulation/processing. The possibilities for in situ analysis are not quite the same, particularly in a temporal manner, as those afforded by the droplet-LMJ-SSP, which has the added benefit of automation and integrated heat mapping capabilities. Nevertheless, rapid plug extractions likely probe the chemistry of fungal cultures in a similar manner.

An advantage of the droplet-LMJ-SSP is that the fungal culture survives the analysis, except in the immediate area of the microextraction (typically ~1 mm). Hence, a promising application for the droplet-LMJ-SSP is examining the timing of secondary metabolite biosynthesis, particularly for the optimization and eventual scale up of drug leads. One concern, particularly from the mycologist on our team, was contamination from fungal spores in the air that could arise when the Petri dish was exposed repeatedly. In sampling dozens of fungal cultures, this occurred in only one instance. Approximately a week after the culture G87 was sampled, a contaminant appeared in the Petri dish. For the purposes of dereplication and heat mapping experiments, this was not an issue, as the cultures were analyzed immediately after opening. However, for temporal studies, this must be considered. Indeed, our current protocol involves first opening a plate in a laminar flow hood, such that fungi that sporulate prolifically do not contaminate the MS facility and instruments; for nonsporulating fungi (i.e., hyphal/mycelia forms) this is not a major concern. Moreover, the plates are exposed only immediately prior to analysis. Since the droplet-LMJ-SSP is a separate instrument from the LC-MS, a possible solution if contamination (either of the sample or of the facility) was a serious concern would be to place the droplet-LMJ-SSP in a laminar flow hood, coupling the instruments together with longer tubing.

Finally, although heat mapping experiments obtain a large amount of information, they are not a replacement for MS imaging experiments. For instance, heat mapping experiments take a longer period of time due to the addition of chromatography. More importantly, the resolution (0.7–1.0 mm) of the current droplet-LMJ-SSP is not as high as it is for other imaging techniques (20–200  $\mu\text{m}$ ) that must be considered when looking for precise changes in distribution of secondary metabolites or biomarkers in other matrices. However, the droplet-LMJ-SSP has the ability to map the spatial distribution of isomers, something not currently possible with other imaging platforms. Also, because heat mapping is not a continuous flow, it has the ability to readjust to the various topographies that are encountered, which is a particular challenge with routine sampling of fungal cultures.(13) Additionally, heat mapping provides semiquantitative results, as its true potential as an analytical technique has been neither evaluated nor optimized due to challenges, such as consistency in droplet recovery. Thus, MS imaging and heat mapping

experiments should be viewed as complementary techniques, rather than a substitution for one another.

In conclusion, coupling a droplet–LMJ–SSP with a UPLC–PDA–HRMS–MS/MS system advances ambient ionization techniques with the inclusion of chromatography. Secondary metabolites were characterized with more confidence due to the mutually supportive data that were obtained. Furthermore, cultures were dereplicated directly, with no time spent on sample preparation, seamlessly integrating the current database of retention times, PDA, HRMS, and MS/MS at the level of the extract. The robustness and simplicity of using the droplet–LMJ–SSP make it a powerful and effective tool for natural products research. Besides our immediate needs in natural products drug discovery, we can envisage applications to probe questions of biosynthesis and chemical ecology.

## Experimental Section

### General Experimental Procedures

The droplet–LMJ–SSP(19-23) capabilities were acquired via collaboration with the Organic and Biological Mass Spectrometry Group at Oak Ridge National Laboratory, who assisted in the conversion of a CTC/LEAP HTC PAL autosampler (LEAP Technologies Inc.) into an automated droplet–LMJ–SSP system by using in-house-developed software dropletProbe Premium. Extractions were performed using Fisher Optima LC/MS grade solvents consisting of 50:50 MeOH–H<sub>2</sub>O. Variations of tested solutions included 30:70 CH<sub>3</sub>CN–H<sub>2</sub>O, 50:50 CH<sub>3</sub>CN–H<sub>2</sub>O, 30:70 MeOH–H<sub>2</sub>O, and 50:50 MeOH–H<sub>2</sub>O. There was no discernible difference in metabolite extraction between the CH<sub>3</sub>CN and MeOH mixtures; therefore all experiments proceeded with 50:50 MeOH–H<sub>2</sub>O. Higher organic ratios often resulted in unsuccessful liquid microjunction formation, as previously reported.<sup>(21)</sup> An initial 5  $\mu$ L of solvent was drawn into the syringe. Droplets of 4  $\mu$ L were dispensed onto the surface of the sample at a rate of 2  $\mu$ L/s, held on the surface for 2 s, and withdrawn back into the syringe at the same rate. This extraction process was repeated a total of three times for a single spot prior to injection into the UPLC–MS system.

The droplet–LMJ–SSP was coupled with a Waters Acquity ultraperformance liquid chromatography (UPLC) system (Waters Corp.) to an MS. The initial testing of the applicability of the droplet–LMJ–SSP on fungal cultures was coupled to an AB Sciex TripleTOF 5600+ at Oak Ridge National Laboratory, but the majority of the analyses was performed on a Thermo QExactive Plus MS (ThermoFisher) at UNCG. The QExactive Plus was adjusted to collect data from 150 to 2000 m/z at a resolution of 70 000. The HCD fragmentation used a normalized collision energy of 35 for all compounds. The voltage for both positive and negative ionization modes was set to 3.7 kV, with a nitrogen sheath gas set to 25 arb, and an auxiliary gas set to 5 arb. The S-Lens RF level was set to 50.0 with a capillary temperature at 350 °C. The flow rate of the UPLC was set to 0.3 mL/min using a BEH C18 (2.1  $\times$  50 mm  $\times$  1.7  $\mu$ m) equilibrated at 40 °C. The mobile phase consisted of Fisher Optima LC–MS grade CH<sub>3</sub>CN–H<sub>2</sub>O (acidified with 0.1% formic acid), starting at 15% CH<sub>3</sub>CN and increasing linearly to 100% CH<sub>3</sub>CN over 8 min. It was held at 100% CH<sub>3</sub>CN for 1.5 min before returning to starting conditions for re-equilibration. The PDA was set to acquire from 200 to 500 nm with 4 nm resolution.

## Fungal Strain Identification

For the identification of strains used in this study, the internal transcribed spacers (ITS) region and a portion of the 28S rRNA gene of the nuclear RNA operon were sequenced. Amplicons and sequences for the ITS1–5.8S–ITS2 region were generated using primers ITS1F/ITS5 and ITS4, and 28S rRNA gene sequence data were obtained for the first two divergent domains (D1/D2) using primers LROR and LR3. Methods used for strain identification and phylogenetic analysis have been detailed previously.(17, 31, 33, 35, 36, 44)

The ITS region was used for barcoding of fungal species by searching against nBLAST with the RefSeq database as well as the regular NCBI database; uncultured/environmental sequences were excluded from the BLAST search. The ITS region was used for species identification, while a portion of the 28S was used for phylogenetic analysis.

## Software

Images of each culture were acquired using an Epson Perfection v370 scanner controlled by dropletProbe Premium. The location of the sampling area and the scanned images were calibrated to correlate the X and Y coordinates, and dropletProbe Premium automatically marked the scanned images with a crosshair at the spots where extraction sampling occurred. The creation of heat maps was also performed using the dropletProbe Premium software by correlating the intensities of specified molecular ions ( $\pm 5$  ppm) and retention times to the selected spots on the scanned images.(22, 23) ACD MS Manager with add-in software IntelliXtract (Advanced Chemistry Development Inc.) was used for the primary analysis of the LC–MS data for dereplication. This software was used as detailed previously.(7)

## Custom Sample Trays

A customized tray 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 held a small or large size Petri dish and a solvent vial and had a needle block position (Figure S8, Supporting Information).

## Supporting Information

Images of key fungi described in this paper along with relevant chromatograms and mass spectra. A representative database of 30 fungal secondary metabolites including molecular formula, retention time, UV/vis absorption maxima, HRMS, and MS/MS of either positive or negative ionization modes. A schematic design and a photograph of plates prototyped on a 3D printer. A video of the droplet–LMJ–SSP sampling a fungal culture and directly injecting the extract into the LC–MS system. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00268.

The authors declare no competing financial interest.

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