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RESEARCH ARTICLE

Direct Analysis of Triterpenes from High-Salt Fermented Cucumbers Using Infrared Matrix-Assisted Laser Desorption Electrospray Ionization (IR-MALDESI)

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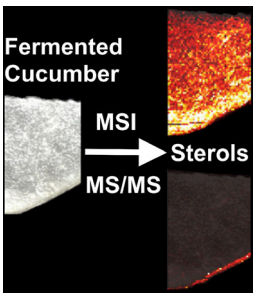

Abstract. High-salt samples present a challenge to mass spectrometry (MS) analysis, particularly when electrospray ionization (ESI) is used, requiring extensive sample preparation steps such as desalting, extraction, and purification. In this study, infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) coupled to a Q Exactive Plus mass spectrometer was used to directly analyze 50-μm thick slices of cucumber fermented and stored in 1 M sodium chloride brine. From the several hundred unique substances observed, three triterpenoid lipids produced by cucumbers, β-sitosterol, stigmasterol, and lupeol, were putatively identified based on exact mass and selected for structural analysis. The spatial distribution of the lipids were imaged, and the putative assignments were confirmed by tandem mass spectrometry performed directly on the same cucumber, demonstrating the capacity of the technique to deliver confident identifications from highly complex samples in molar concentrations of salt without the need for sample preparation.

Keywords: Food preservation, IR-MALDESI, Mass spectrometry imaging, Phytosterols, Direct analysis, HRAM, Fermented vegetable composition, Q Exactive

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Introduction

For the past two decades, electrospray ionization (ESI) has been used extensively as a soft ionization source particularly suited for analysis of biological specimens because of the ease of coupling liquid chromatography (LC) separation to mass spectrometry (MS) detection. LC-MS has become widely used for qualitative and quantitative analysis of many biomolecules in the food and agriculture sciences. However, there are a number of limitations associated with ESI-MS methods that have yet to be fully overcome. For chemical analysis of complex biological systems, the samples are often subjected to a long and laborious preparation process to extract and purify analytes of interest and remove interfering contaminants [1–9]. The presence of high levels of low volatility substances such as inorganic salts is particularly destructive to the ESI ionization process, as they inhibit the transfer of ions from the electrospray droplets to the gas phase [10]. Therefore, it is often necessary to desalt samples before analysis by mass spectrometry [4, 6, 11].

One way of circumventing some of these issues is by using ESI for post-ionization by introducing the sample solution into the electrospray plume after its formation, as is done in a number of published methods, including desorption electrospray ionization (DESI) [12], fused-droplet ESI (FD-ESI) [13], and matrix-assisted laser desorption electrospray ionization (MALDESI) [14]. The salt tolerance of DESI and FD-ESI has been characterized in detail for pure analytes in solvents with salt content as high as 2 M [13,

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15]. It may be hypothesized that compatibility with high salt samples is an inherent property of all ESI post-ionization methods, including MALDESI. Other strategies have been successfully developed to counter the effect of ion suppression by making fundamental changes to the electrospray, including nanoelectrospray ionization (nanoESI) [16] and probe electrospray ionization (PESI) [17].

MALDESI is an ambient ionization source where a pulsed laser is used to ablate material from a sample surface that is subsequently captured by an orthogonal electrospray plume where it is ionized [14]. The most current version of MALDESI employs a mid-infrared laser for desorption. The energy of the laser pulse is absorbed by the endogenous water present in the sample with the optional addition of ice as an external matrix. The method has in this specific application been abbreviated as IR-MALDESI [18]. In our laboratory, the instrument is routinely used for mass spectrometry imaging (MSI) of biological tissues without desalting or other chemical processing, and has proven to be useful for analyzing the spatial distributions of metabolites and drugs in tissue specimens [19, 20]. For an estimation of the salt content of the typical MALDESI sample, osmolalities of healthy mammalian fluids range around 0.3 osmoles/kg, roughly equivalent to 0.15 M sodium chloride [21]. Herein, we demonstrate the capacity of IR-MALDESI to directly analyze fermented cucumber tissue stored in molar-level sodium chloride solutions and characterize biologically interesting molecules based on accurate mass as well as tandem MS.

Experimental

Materials and Methods

LC-MS-grade methanol and water were purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid (MS-grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). High purity (99.999%) nitrogen gas for the higher energy collision (HCD) cell was purchased from Arc3 Gases (Raleigh, NC, USA).

Cucumber Fermentation

Fresh, size 2B (3.5–3.8 cm diameter) pickling cucumbers were obtained from a local processor and packed into 32 oz. (946 mL) jars. A brine equilibrating to 1 M NaCl, 0.025 M acetic acid, and 4 mM potassium sorbate was added to the cucumbers at a 55:45 (w/w) cucumber to brine ratio. The cucumbers were inoculated with a starter culture at 10^6 CFU/mL *Lactobacillus plantarum* (LA0445, USDA-ARS, Food Science Research Unit, Raleigh, NC culture collection). *L. plantarum* starter culture was grown at 30 °C in deMan, Rogosa, and Sharpe (MRS; Becton, Dickinson and Co.) broth to a population of approximately 10^9 CFU/mL, centrifuged to pellet the cells, and resuspended in saline. After inoculation, the jars were sealed and incubated at 28 °C for 100 d to simulate a typical fermentation and bulk storage time. The cucumbers were fully fermented as evidenced by low residual sugars, a pH of 3.2, and ~120 mM lactic acid. A transverse 6.7 mm slice from the center of each cucumber was stored in the fermentation brine at 4 °C until analyzed.

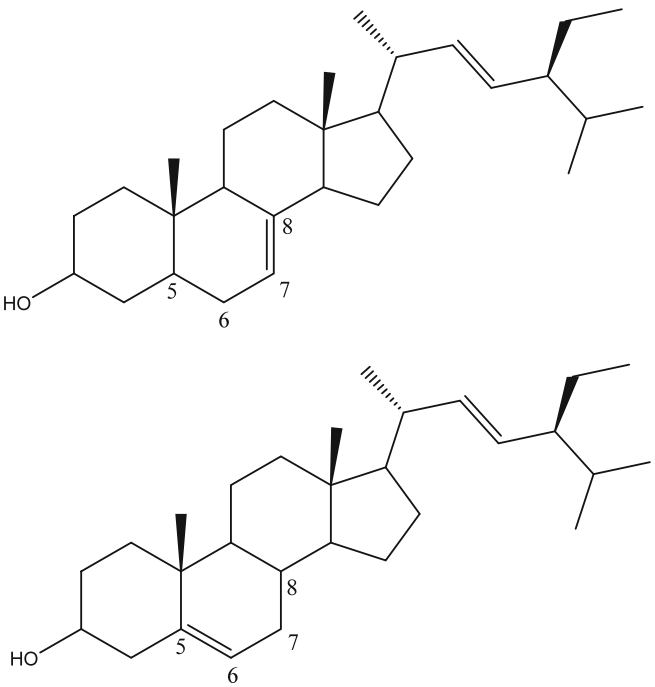
Sample Preparation

A single lobe was removed from an axial section of the fermented cucumbers using a precleaned single edge blade, and was further sectioned into 50-µm thick slices using a Leica CM1950 cryostat (Buffalo Grove, IL, USA). This thickness was found to be the lowest practical setting, due to the fragility of the frozen cucumber. The samples were thaw-mounted onto precleaned glass microscope slides (VWR, Radnor, PA, USA) and analyzed without further preparation. Samples for MS/MS analysis were stored mounted on slides at –20 °C until time of analysis.

IR-MALDESI Mass Spectrometry

Direct full MS analysis (MS1) of a whole slice was performed immediately following preparation. A 50:50 mixture of methanol:water with 0.2% formic acid was used as the electrospray solvent. No external matrix was added, as unlike for animal tissue samples, a preliminary experiment had indicated no added benefit of depositing a layer of ice before analysis (data not shown). This is presumably due to the high content of endogenous water in the cucumber itself.

All experiments were conducted at the ambient temperature and pressure of the laboratory. A mid-IR laser (IR-Opolette 2371; Opotek, Carlsbad, CA, USA) at a wavelength of 2.94 µm was used to desorb material from the surface. Two 7-ns pulses at a 20 Hz repetition rate were used to ablate the material for



Scheme 1. Comparison of molecular structures. Top – Δ7-Stigmasterol (spinasterol); Bottom – Δ5-Stigmasterol

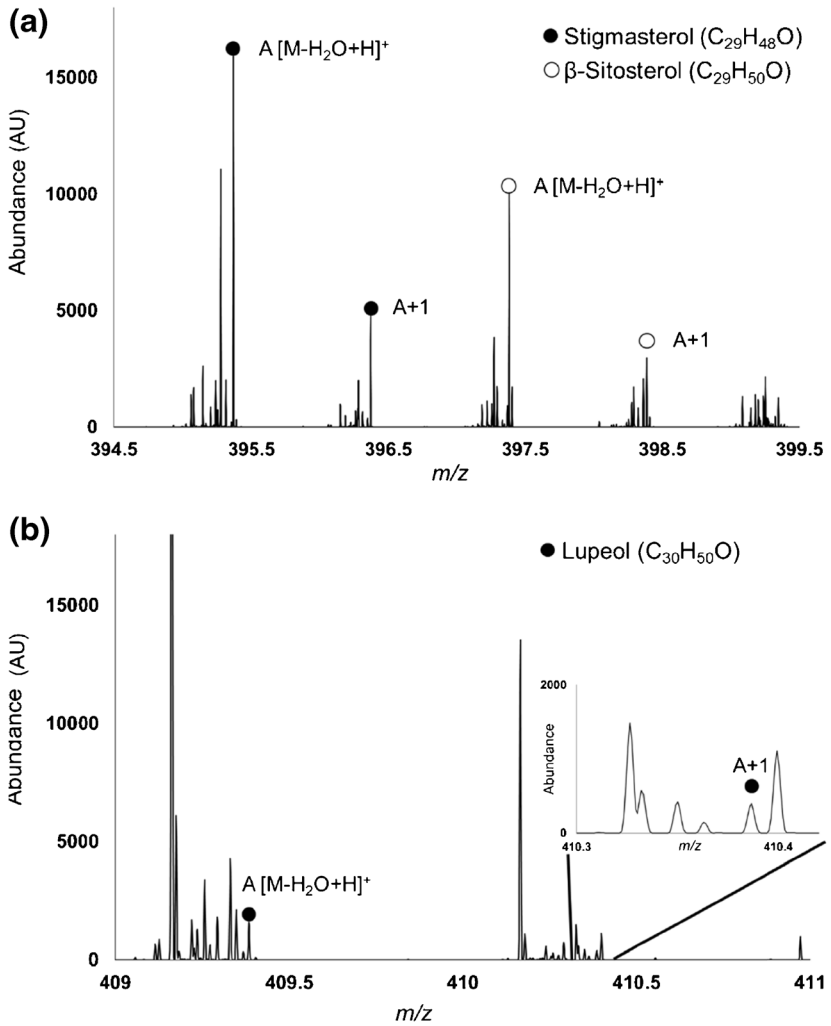


Figure 1. Average of 232 separately acquired MS1 spectra over the whole fermented cucumber tissue with magnification around regions of interest. The full spectrum is supplied as Supplementary Figure S1. Black and white circles indicate theoretical isotopic distributions. All assigned peaks are within 1 ppm of their theoretical *m/z*. **(a)** Zoomed-in region of *m/z* 395–399 with relative abundances and isotopic distributions of stigmasterol and sitosterol. **(b)** Magnification of *m/z* 409–411 showing isotopic distribution of lupeol. Inset shows further magnification of range around the A+1 isotope

each spectrum acquired. The IR-MALDESI source was coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) and the *m/z* range of 200–800 was measured with resolving power of 140,000 (FWHM, *m/z* 200) in positive ion mode. Due to the pulsed nature of IR-MALDESI, the automatic gain control (AGC) feature was disabled. The ions generated in both ablation events were collected in the C-trap for a fixed injection time of 110 ms, and were subsequently measured in a single Orbitrap acquisition. Electrospray solvent was supplied at a flow rate of 2.0 µL/min. The electrospray driving voltage was set to 4 kV, and the capillary inlet temperature was held at 275 °C.

MS/MS experiments were carried out using the same method as described above, with the instrument in all-ion fragmentation (AIF) mode. Ions of interest were isolated using a 1.0 Th window and fragmented using HCD with normalized collisional energy (NCE) set to 20%. Each target molecule was sampled at around 200 scans/molecule across the tissue so as to include both mesocarp (flesh) and exocarp (skin) tissue. Mass spectra were acquired in the ranges of *m/z* 50–200 and 100–400.

Data Analysis

Mass spectra were analyzed using Xcalibur (Thermo Scientific, Bremen, Germany). For MSI analysis, the raw files were converted to mzml format using MSConvert, part of the ProteoWizard toolkit [22], and from mzml to imzml format using imzMLConverter [23].

Ion images were generated using MSiReader [24] ver. 0.06 with a *m/z* bin width of 5 ppm (±2.5 ppm). The peak picking function in MSiReader was used to generate a list of masses present in the cucumber tissue, and this list was cross-referenced with the METLIN database ver. c1.1 (beta) [25]. From the resulting putative identifications, the sterol lipids stigmasterol and β-sitosterol as well as the related triterpenoid

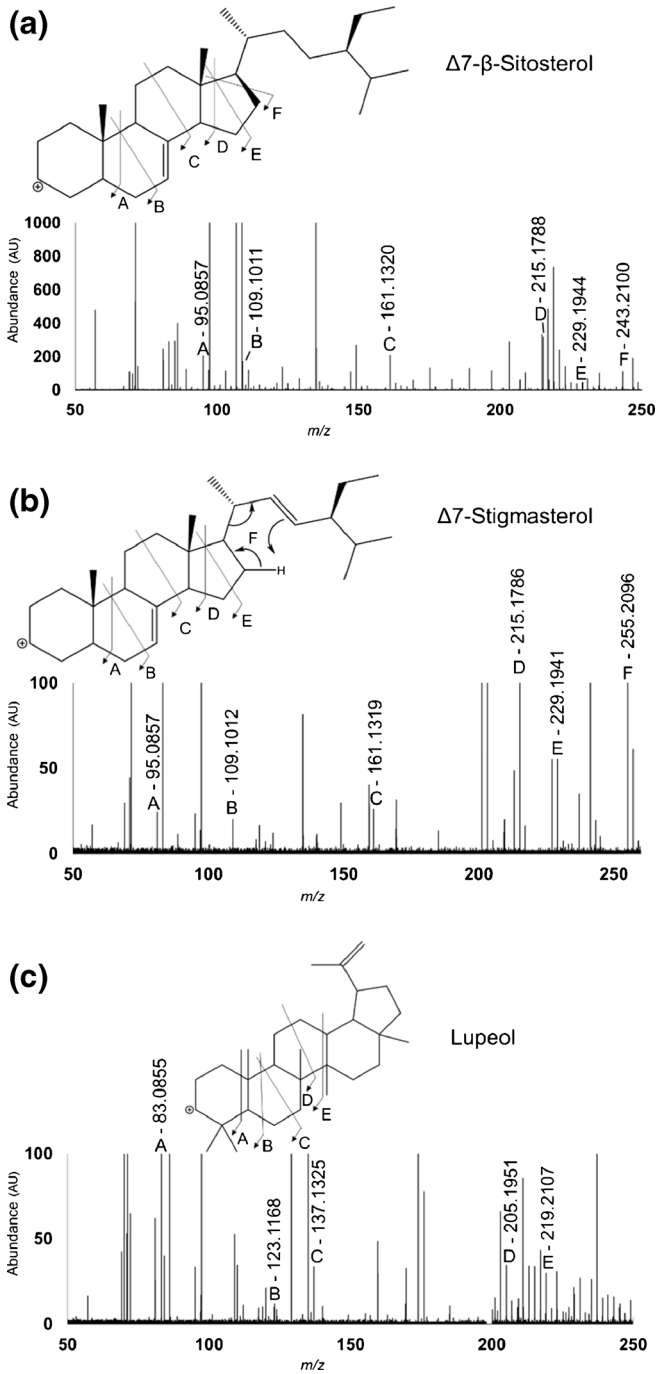


Figure 2. AIF spectra of **(a)** *m/z* 397.4 ± 0.5 (β-sitosterol); **(b)** *m/z* 395.4 ± 0.5 (stigmasterol); **(c)** *m/z* 409.4 ± 0.5 (lupeol) with proposed fragmentation pathways illustrated

lupeol were selected for further structural analysis with MS/MS.

Results and Discussion

MS1 Survey

The initial full imaging scan over a section of cucumber yielded 4500 individual MS1 spectra, each corresponding to the ablation of a 100 × 100 μm area. An unannotated raw spectrum averaged over multiple on-tissue scans is provided as Supplementary Figure S1 in the Electronic Supplementary Information to show the complexity and quality of the data collected. The entire dataset after processing with the routine imaging workflow detailed in the experimental section was interrogated for masses correlating spatially with the tissue, and a list of masses correlated to the on-tissue region was generated using the peak picking tool within MSiReader. The list was used to guide further inquiry, but should not be thought of as a comprehensive survey of unique identities due to lack of filtering for false hits such as may result from selecting multiple isotopes of the same molecule.

To confirm the identity of the putative assignments, three species were selected for structural analysis. The phytosterols stigmasterol and β-sitosterol (*m/z* 395.367 and 397.383, respectively) were chosen as targets because of their distinctiveness to cucumbers. Specifically, the family of *Cucurbitaceae*, including cucumbers, pumpkins, and squash, is well known to accumulate the Δ7 isomer of phytosterols rather than the Δ5 form, which is the more common form found in nature [26, 27]. The structural difference is illustrated for stigmasterol in Scheme 1. The two related phytosterols are only differentiated by a single unsaturation in the side chain. An additional ion at *m/z* 409.383, believed to be the dehydrated ion of one of several triterpenoids with the molecular formula $C_{30}H_{50}O$ (lupeol; α-, β-amyrin; cycloartenol), was selected for structural analysis because of its distinct observed localization to the exocarp of the cucumber.

For all three molecules, the most abundant molecular ion was the dehydrated form $[M + H - H_2O]^+$. Masses consistent with singly protonated and sodium adducted ions were present in the spectra at less than 10% abundance relative to the dehydrated peak in all cases, which is consistent with typical ionization patterns for sterols and related molecules [23]. Annotated mass spectra of the mass ranges containing the analytes of interest are shown in Figure 1, where A and A + 1 peaks are labeled. The complex environment of the peak clusters exemplifies the need for high mass resolving power to achieve confident identification of molecules from direct analysis of biological tissue samples. The spectra in Figure 1 represent averages over a region including both endocarp and exocarp tissue.

Structural Determination with Tandem Mass Spectrometry

Tandem mass spectra were collected from different sections of the same fermented cucumber. The results are shown in Figure 2. All fragments were found within 3 ppm of their theoretical masses and are well resolved from a significant background of fragments, presumably from the cluster of unrelated ions included in the 1 Th isolation window (Figure 1). The spectra were additionally compared with fragmentation patterns observed by others

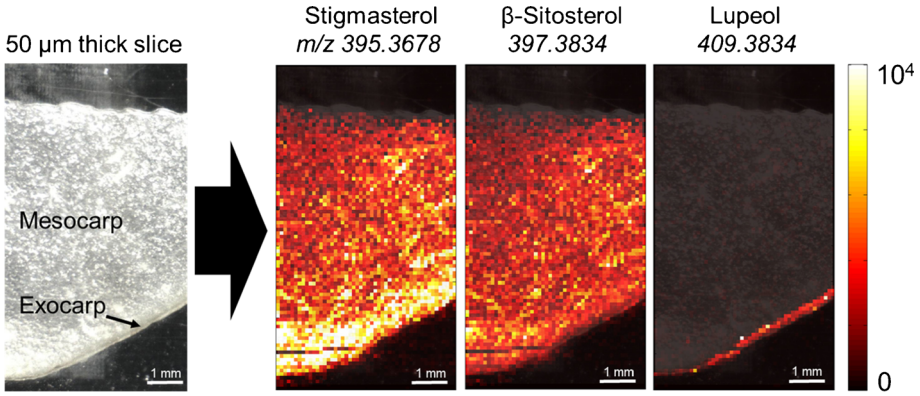


Figure 3. MS images for stigmasterol, β-sitosterol, and lupeol showing spatial distribution in exocarp (skin) and mesocarp (flesh) of fermented cucumber. The image on the left is a microscope image of the sample mounted on a glass slide. The same image has been overlaid on top on the mass images as a visual reference. All images have a lateral resolution of 100 µm with a mass bin width of 5 ppm

using more conventional methods of coupling separations (TLC and LC) with MS detection [28–30]. The full comparison was found to support the proposed identifications, and can be found in the Electronic Supplementary Information as Supplementary Table S1.

Upon examination of fragments stemming from cleavages in the main ring structure of the two sterols, it can be seen that the fragments corresponding to cleavage of the position 9–10 bond combined with either of the 5–6 or 6–7 bonds in the Δ7 sterols are both present, whereas neither of the corresponding fragments for the Δ5 isomer are observed. Sitosterol and stigmasterol can be easily distinguished by the presence of a fragment at *m/z* 255.211, attributed to the loss of the side chain though a remote hydrogen rearrangement reaction that is possible in stigmasterol but not in β-sitosterol (Figure 2).

The triterpenoids associated with the 409.383 peak are known to have very similar fragmentation patterns [28–30], and have all been previously observed in cucumbers [31]. However, the lack of a strong peak at *m/z* 191, as well as the equal abundance of the peaks at 257 and 259, indicate that cycloartenol is not a major constituent [28, 29]. The ion at *m/z* 219.210 is consistent with either the charge-retained ring cleavage of lupeol as annotated in Figure 2c, or a post-protonated fragment of retro-Diels-Alder decyclization at the 12=13 double bond in amyrin (not shown). The latter would be expected to be accompanied by a signal from the charge-retained fragment of *m/z* 191.180 ($C_{14}H_{23}^{\bullet+}$), which is not observed. Based on these data, lupeol is assumed to be the principal isomer in the fermented cucumber sample and *m/z* 409.383 is annotated as such in all figures and diagrams.

Mass Spectrometry Imaging

Images of spatial distribution of each investigated species in the full MS dataset are shown in Figure 3. Also included in the Electronic Supplementary Information as Supplementary Figure S2 is an image of the abundance ratio of β-sitosterol to stigmasterol, showing near uniformity over the whole tissue. Triterpenoids, including sterols, are well known for their role in controlling the fluidity and permeability of cell membranes [32–34], and it has been demonstrated that the ratio of sterols to phospholipids in plant cells can vary between tissue type and age [35, 36]. It is also established that some plants respond to environmental stress such as temperature change or salinity by adjusting the sterol composition to control membrane properties [36–38]. The ability to quickly and accurately determine lipid ratios directly from the tissue without sample preparation steps could see various applications in the fields of plant pathology and nutrition.

In Figure 3, it can be seen that sterol content is roughly constant throughout the cucumber, whereas the level of lupeol and/or isomeric pentacyclic triterpenoids is much higher in the exocarp, likely due to their incorporation in the protective layer of cuticular wax, which is known to contain relatively high levels of triterpenoids [39, 40]. Identically performed imaging of raw cucumber (included in the Electronic Supplementary Material as Supplementary Figure S3) shows the same trends in ion distribution, indicating that the presence of 1 M NaCl and the bacterial fermentation process does not significantly alter the ability of this technique to detect and visualize native triterpenoid lipid distribution.

Conclusions

IR-MALDESI is capable of analyzing biologically important molecules directly from complex biological samples treated with molar concentration of salts in the context of an imaging experiment with minimal sample preparation. As such, it has potential use as a complement to conventional chromatography-based analysis of salt-rich foods, such as fermented vegetables, which presently require laborious preparation and desalting before analysis. An added benefit to IR-MALDESI is the inherent suitability for spatial analysis (imaging), which can provide deeper insight into the function of biological systems. The use of high resolution accurate mass (HRAM) spectrometry allows putative peak assignments based

on observed mass as well as confident assignments using tandem MS without prior separation.

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