



● Interrogation of the Spatial Metabolome of *Ginkgo biloba* with high-resolution MALDI and LDI Mass Spectrometry Imaging

MALDI Imaging was used to unravel distinct spatial metabolomics in leaf cross sections.

Introduction

Ginkgo biloba is the only extant species in the division Ginkgophyte, and so is called a "living fossil". Leaves of the *Ginkgo biloba* are a rich resource for bioactive products for treatment of diseases associated with peripheral circulation, memory dysfunction, etc. Numerous

modern techniques have been used to study *Ginkgo biloba*, trying to profile metabolites to help understand biosynthesis pathways from the ginkgo plant; ex., gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE), GC/LC coupled mass spectrometry (MS), and nuclear magnetic resonance (NMR)[1]. However,

little is known about the distribution of various types of metabolites due to tissue homogenization. There are at least ten basic tissue types and more than 15 cell types[2] that are heterogeneously distributed in the plant. Current plant imaging techniques, such as light or electron microscopy, have been used to study the distribution of chemical

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components in plant tissues, but these approaches require molecular labels and are ineffective at mapping unlabeled compounds.

MALDI imaging has been extensively used for imaging of proteins, peptides, lipids and metabolites with no labeling required[3]. In this study, adapted from our recent article in Plant, Cell and Environment [4], we have used two MALDI platforms to map constituent compounds in *Ginkgo biloba*: the ultrafleXtreme MALDI-TOF/TOF and solariX MRMS. The faster ultrafleXtreme was used as a screening tool to optimize the matrix, detection mass range, spatial resolution, etc. The solariX MRMS offers greater molecular specificity and was used to collect ginkgo leaf images and representative spectra from various ginkgo cross leaf sections. Numerous species including flavonoid aglycones, bi-flavonoids, flavonoid glycosides, biginkgosides, ginkgolides etc. were visualized in ginkgo leaf for

the first time. High mass accuracy and in situ MSMS measurements were used to identify metabolites.

Materials and Methods

Chemicals and plant samples

Formic acid, acetonitrile, trifluoroacetic acid (TFA), water (all LCMS grade) 2,5-dihydroxybenzoic acid (DHB), α -cyano-4-hydroxycinnamic acid (CHCA) and 9-aminoacridine (9-AA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Ginkgo biloba* leaves were collected from the arboretum at the University of Illinois at Urbana-Champaign, USA.

Sample preparation for MALDI imaging

Fresh *Ginkgo biloba* leaves were immediately embedded in 10% gelatin (W/V) solutions. Tissues kept in the molds were transferred to a -80°C freezer for 30 min before sectioning

using a cryostat (Leica, Germany) with deionized water as the adhesive. 16- μ m thick tissues were obtained at -20°C and mounted on indium tin oxide (ITO)-coated glass slides, followed by a 10 min dehydration process using a vacuum desiccator. A Zeiss Axio M2 microscope (Zeiss, Germany) was used to obtain optical images of the sections.

Wet spraying

A home-built matrix application system was used to apply matrix by aerosol[5]. Briefly, 50 mg/mL DHB or 15 mg/mL CHCA dissolved in ACN: Water (0.1% TFA) (7:3, V/V) was applied for the positive ion mode MALDI experiments. For negative ion mode MALDI, 10 mg/mL 9-AA dissolved in methanol: water (9:1, V/V) was applied.

Dry spraying

Additionally, DHB was sublimed using a home-built apparatus.

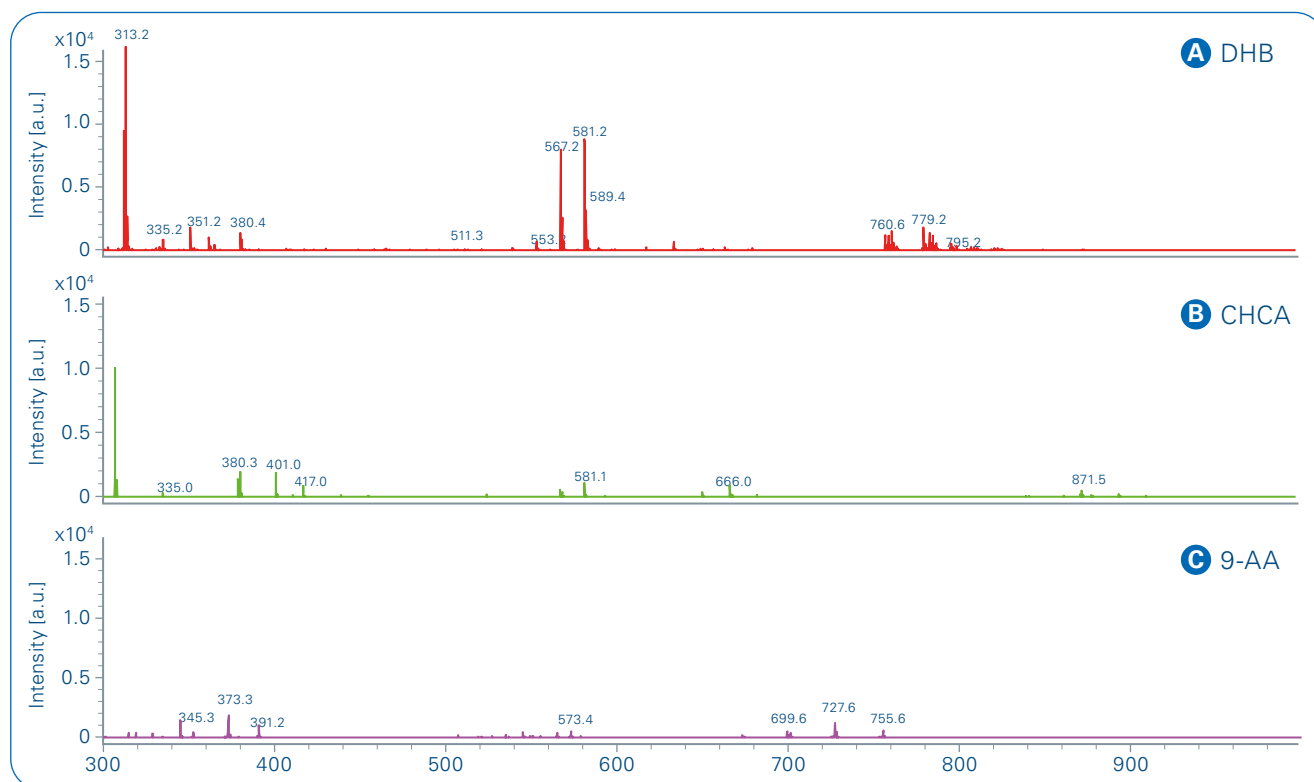


Figure 1: Matrix optimization. Representative single-pixel MALDI-TOF mass spectra acquired from a cross section of ginkgo leaf. (A) Positive ion mode with DHB as matrix. (B) Positive ion mode with CHCA as matrix. (C) Negative ion mode with 9-AA as matrix

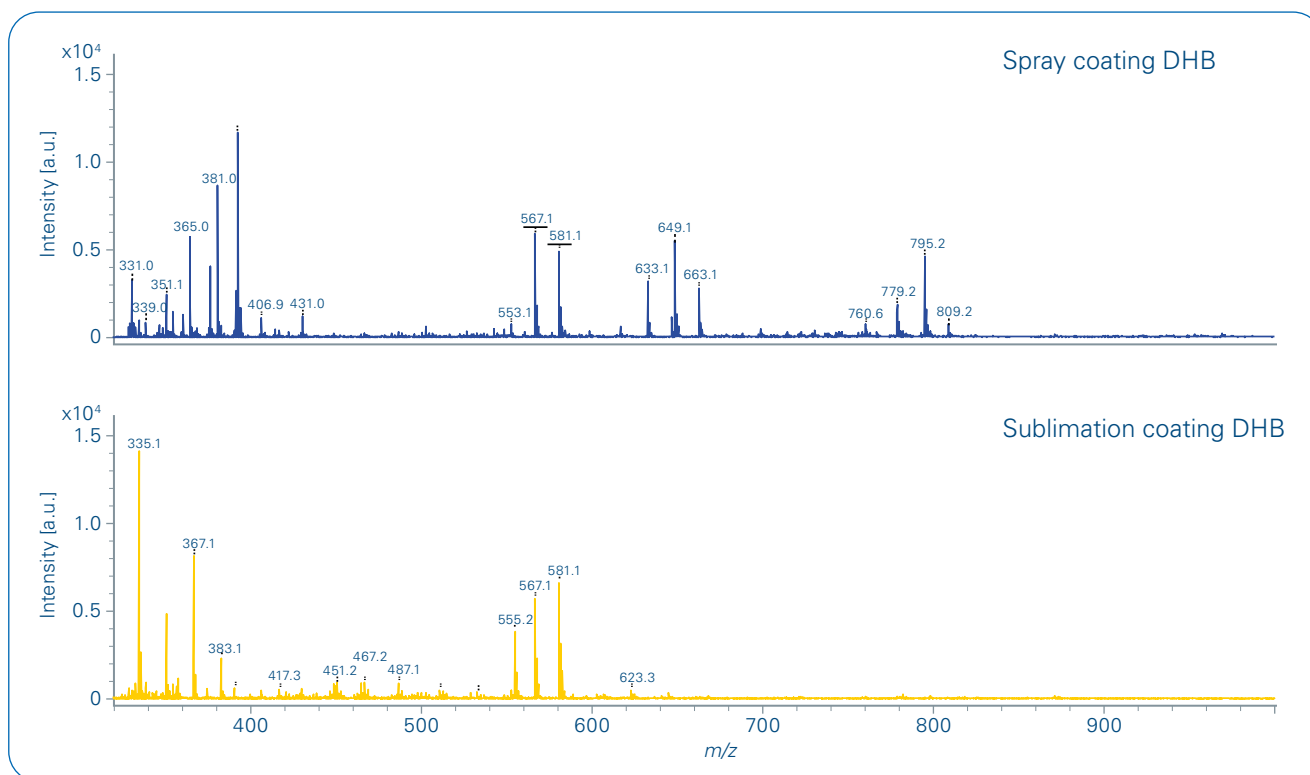


Figure 2: Matrix coating comparison between spray (wet) coating or sublimation (dry) coating

MALDI imaging instrumentation

MALDI imaging was performed using a 7T solariX MRMS mass spectrometer (Bruker Daltonik, Germany) equipped with a dual ESI/MALDI source and a Smartbeam II laser. An m/z range of 150–2000 was acquired with single-scan spectra consisting of 100 accumulated “Small” laser shots at 1 kHz. MALDI images were acquired at a 50 μm pitch.

An ultrafleXtreme MALDI TOF/TOF mass spectrometer was used to optimize the matrix selection and experimental design in MS and MS/MS mode. Data were analyzed using flexAnalysis 3.4, and Data Analysis 4.0. flexImaging 4.1 was used to produce a peak list linked to 2D tissue maps for visualization. (Bruker Daltonik, Germany).

Results

To choose the best matrix to detect metabolites in ginkgo leaves, three

matrices (DHB and CHCA for positive ion mode, and 9-AA for negative ion mode) were tested on tissue using the ultrafleXtreme and wet spraying technique. The total average spectra are shown in Figure 1. Figure 1a spectrum has the most signals, which were identified as biflavonoids, flavonoid glycosides, and lipids (data not shown). Therefore, DHB was selected for analyzing ginkgo leaf sections in positive ion mode.

Next, the two coating systems (wet and dry spraying) were also compared to determine which one produces the optimized signal intensity and most analyte signals, as well as the lowest metabolite delocalization. In Figure 2, more ion signals were detected using the wet spraying system than sublimation with DHB matrix in positive ion mode using the ultrafleXtreme. Most of the ion signals were later identified as biflavonoid-associated ions based on accurate mass using the solariX MALDI-MRMS and/or MALDI TOF/TOF using the

ultrafleXtreme (data not shown). Based on these observations, the wet spraying preparation using the home-built sprayer was used to collect all imaging data from leaf sections.

After the experimental conditions were optimized, the solariX was used to collect single pixel spectra from various leaf cross sections. In addition to delivering much higher mass resolving power, the MRMS ion source produces less interference from matrix ions in the lower molecular mass range. An optical image of one of the *Ginkgo biloba* sections analyzed is shown in Figure 3. Representative spectra in positive and negative ion modes taken from various locations on leaf sections from the upper epidermis, mesophyll and secretory cavity are shown in Figure 4. Compounds were identified based on accurate sub ppm mass accuracy. Flavonoid glycosides (Figure 4a) were easily resolved from the upper epidermis surface in protonated ions

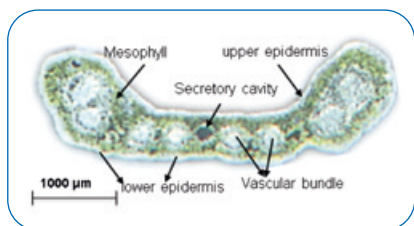


Figure 3: Optical images of *Ginkgo biloba* leaf

as well as in sodium- and potassium-adduct ions. Relatively large molecules were also detected at about m/z 1500 Da from the upper epidermis and these were identified as dimers of flavonoid aglycones in potassium ion form (Figure 4b). LDI was also explored to detect

UV-absorbing flavonoid aglycones in negative mode. Three pairs of flavonoid aglycone peaks were able to be observed from the upper epidermis at ~ 1 Da apart, ex., m/z 284.0331 and 285.0409, m/z 300.0279 and 301.0359, m/z 314.0437 and 315.0517 (Figure 4c). These were

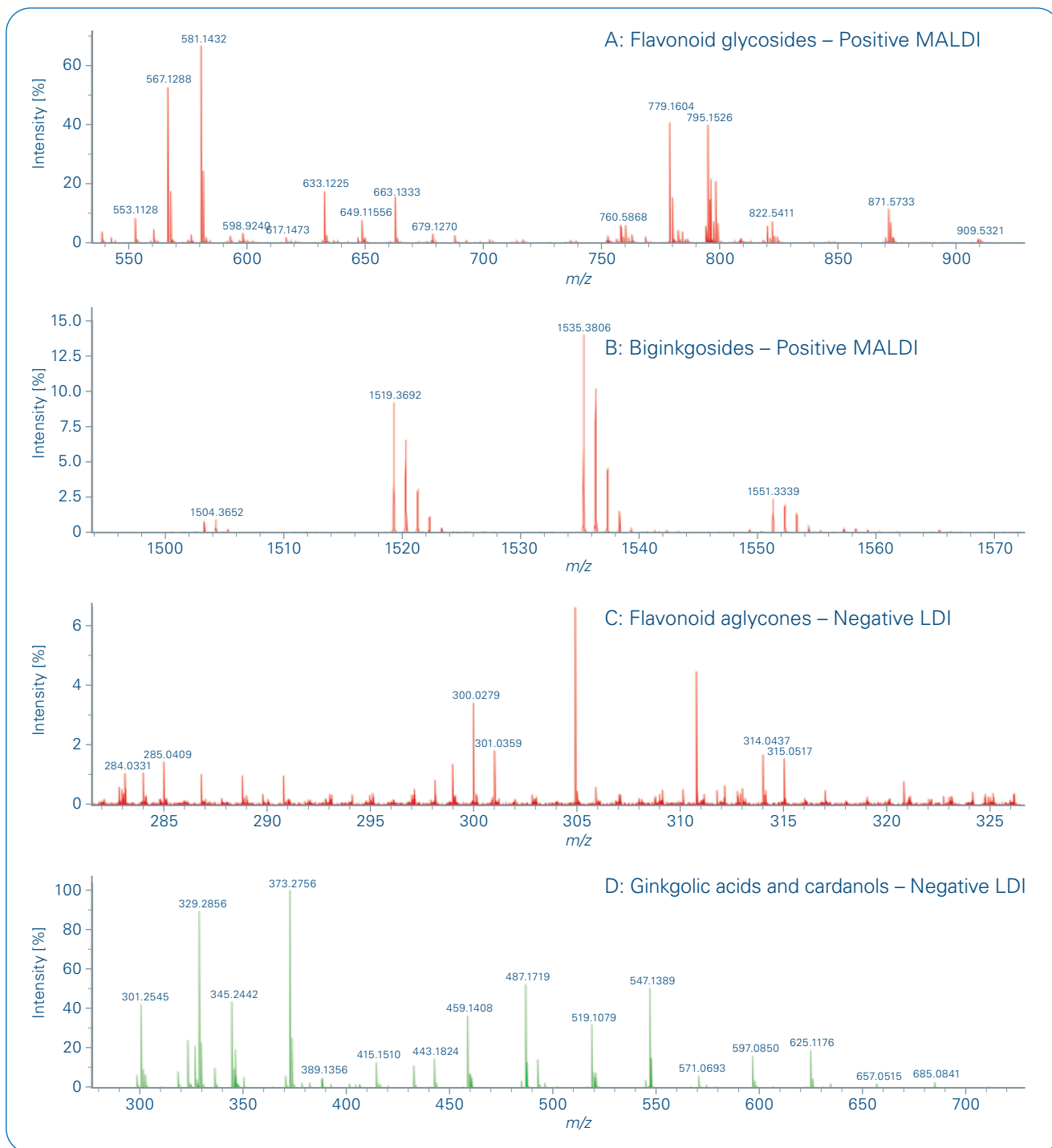


Figure 4: Representative single-pixel MALDI/LDI mass spectra. Identified compounds are labeled with measured mass

identified as deprotonated ions [M-H]⁻ and radical product ions [M-H-H][•] due to the presence of a free 4'-OH group on the B-ring of some aglycones. A negative ion mode LDI mass spectrum of ginkgolic acids and cardanols obtained from the secretory cavity is shown in Figure 4d.

MALDI-TOF imaging technology has been used to reveal heterogeneous

distribution of metabolites in the ginkgo leaf. In Figure 5 several ion images (including flavonoid aglycones, bi-flavonoids, flavonoid glycosides, and biginkgosides) with different localizations were visualized using the flexImaging software. The majority of ions mainly accumulated in the upper and lower epidermis. Notably, flavonoids have only been reported very recently due to their

low abundance in plants. The reason is because typical LCMS needs to homogenize whole samples, and therefore, these compounds are usually not detected due to their low amounts in mixtures. MALDI imaging is well suited for acquiring very localized distributions from plant sections or other materials. Also observed in Figure 5, several ions were found to be localized in higher abundance on the upper epidermis than the lower side, such as *m/z* 1551.34 and *m/z* 1535.35 (biginkgosides). The data is consistent with the heterogeneous distribution of chalcone synthase bioactivity (a key enzyme in flavonoid biosynthesis), which is mainly present in the upper epidermis. Some phosphocholines (PCs) were detected in mesophyll layers and secretory cavities (ex., *m/z* 796.52); these lipid-associated compounds have been reported to act as second messengers in plant cells[6] and were observed mainly in secretory cavities.

In this study, we have demonstrated the use of MALDI imaging technology to visualize metabolites in leaf cross sections.

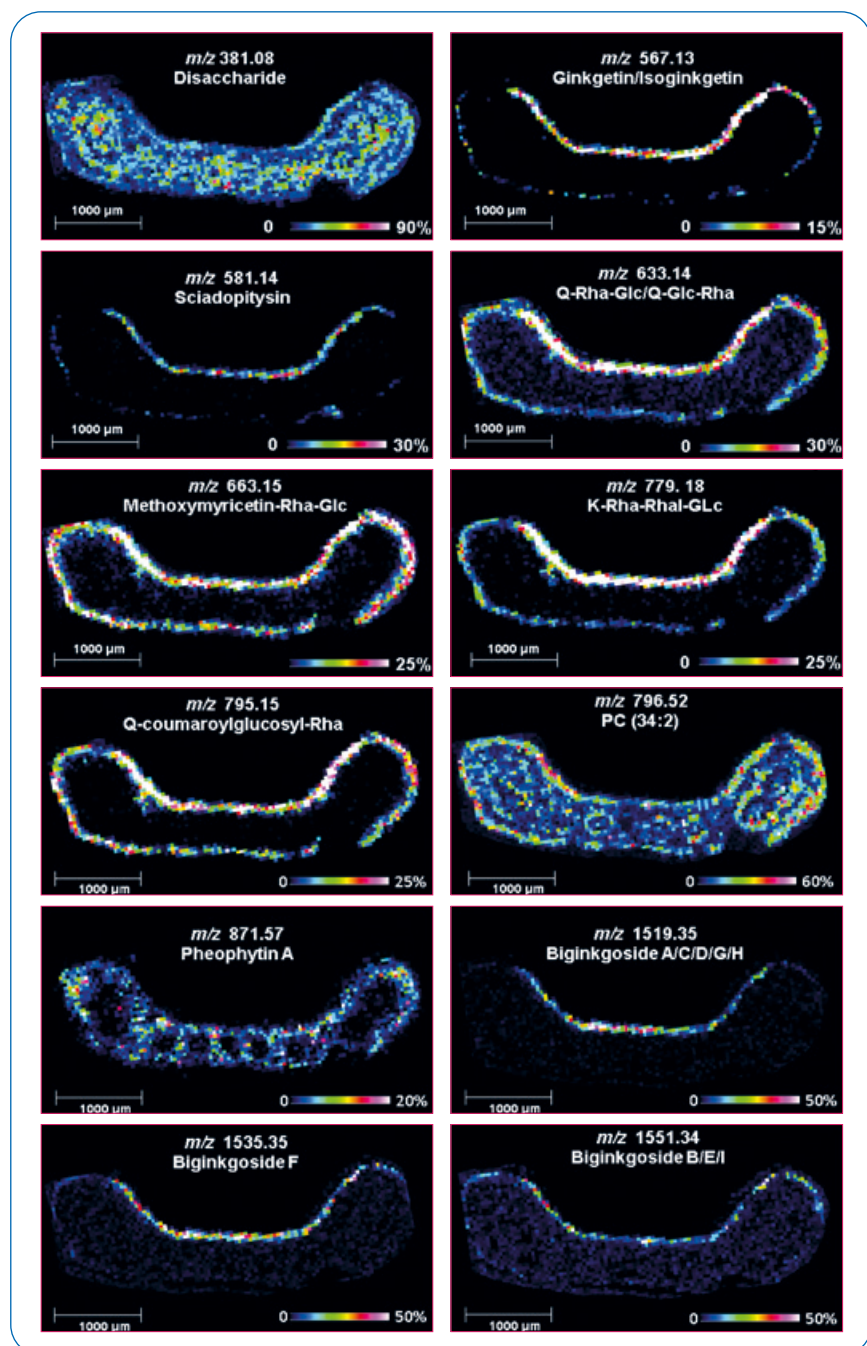


Figure 5: MALDI images of selected ions including flavonoid aglycones, biflavonoid, flavonoid glycosides, etc.

Conclusions

- MALDI Imaging is shown to be a powerful tool to visualize ginkgo leaf metabolites for the first time.
- These ion images could improve the understanding of the distinct functions of individual species, and their biofunctions in plant growth and development, as well as abiotic and biotic stress response.



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