analytical chemistry

Article

Cite This: *Anal. Chem.* 2019, 91, 8326−8333

pubs.acs.org/ac

Direct Depolymerization Coupled to Liquid Extraction Surface Analysis-High-Resolution Mass Spectrometry for the Characterization of the Surface of Plant Tissues

Chiara Giorio,*[,†,‡] Edwige Moyroud,[§] Beverley J. Glover,[∥] and Markus Kalberer[†,⊥]

[†]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

[‡]Department of Chemical Sciences, University of Padua, via Marzolo 1, 35131 Padova, Italy

[§]The Sainsbury Laboratory, Cambridge University, Bateman Street, Cambridge CB2 1LR, United Kingdom

[∥]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom

[⊥]Department of Environmental Sciences, University of Basel, Klingelbergstrasse 27, 4056 Basel, Switzerland

Supporting Information

ABSTRACT: The cuticle, the outermost layer covering the epidermis of most aerial organs of land plants, can have a heterogeneous composition even on the surface of the same organ. The main cuticle component is the polymer cutin which, depending on its chemical composition and structure, can have different biophysical properties. In this study, we introduce a new on-surface depolymerization method coupled to liquid extraction surface analysis (LESA) high-resolution mass spectrometry (HRMS) for a fast and spatially resolved chemical characterization of the cuticle of plant tissues. The method is composed of an on-surface saponification, followed by extraction with LESA using a chloroform−acetonitrile−water (49:49:2) mixture and direct HRMS detection. The method is also compared with LESA-HRMS without prior depolymerization for the analysis of the surface of the petals of *Hibiscus richardsonii* flowers, which have a ridged cuticle in the proximal region and a smooth cuticle in the distal region. We found that on-surface saponification is effective enough to depolymerize the cutin into its monomeric constituents thus allowing detection of compounds that were not otherwise accessible without a depolymerization step. The effect of the depolymerization procedure was more pronounced for the ridged/proximal cuticle, which is thicker and richer in epicuticular waxes compared with the cuticle in the smooth/distal region of the petal.

The epidermal layer of most aerial organs of land plants is covered with a cuticle that acts as a permeable barrier against water vapor loss and provides protection against external stressors.[1,2] The main component of the cuticle is cutin, a lipid polymer which can have different biophysical properties depending on its precise chemical composition and structure.[3] As an example, arrays of regularly spaced nanoscale ridges, or striations, were found in the cuticle on the flat epidermal cells of *Hibiscus trionum* (also known as Venice mallow or flower-of-an-hour) and many species of tulips. These cuticular striations act as a diffraction grating and create an iridescent effect where the color of the petal surface varies with the observation angle.[4−7] Such optical properties have been shown to be salient to pollinators;[6−8] however, the specific mechanisms by which plants can create striations and diffraction gratings on their surface are not well understood. As such mechanisms could be chemistry-driven, an in-depth chemical characterization of both the striated and nonstriated portions of the surface of the same petal would be key to identify the compounds underpinning the chemical process. Previous studies showed a heterogeneous composition of the cuticle even on the same organ.[2,9−11] Thus, there is a need to perform a spatially resolved characterization of the cuticle chemistry on the surface of the same organ.

Direct surface analysis techniques like desorption electrospray ionization mass spectrometry (DESI-MS) and liquid extraction surface analysis mass spectrometry (LESA-MS) have proved useful to gain insights into the composition of plant surfaces.[12,13] DESI-MS can provide a higher spatial resolution compared with LESA-MS (100−200 μm and ∼1 mm for DESI-MS and LESA-MS, respectively), but LESA-MS allows the control of extraction time.[12,13] Moreover, while a standard application of DESI-MS or LESA-MS is suitable for a fast, spatially resolved analysis, it is mainly sensitive to metabolites or free cutin monomers present on the surface, but it is not able to provide information on the composition of the cutin

Received: March 1, 2019
Accepted: May 24, 2019
Published: May 24, 2019

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

polymer or to detect metabolites deeply embedded in the cutin matrix.

In order to characterize the cutin polymer with mass spectrometry, it is necessary to depolymerize it to break down the macromolecules into their monomeric constituents. Typically, this is done by extracting and depolymerizing bulk samples of cutin, thus losing any spatial resolution on the same tissue and risking contaminations from compounds coming from the bulk of the sample rather than the surface only.[14−20] Another option is to mechanically strip off[21] the cuticle before extraction and depolymerization. This procedure, however, is not always possible. For example, on tissues such as petals, that are fragile, it does not completely circumvent the problem of contamination from tissues under the cuticle surface. Therefore, there is a need for a fast and spatially resolved depolymerization approach that selectively targets only the surface.

In this study, we introduce a new method for the characterization of the surface of plant tissues by a direct on-surface depolymerization of the cuticle followed by LESA-MS analysis. Depolymerization was done by adapting a method proposed by Mendez-Millan et al.[22] for bulk samples which was modified here into a fast and direct approach that provides spatially resolved characterization on the surface of the same organ. This method was successfully applied to the characterization of the cuticle of the petals of *Hibiscus richardsonii*,[23,24] a flower characterized by a ridged/proximal and a smooth/distal portion (Figure 1). Cuticular ridges are characterized by a submicrometer distance between a crest and its following trough (Figure 1), thus neither DESI-MS nor LESA-MS provide sufficient spatial resolution to characterize the intraridge chemical composition. However, the ridged/proximal and smooth/distal portions both extend for centimeters on a petal surface so both DESI-MS and LESA-MS would allow one to analyze separately the chemical composition of each of the two portions of the petals. LESA-MS additionally allows the control of extraction time and potentially allows incorporation of a depolymerization step into an automatic routine. Chemical composition of the different portions of the petals are here compared and discussed to gain insights concerning the compounds that may play a role in the formation of cuticular ridges on the surface of the petals.

MATERIALS AND METHODS

Plant Growth Conditions. Seeds of *Hibiscus richardsonii* (Voucher AK251841, Mayor Island (Tuhua), New Zealand) were obtained from Dr. Brian G. Murray (University of Auckland). Plants were grown to flowering in Levington M3 compost in a controlled greenhouse environment at 26 °C with a 16 h/8 h light/dark regime.

Sample Preparation and Analysis. *Sample Preparation.* Sample preparation was done according to the procedure already described in a previous study.[13] Briefly, petals of *Hibiscus richardsonii* were detached from the flowers using tweezers, cleaned with a dry white nylon brush and a gentle stream of N_2, and placed on a movable liquid extraction surface analysis (LESA, Triversa NanoMate Advion, Ithaca, NY) sample stage covered with clean aluminum foil.[13] On some petals, LESA was done using a non-polar (chloroform−acetonitrile−water (49:49:2)) solvent mixture or a polar (acetonitrile−water (90:10)) solvent mixture without prior depolymerization, respectively.[13] Formic acid (0.1%) was added to the water used for preparation of the extraction mixtures in order to increase spray stability and ionization efficiency.[13] Other petals were subjected to depolymerization before analysis.

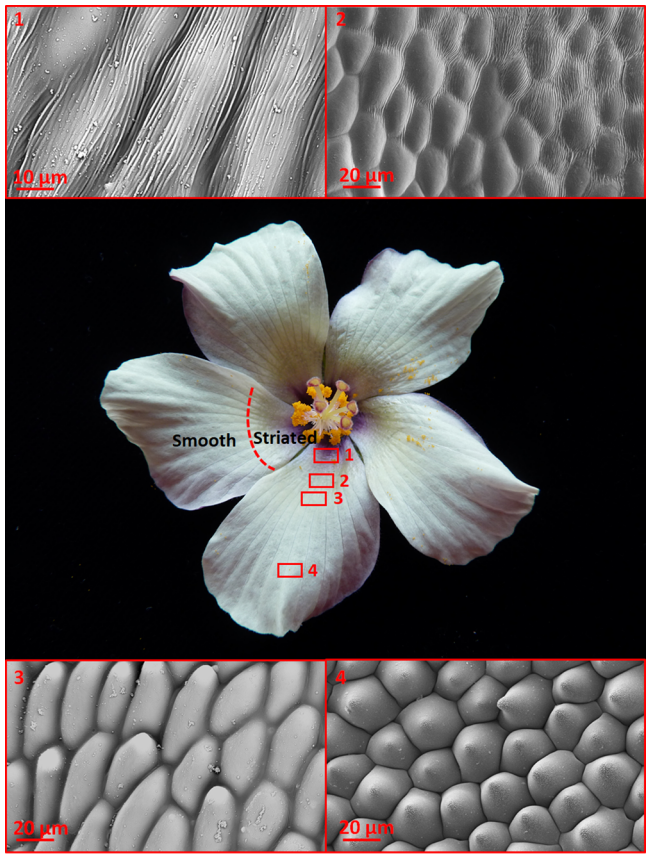

Figure 1. Picture of a *Hibiscus richardsonii* flower and scanning electron microscopy (SEM) images of (1) the striated proximal region, (2) the junction between the smooth and striated regions, (3) the smooth region next to the junction, and (4) the smooth distal region.

Depolymerization. Depolymerization of the cutin was done via saponification directly on the surface of the petals through a simplified procedure adapted from the method used by Mendez-Millan et al.[22] A droplet of about 20 μL volume of reagent mixture (6% KOH in 10:90 water−methanol) was placed on the smooth/distal surface, and another droplet was placed on the ridged/proximal surface of the petals using a Pasteur pipet. The petals were left at room temperature for 30 min for depolymerizing the cutin and drying of the solvent on the petal surface before analysis. Immediately after depolymerization, liquid extraction surface analysis was done as described in the section LESA-HRMS Analysis using the non-polar chloroform−acetonitrile−water (49:49:2) mixture[13] to minimize solubilization of KOH, which could potentially cause corrosion of MS internal components. Nevertheless, the non-polar mixture is also the most suitable mixture for solubilization of cutin and wax monomers.

LESA-HRMS Analysis. LESA-HRMS analysis was done on both depolymerized and nondepolymerized samples according to the procedure already described in a previous study.[13] Briefly, 3 μL of extraction mixture was deposited at a height of 1.4 mm from the sample plate, and the liquid junction was maintained for 30 and 45 s for the non-polar and polar

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

extraction mixtures, respectively; the droplets containing the dissolved analytes were then aspirated at a height of 1.2 mm from the sample plate and infused directly in a chip-based nanoelectrospray ionization (nanoESI) source (Triversa NanoMate Advion, Ithaca, NY) operating in negative ionization mode. The NanoMate temperature was set at 16 °C to reduce solvent evaporation, especially when the non-polar mixture was used, and to allow for longer extraction times to be used compared with previous studies.[25,26] A direct contact of the tip with the sample surface, rather than forming a liquid microjunction, could also allow for longer extraction times[27,28] but was not explored in our study. Blanks were analyzed by repeating the same procedure (depolymerization and extraction or extraction only) on the clean aluminum foil. Mass spectrometry analysis of the LESA extracts was done with a LTQ Velos Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) with a resolution of 100 000 at m/z 400 and a typical mass accuracy within ±2 ppm. Samples were sprayed at a gas (N_2) pressure of 0.80 psi, ionization voltage of −1.4 kV (negative ionization mode), and with a transfer capillary temperature of 210 °C. Data were acquired in full scan in the m/z ranges 80−600 and 150−1000 and auto MS/MS analysis on the five most intense peaks with a collision-induced dissociation (CID) energy of 35 (normalized collision energy). Concerning data processing, molecular formulas were assigned using Xcalibur 2.1 (Thermo Scientific, Bremen, Germany) within a ±5 ppm error and under the following restrictions: number of ^{12}C = 1−100, ^{13}C = 0−1, H = 1−200, O = 0−50, N = 0−2, ^{32}S = 0−1, and ^{34}S = 0−1. Data were then filtered using a Mathematica 10 (Wolfram Research Inc., U.K.) code developed in-house and already described elsewhere,[29] which uses a series of rules (e.g., nitrogen rule, isotope ratios) and element ratios (O/C ≤ 2, H/C ≥ 0.3, H/C ≤ 2.5, N/C ≤ 0.5, S/C ≤ 0.2) to determine a list of chemically meaningful formula assignments. More details about instrumental settings, calibrations, and data processing procedures can be found elsewhere.[13,29,30] The following discussion refers to CHO compounds only, which are the most relevant cuticle building-blocks[11] and represent almost entirely the compounds detected in this study.

Statistical Analysis. Principal component analysis (PCA) was done using Statistica 10 (StatSoft Inc., Tulsa, OK) on 16 samples, representing different spots analyzed (with direct depolymerization followed by LESA-HRMS) on the ridged/proximal and smooth/distal portions of the petals, and 587 active variables, describing the absolute intensities of the predominant ions (most intense) in the mass spectra.

RESULTS AND DISCUSSION

The new cuticle characterization method developed here was adapted from Mendez-Millan et al.[22] to translate a bulk saponification procedure into a direct/on surface saponification of the cutin prior to LESA-HRMS analysis. Saponification was chosen over transesterification and CuO oxidation because it was the most effective method for analysis of the cutin of maize[22] and because it produces free fatty acid anions that can be easily ionized with LESA-HRMS. The saponification method used by Mendez-Millan et al.[22] consisted of (i) removal of free-lipids with dichloromethane extraction in an ultrasonic bath, (ii) reflux for 18 h with a solution of 6% KOH in methanol−water (90:10), (iii) filtration and washing of the residues with methanol, (iv) acidification, (v) liquid−liquid extraction with dichloromethane, and (vi) concentration in a rotary evaporator. Compared with the method from Mendez-Millan et al.,[22] the method we proposed here is much faster (30 min vs 18 h), easier (single step method), and spatially resolved. It also uses much less solvent (~20 μL vs hundreds of milliliters used in several extraction steps in addition to the reflux) and could potentially be incorporated into an automatic routine with LESA-HRMS analysis. However, the method used by Mendez-Millan et al.[22] is exhaustive and quantitative, in contrast to the qualitative but fast, spatially resolved and direct method proposed here.

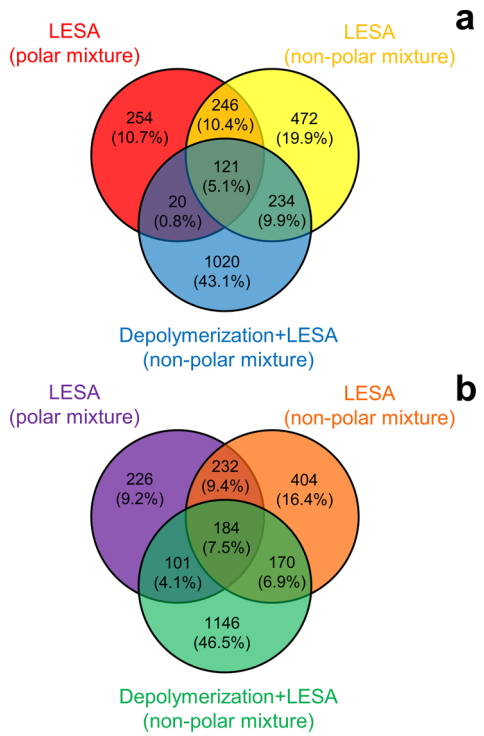

Figure 2. Venn diagrams showing the overlap and specificity of the different methods in terms of number of assigned molecular formulas for the smooth/distal (a) and ridged/proximal (b) portions of the petals.

Another important aspect concerns the possible contamination arising from the tissues under the cuticle surface. Unlike the method we described here, bulk extraction methods, as those used by Mendez-Millan et al.[22] and others,[14−20] are used on the whole sample, and not just the surface, and therefore are intrinsically a mixture of the cuticle and other compartments of the petal where unique signatures of the cuticle are more difficult to isolate. Alternatively, the surface of the petal could be stripped off mechanically and subjected to depolymerization.[21] However, fragile tissues, like petals, can easily break during such procedure therefore failing to prevent the problem of contaminations coming from the underlying tissues.

The results obtained with the new method developed here are compared with LESA-HRMS analysis done using a procedure without saponification, as used in previous studies.[13,31] This comparison allows the assessment of whether a direct depolymerization is efficient enough to bring new insights into the composition of the cuticle of *Hibiscus richardsonii* petals in both the smooth/distal and the ridged/proximal portions.

The HRMS analysis allows for an unambiguous determination of molecular formulas for the peaks detected in the

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

Table 1. Total Number of Peaks Detected (N), Total Ion Current (TIC), Average Double Bond Equivalents (DBE), O/C, H/C, and Carbon Oxidation State ($\overline{OSc}$) of All Formulas in the Mass Spectra from the Smooth/Distal and Ridged/Proximal Portions of the Petals of *Hibiscus richardsonii* Derived from the Three Different Extraction Methods

portion of the petals	extraction methods (extraction solvent)	N	TIC (a.u.)	DBE	O/C	H/C	$\overline{OSc}$
smooth/distal	LESA (polar mixture)	641	3.83×10^7	10	0.58	1.32	−0.15
smooth/distal	LESA (non-polar mixture)	1073	5.48×10^6	7	0.45	1.45	−0.56
smooth/distal	depolymerization + LESA (non-polar mixture)	1395	6.07×10^7	8	0.29	1.58	−1.01
ridged/proximal	LESA (polar mixture)	743	6.44×10^6	8	0.46	1.49	−0.57
ridged/proximal	LESA (non-polar mixture)	990	3.77×10^6	7	0.46	1.43	−0.52
ridged/proximal	depolymerization + LESA (non-polar mixture)	1601	1.75×10^8	6	0.25	1.67	−1.16

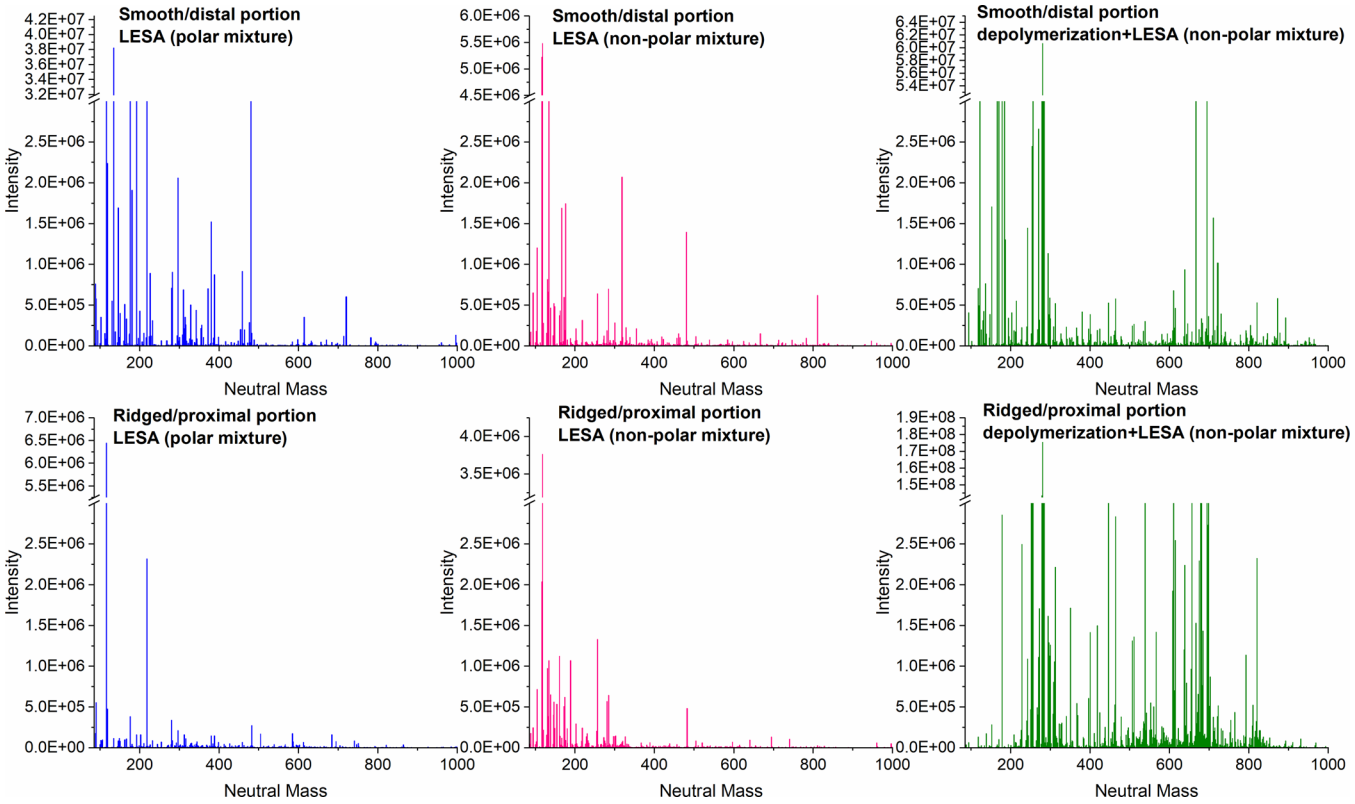

Figure 3. Mass spectra of the smooth/distal (top panels) and ridged/proximal (bottom panels) portions of the petals obtained using the three different extraction methods.

mass spectra following the method described above. Figure 2 shows the overlap and specificity of molecular formulas obtained with the different methods used in this study. While some molecular formulas were identified by all methods, the vast majority of compounds were only detected by a single method. In particular, the newly proposed method using depolymerization coupled to LESA-HRMS analysis provided 1020 new molecular formulas for the smooth/distal region and 1146 new molecular formulas for the ridged/proximal portion of the petals that were not otherwise accessible without depolymerization.

The total number of peaks with an assigned formula, the total ion current (TIC), together with average O/C, H/C, double bond equivalents (DBE), and carbon oxidation state ($\overline{OSc}$) for the smooth/distal and ridged/proximal portions are reported in Table 1 for all extraction procedures tested. Numbers reported consider three different spots on each portion (smooth/distal vs ridged/proximal) of the surface of the petals, for at least three petals coming from at least two different flowers. Table 1 shows that not only more peaks were detected with the depolymerization method but also TIC was higher, for both the smooth/distal and ridged/proximal portions of the petals by doing a depolymerization prior to LESA-HRMS analysis. The effect of the depolymerization is more prominent for the ridged/proximal portion where the number of detected peaks increased roughly 2-fold compared with LESA-HRMS analysis without depolymerization and the TIC increased by about 2 orders of magnitude. This is evident also from Figure 3, showing the mass spectra of the smooth/distal and ridged/proximal portions of the petals obtained with LESA-HRMS with and without prior depolymerization, respectively. In particular, the depolymerization allowed us to extract many more compounds with high molecular weights around 200−400 Da and 600−800 Da compared with LESA-HRMS analysis without prior depolymerization.

The van Krevelen diagram in Figure 4 shows the H/C and O/C ratio distributions of the molecular formulas detected using the three extraction methods. It can be seen from Figure 4 that the majority of peaks detected after depolymerization of the cutin are distributed in the region of lipids (red square),

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

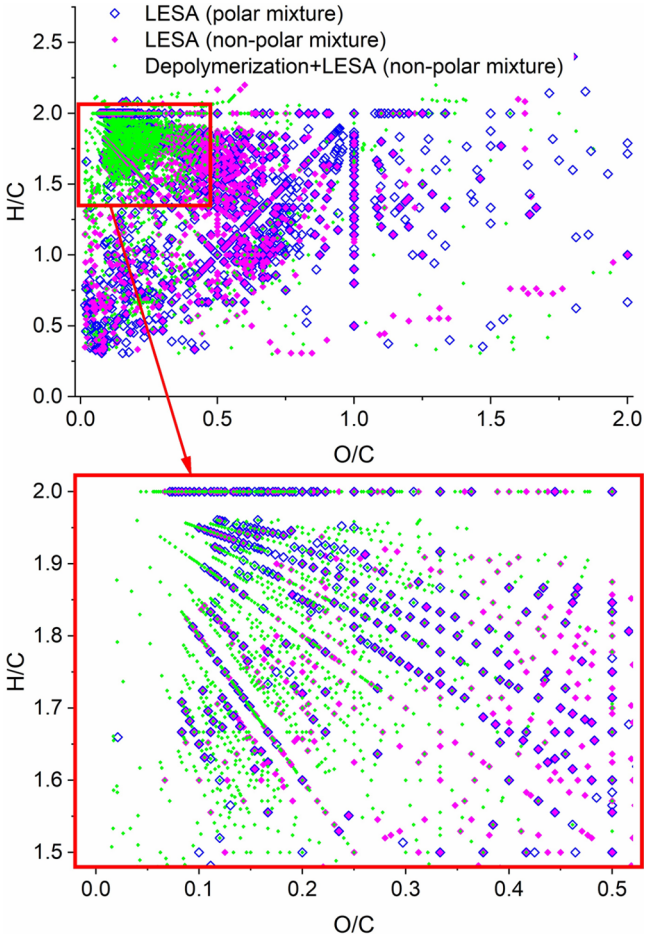

Figure 4. Van Krevelen diagram showing the distribution of all molecular formulas detected on the smooth/distal and the ridged/proximal portions (combined) of the petals of *Hibiscus richardsonii* using different extraction solvents, with or without prior depolymerization. The red square (and zoomed region on the bottom part of the figure) indicates the area of lipids, compounds that were more efficiently extracted after depolymerization of the cutin.

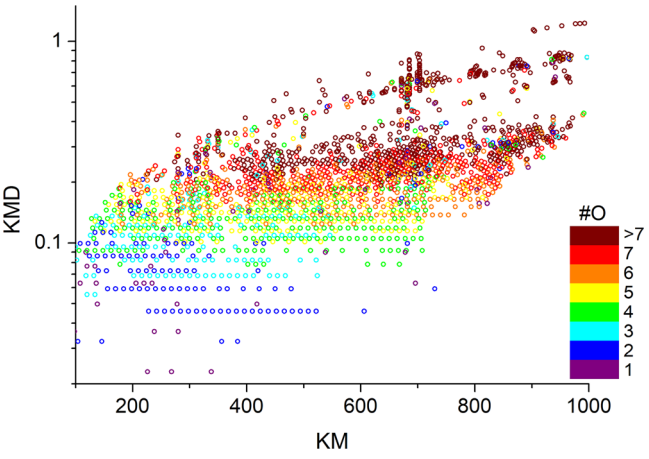

Figure 5. Kendrick mass defect plot in $-CH_2$ base showing the distribution of all molecular formulas detected on the petals of *Hibiscus richardsonii* (smooth/distal and ridged/proximal portions combined) with prior depolymerization. The color scale shows the number of oxygen atoms in the formulas.

the region corresponding to low O/C and high H/C. This is also confirmed by the data reported in Table 1, where it is evident that, on average, molecular formulas in the depolymerization extracts have a lower O/C, lower DBE (for the ridged part), and higher H/C, which are typical of lipid compounds. It is also worth noticing from Figure 4 that not all compounds detected using LESA-HRMS without depolymerization are also detected after depolymerization. This might be due to a degradation of plant metabolites and/or suppression of the signal of those compounds because of competitive ionization in the source of the mass spectrometer.

The Kendrick mass defect plot in Figure 5 helps to identify homologous series of compounds having the same number of rings/double bonds and heteroatoms but different chain lengths. Main series detected through depolymerization coupled to LESA-HRMS analysis are long-chain fatty acids, hydroxy fatty acids, dihydroxy fatty acids, and monounsaturated hydroxy fatty acids (blue, light-blue, and green series of horizontal data points in Figure 5), which are all known components of plant epicuticular and intracuticular waxes.[2,11,13] A list of the main compounds detected using the new method is reported in Table 2, including tentative assignments based on the molecular formulas or MS/MS spectra where available. The majority of the compounds detected exclusively after depolymerization are long-chain fatty acids and high-molecular weight compounds tentatively associated with monogalactosyldiacylglycerol (MGDG) lipids.

More specifically, the most intense peaks in the mass spectra, especially for the ridged/proximal portion, correspond to saturated C16 and C18 fatty acids, monounsaturated C16 and C18 fatty acids, and diunsaturated C16 and C18 fatty acids (Figure 6), which are well-known monomers of the cutin, epicuticular, and intracuticular waxes.[2,11,13] In addition to the monomeric cutin units and waxes, some dimers can be seen in the high molecular weight region of the mass spectra (Figure 6). A more exhaustive list of the main molecular formulas detected that could be associated with cuticle constituents is reported in Table S1 in the Supporting Information. Conversely, the smooth/distal portion of the petal is richer, on average, in lower molecular weight compounds (around m/z 150−200, see Figure 6), for example, those with molecular formulas $C_7H_6O_2$, $C_9H_{10}O_3$, $C_7H_6O_5$, and $C_{10}H_{16}O_3$, which may be associated with plant metabolites.

Next, we performed a principal component analysis (PCA) on the 587 most intense peaks in the mass spectra obtained by analyzing the ridged/proximal and smooth/distal portions of the petals using the new method with prior depolymerization. This analysis confirms a markedly different composition of the cuticle in the two portions of the petals (Figure 7). In fact, the samples taken from the ridged/proximal portions are scattered on the top part of the score plot (Figure 7a), while the samples taken from the smooth/distal portions of the petals are clustered toward the bottom-right part of the score plot (Figure 7a). As shown by the loading plot in Figure 7b, the differences between the ridged/proximal and the smooth/distal portions can be ascribed mainly to a few compounds with molecular formulas $C_{10}H_{10}O_3$, $C_{16}H_{30}O_2$, $C_{18}H_{30}O_2$, $C_{18}H_{32}O_2$, $C_7H_6O_2$, $C_9H_{10}O_3$, $C_{10}H_{16}O_3$, and $C_{46}H_{78}O_4$. The ridged/proximal portion is richer in $C_{10}H_{10}O_3$ (methoxycinnamic acid), $C_{16}H_{30}O_2$ (palmitoleic acid), $C_{18}H_{30}O_2$ (linolenic acid), and $C_{18}H_{32}O_2$ (linoleic acid) while the smooth/distal portion is richer in $C_7H_6O_2$ (salicylaldehyde), $C_9H_{10}O_3$ (dihydro-coumaric acid), $C_{10}H_{16}O_3$ (pinonic acid), and

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

Table 2. List of Main Compounds (Most Intense Peaks in the Mass Spectra) Detected on the Surface of the Petals with LESA-HRMS with Prior Depolymerization[a]

neutral formula	theoretical mass	DBE	MS/MS analysis[b]	tentative assignment[c]	database
$C_5H_{10}O_3$	**118.0630**	**1**		**hydroxyvaleric acid**	**PubChem**
$C_7H_6O_2$	122.0368	5		salicylaldehyde	PubChem
$C_6H_{12}O_3$	**132.0786**	**1**		**hydroxyhexanoic acid**	**PubChem**
$C_8H_8O_2$	**136.0524**	**5**		**phenylacetic acid**	**PubChem**
$C_8H_8O_3$	**152.0473**	**5**		**vanillin**	**PubChem**
$C_9H_{10}O_3$	166.0630	5		dihydro-coumaric acid	PubChem
$C_7H_6O_5$	170.0215	5		gallic acid	PubChem
$C_{10}H_{10}O_3$	178.0630	6	162.03/145.03/133.03/118.04	methoxycinnamic acid	PubChem
$C_8H_8O_5$	**184.0372**	**5**		**3,4-dihydroxymandelic acid**	**PubChem**
$C_{10}H_{16}O_3$	**184.1099**	**3**		**pinonic acid**	**PubChem**
$C_{10}H_{18}O_3$	**186.1256**	**2**		**oxodecanoic acid**	**PubChem**
$C_{11}H_{12}O_4$	208.0736	6	192.04/179.03	not found	
$C_{15}H_{16}O_2$	**228.1150**	**8**		**bisphenol A**	**PubChem**
$C_{14}H_{28}O_2$	228.2089	1		myristic acid	PubChem
$C_{13}H_{20}O_4$	**240.1362**	**4**		**diethyl diallylmalonate**	**PubChem**
$C_{15}H_{30}O_2$	242.2246	1		pentadecanoic acid	PubChem
$C_{16}H_{28}O_2$	252.2089	3		hexadecadienoic acid	PubChem
$C_{16}H_{30}O_2$	254.2246	2		palmitoleic acid	PubChem
$C_{16}H_{32}O_2$	256.2402	1	237.22	palmitic acid	PubChem
$C_{17}H_{30}O_2$	**266.2246**	**3**		**heptadec-2-ynoic acid**	**PubChem**
$C_{16}H_{30}O_3$	**270.2195**	**2**		**keto palmitic acid**	**PubChem**
$C_{17}H_{34}O_2$	270.2559	1		heptadecanoic acid	PubChem
$C_{18}H_{30}O_2$	278.2246	4	259.21/233.23/179.18	linolenic acid	PubChem
$C_{18}H_{32}O_2$	280.2402	3	261.22/234.23	linoleic acid	PubChem
$C_{18}H_{34}O_2$	282.2559	2		oleic acid	PubChem
$C_{18}H_{36}O_2$	284.2715	1	265.25	stearic acid	PubChem
$C_{18}H_{30}O_3$	294.2195	4		hydroxylinolenic acid	PubChem
$C_{19}H_{36}O_2$	**296.2715**	**2**		**methyl oleate**	**PubChem**
$C_{20}H_{34}O_2$	**306.2559**	**4**		**eicosatrienoic acid**	**PubChem**
$C_{20}H_{38}O_2$	**310.2872**	**2**		**eicosenoic acid**	**PubChem**
$C_{20}H_{40}O_2$	312.3028	1		arachidic acid	PubChem
$C_{20}H_{40}O_3$	**328.2977**	**1**		**glycol stearate**	**ChemSpider**
$C_{22}H_{38}O_3$	**350.2821**	**4**		**furancarboxylic acid, heptadecyl ester**	**PubChem**
$C_{22}H_{38}O_4$	**366.2770**	**4**		**dicyclohexyl sebacate**	**PubChem**
$C_{17}H_{16}O_{10}$	**380.0744**	**10**		**trimethylenglykol-digalloat**	**PubChem**
$C_{22}H_{42}O_6$	**402.2981**	**2**		**glyceryl lactylpalmitate**	**PubChem**
$C_{29}H_{50}O_8$	**526.3506**	**5**		**steroid**	**PubChem**
$C_{34}H_{66}O_4$	538.4961	2		didodecyl sebacate	PubChem
$C_{30}H_{54}O_8$	**542.3819**	**4**		**sorbitan, trioctanoate**	**PubChem**
$C_{34}H_{64}O_7$	**584.4652**	**3**		**sorbitan, ditetradecanoate**	**PubChem**
$C_{36}H_{70}O_9$	**646.5020**	**2**		**1-(O-α-D-glucopyranosyl)-3-keto-(1,27R,29R)-triacontanetriol**	**LipidMAPS**
$C_{37}H_{68}O_9$	**656.4863**	**4**		**parisin**	**PubChem**
$C_{37}H_{68}O_{10}$	**672.4813**	**4**		**MGDG(28:1)[d]**	**LipidMAPS**
$C_{37}H_{70}O_{10}$	674.4969	3		MGDG(28:0)	LipidMAPS
$C_{39}H_{66}O_9$	**678.4707**	**7**		**1,3,5-tris(10-carboxydecyloxy)benzene**	**PubChem**
$C_{39}H_{68}O_9$	**680.4863**			**triethoxycholesterol galactose**	**PubChem**
$C_{39}H_{72}O_9$	**684.5176**	**4**		**nonatriaconta-10,17,24-trien-3-one**	**PubChem**
$C_{46}H_{78}O_4$	694.5900	8		hexadecanoyloleanolic acid	PubChem
$C_{39}H_{68}O_{10}$	**696.4813**	**6**		**MGDG(30:3)**	**LipidMAPS**
$C_{39}H_{70}O_{10}$	698.4969	5		MGDG(30:2)	LipidMAPS
$C_{39}H_{72}O_{10}$	**700.5126**	**4**		**MGDG(30:1)**	**LipidMAPS**
$C_{39}H_{74}O_{10}$	**702.5282**	**3**		**MGDG(30:0)**	**LipidMAPS**
$C_{40}H_{72}O_{10}$	**712.5126**	**5**		**MGDG(31:2)**	**LipidMAPS**
$C_{40}H_{74}O_{10}$	**714.5282**	**4**		**MGDG(31:1)**	**LipidMAPS**
$C_{47}H_{86}O_5$	**730.6475**	**5**		**1-docosanoyl-2-(10Z,13Z,16Z-docosatrienoyl)-*sn*-glycerol**	**LipidMAPS**
$C_{48}H_{92}O_{10}$	**828.6691**	**3**		**MGDG(39:0)**	**LipidMAPS**

[a]Compounds that were only detected with prior depolymerization are highlighted in bold. [b]Fragment ions detected in negative ionization with LESA-HRMS. [c]Please refer to Table S2 for the links. [d]MGDG(x:y) refers to the monogalactosyldiacylglycerol lipid class with x carbon atoms and y degree of unsaturation of the fatty acid chain.

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

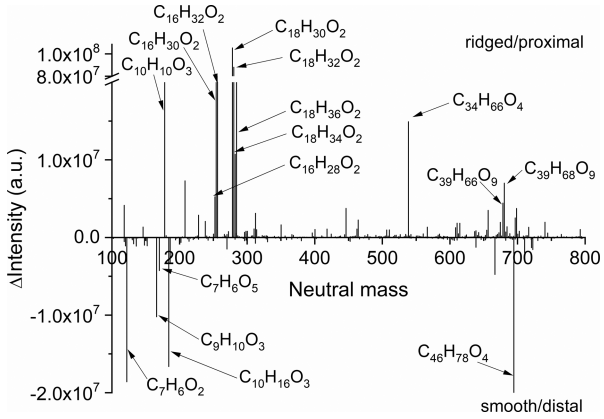

Figure 6. Difference between the mass spectra of the ridged/proximal portion and the mass spectra of the smooth/distal portion of the petals of *Hibiscus richardsonii* analyzed with the new method using direct depolymerization followed by LESA-HRMS using the nonpolar solvent mixture.

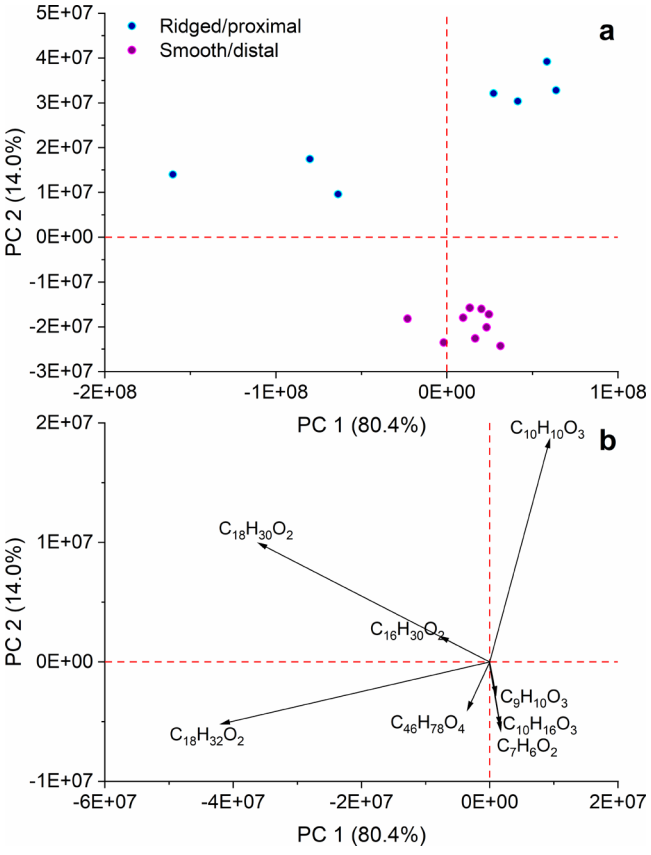

Figure 7. Scores (a) and loadings (b) of the first two principal components, explaining 94.4% of the variance of the data set, obtained from the principal components analysis applied to the 587 most intense peaks in the mass spectra of the ridged/proximal and smooth/distal portions of the petals analyzed with the new method proposed here, i.e., direct depolymerization followed by LESA-HRMS using the nonpolar solvent mixture. The loading plot only shows the compounds that contributed the most to differentiate the ridged/proximal from the smooth/distal portion of the petal.

$C_{46}H_{78}O_4$ (hexadecanoyloleanolic acid). Whether these compounds (tentatively assigned based on the molecular formulas and MS/MS spectra where available) may play a role in the formation of ridges on the surface of the proximal portion of the petals of *Hibiscus richardsonii* will need to be investigated in future studies.

These results show that the direct depolymerization method presented here is able to provide a qualitative composition of the surface of the petals with the possibility of capturing important differences in the chemical composition of different regions of the petal cuticle (smooth/distal vs ridged/proximal).

CONCLUSIONS

We presented a new analytical method using direct/on surface depolymerization coupled with LESA-HRMS for the detailed characterization of the surface of plant tissues. This method was used for the chemical characterization of the cuticle of *Hibiscus richardsonii* petals, whose surface is characterized by a ridged/proximal region and a smooth/distal portion.

The on-surface saponification proved to be effective to depolymerize the cutin to obtain qualitative analysis of cuticular constituents. Comparison with direct analysis without prior depolymerization showed that (i) depolymerization was effective to break down the cutin polymers into their monomeric constituents, (ii) the signal of other compounds was suppressed, probably due to competitive ionization and degradation. For these reasons, it would be beneficial to analyze the petal cuticle via LESA-HRMS analysis both with and without prior depolymerization in order to obtain a more complete picture of the surface composition. The depolymerization step proposed here is compatible with the Triversa NanoMate and could potentially be incorporated into an automated procedure, which is currently not possible due to software limitations.

The fast and spatially resolved depolymerization approach presented here, that selectively targets only the surface, allowed detection of a set of cuticular compounds that were not otherwise accessible without a depolymerization step. The effect of the depolymerization was more pronounced for the ridged/proximal portion of the petal, which has a thicker cuticle and is richer in epicuticular waxes compared with the smooth/distal portion. Our results are consistent with previous studies that showed that the composition of the plant cuticle can be chemically and morphologically heterogeneous even between different regions of the same petal.[13,31] Further experiments are necessary to establish whether there is a link between chemical composition of the cuticle and patterning differences of plant surfaces.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b01094.

Most intense peaks detected and possibly associated with cuticle components (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: chiara.giorio@unipd.it.

ORCID

Chiara Giorio: 0000-0001-7821-7398

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333

■ ACKNOWLEDGMENTS

The authors would like to thank Dr. B.G. Murray for the generous gift of *Hibiscus richardsonii* seeds. This work was funded by the European Research Council (ERC Consolidator Grant 279405) to M.K., the Herchel Smith Fund and the Gatsby Charitable Foundation to E.M., and by a BBSRC Grant BB/P001157/1 to B.J.G. and M.K.

■ REFERENCES

(1) Fernández, V.; Guzmán-Delgado, P.; Graça, J.; Santos, S.; Gil, L. *Front. Plant Sci.* **2016**, *7* (March), 1−14.
(2) Jetter, R.; Kunst, L.; Samuels, A. L. Composition of Plant Cuticular Waxes. In *Annual Plant Reviews*; Riederer, M., Müller, C., Eds.; Blackwell Publishing Ltd.: Oxford, U.K., 2006; Vol. *23: Biology of the Plant Cuticle*, pp 145−181, DOI: 10.1002/9780470988718.ch4.
(3) Dominguez, E.; Heredia-Guerrero, J. A.; Heredia, A. *New Phytol.* **2011**, *189*, 938−949.
(4) Vignolini, S.; Moyroud, E.; Glover, B. J.; Steiner, U. *J. R. Soc., Interface* **2013**, *10* (87), 20130394.
(5) Vignolini, S.; Moyroud, E.; Hingant, T.; Banks, H.; Rudall, P. J.; Steiner, U.; Glover, B. J. *New Phytol.* **2015**, *205* (1), 97−101.
(6) Moyroud, E.; Wenzel, T.; Middleton, R.; Rudall, P. J.; Banks, H.; Reed, A.; Mellers, G.; Killoran, P.; Westwood, M. M.; Steiner, U.; et al. *Nature* **2017**, *550* (7677), 469−474.
(7) Whitney, H. M.; Kolle, M.; Andrew, P.; Chittka, L.; Steiner, U.; Glover, B. J. *Science (Washington, DC, U. S.)* **2009**, *323* (5910), 130−133.
(8) Whitney, H. M.; Reed, A.; Rands, S. A.; Chittka, L.; Glover, B. J. *Curr. Biol.* **2016**, *26* (6), 802−808.
(9) Nawrath, C. *Curr. Opin. Plant Biol.* **2006**, *9* (3), 281−287.
(10) Jeffree, C. E. The Fine Structure of the Plant Cuticle. In *Annual Plant Reviews*; Riederer, M., Müller, C., Eds.; Blackwell Publishing Ltd.: Oxford, U.K., 2006; Vol. *23: Biology of the Plant Cuticle*, pp 11−125, DOI: 10.1002/9780470988718.ch2.
(11) Stark, R. E.; Tian, S. The Cutin Biopolymer Matrix. In *Annual Plant Reviews*; Riederer, M., Müller, C., Eds.; Blackwell Publishing Ltd.: Oxford, U.K., 2006; Vol. *23: Biology of the Plant Cuticle*, pp 126−144, DOI: 10.1002/9780470988718.ch3.
(12) Hemalatha, R. G.; Pradeep, T. *J. Agric. Food Chem.* **2013**, *61*, 7477−7487.
(13) Giorio, C.; Moyroud, E.; Glover, B. J.; Skelton, P. C.; Kalberer, M. *Anal. Chem.* **2015**, *87* (19), 9900−9907.
(14) Lü, S.; Song, T.; Kosma, D. K.; Parsons, E. P.; Rowland, O.; Jenks, M. A. *Plant J.* **2009**, *59* (4), 553−564.
(15) Riederer, M.; Schönherr, J. *J. Chromatogr. A* **1986**, *360*, 151−161.
(16) Tsubaki, S.; Sakumoto, S.; Uemura, N.; Azuma, J. *Food Chem.* **2013**, *138* (1), 286−290.
(17) van Maarseveen, C.; Jetter, R. *Phytochemistry* **2009**, *70* (7), 899−906.
(18) Ray, A. K.; Chen, Z.-J.; Stark, R. E. *Phytochemistry* **1998**, *49* (1), 65−70.
(19) Bonaventure, G.; Beisson, F.; Ohlrogge, J.; Pollard, M. *Plant J.* **2004**, *40* (6), 920−930.
(20) Gérard, H.; Pfeffer, P.; Osman, S. *Phytochemistry* **1994**, *35*, 818−819.
(21) Ji, X.; Jetter, R. *Phytochemistry* **2008**, *69* (5), 1197−1207.
(22) Mendez-Millan, M.; Dignac, M.-F.; Rumpel, C.; Derenne, S. *Org. Geochem.* **2010**, *41* (2), 187−191.
(23) Craven, L. A.; de Lange, P. J.; Lally, T. R.; Murray, B. G.; Johnson, S. B. *N. Z. J. Bot.* **2011**, *49* (1), 27−40.
(24) Johnson, S. B.; Craven, L. A. *Plant Prot. Q.* **2013**, *28* (2), 50−56.
(25) Himmelsbach, M.; Varesio, E.; Hopfgartner, G. *Chimia* **2014**, *68* (3), 150−154.
(26) Hall, Z.; Chu, Y.; Griffin, J. L. *Anal. Chem.* **2017**, *89*, 5161−5170.
(27) Almeida, R.; Berzina, Z.; Arnspang, E. C.; Baumgart, J.; Vogt, J.; Nitsch, R.; Ejsing, C. S. *Anal. Chem.* **2015**, *87*, 1749−1756.
(28) Martin, N. J.; Griffiths, R. L.; Edwards, R. L.; Cooper, H. J. *J. Am. Soc. Mass Spectrom.* **2015**, *26*, 1320−1327.
(29) Zielinski, A. T.; Kourtchev, I.; Bortolini, C.; Fuller, S. J.; Giorio, C.; Popoola, O. A. M.; Bogialli, S.; Tapparo, A.; Jones, R. L.; Kalberer, M. *Atmos. Environ.* **2018**, *178*, 129−139.
(30) Zielinski, A. T.; Campbell, S. J.; Seshia, A. A.; Jones, R. L.; Kalberer, M.; Giorio, C. *Aerosol Sci. Eng.* **2018**, *2* (3), 118−129.
(31) Li, B.; Hansen, S. H.; Janfelt, C. *Int. J. Mass Spectrom.* **2013**, *348*, 15−22.

DOI: 10.1021/acs.analchem.9b01094
Anal. Chem. 2019, 91, 8326−8333