



## ● MALDI-TOF Mass Spectrometry Imaging (MSI) for Distribution Analysis of Anthocyanins in Strawberry Fruits

Imaging mass spectrometry (MSI) via matrix-assisted laser desorption / ionization (MALDI) TOF MS enables the simultaneous detection and localization of molecular species across a broad molecular mass range. In biological samples, diverse classes of biomolecules may be sought, including metabolites, sugars, lipids, peptides, and proteins.

Depending on the sample and the compound class(es) to be targeted in a given MALDI-TOF MSI study, sample preparation schemes (potential necessities of structural reinforcement of delicate samples, thickness of sample slicing, and optional washing steps) may vary, as may

the selection of the most suitable MALDI matrix to enable desorption and ionization of the target classes. Sensitivity and spatial resolution are key elements to successful MSI studies, and matrix application directly influences the quality and quantity of the spectra data obtained.

Uniformity of coverage on the sample surface, matrix crystal size, and "extractability" of analytes depend on many variables, including the physical means of application, the selection of application solvent(s), if used, and application temperature.

*Keywords:*  
Food analysis, mass spectrometry imaging, MALDI-TOF MS

The utility of MSI in food analyses has been demonstrated in the identification and quantitation of metabolites, proteins, and pesticides in various food matrices, including fruits and vegetables [1]. In this study, the distribution of anthocyanin species in strawberries (*Fragaria x ananassa*) was characterized by MSI. Anthocyanins are polyphenolic compounds present in strawberries, as in other fruits and vegetables naturally red, purple, or blue toned at maturity. Beyond providing vivid color, anthocyanins have shown to have antioxidative, anti-inflammatory, and antitumor activities [2-4], making this class of compounds of interest for nutraceutical, skin care, and drug research and development. Pelargonidin and cyanidin derivatives are the most representative anthocyanins in strawberries, but this class of compounds includes more than 500 entities [2]. The identification, distribution, and relative quantitation of anthocyanins can support studies of natural species and the development of genetically modified varieties of such fruits for the monitoring and development of desirable anthocyanin content characteristics. In addition to "core" profiling via analysis of longitudinal and cross-sectional slices, analysis of surface slices including the strawberry achenes enables a more complete target evaluation and further demonstrates the utility and flexibility of MSI technology.

## Methods and Materials

### Preliminary Analysis: Preparation and Profiling of Strawberry Extracts

"Tochiotome" strawberry (*Fragaria x ananassa* Duch.) fruits were cultivated in the Strawberry Research Center (Tochigi, Japan), harvested in winter after ripening, and stored at -80°C until use.

Strawberry fruit extracts were prepared by freeze drying and

Table 1: Anthocyanins identified in this study

Precursor ion, (m/z) [M] <sup>+</sup>	Fragment ion, (m/z) [M] <sup>+</sup>	Anthocyanin species
433.1	271	Pelargonidin-3-O-glucoside
449.1	287	Cyanidin-3-O-glucoside
479.1	303	Delphinidin-3-O-glycoside
519.1	271	Pelargonidin-3-O-malonylglucoside
535.1	287	Cyanidin-3-O-malonylglucoside
579.1	271	Pelargonidin-3-O-rutinoside

then homogenized. Homogenates were suspended in 50% methanol (Sigma-Aldrich, St. Louis, MO, USA) and kept under continuous shaking at room temperature for 2 hours. The solutions were centrifuged at 3000 rpm for 15 minutes, and the supernatants were collected as extracts for analysis.

Strawberry extracts (1 µL) were mixed with an equal volume of 40 mg/mL DHB (2,5-Dihydroxybenzoic acid, in 70% aqueous methanol containing 0.1% FA (formic acid). The mixed solution was deposited onto an indium

tin oxide (ITO)-coated glass slide (Bruker Daltonics, Billerica, MA, USA) and dried before MALDI-MS analysis. MALDI-MS and MS/MS analyses were performed using an ultrafleXtreme MALDI time-of-flight (TOF)/TOF MS system (Bruker Daltonics). MS data was collected in positive-ion reflector mode across a m/z range of 100-600 at a repetition rate of 1000 Hz and was externally calibrated. For MS/MS analysis, the selected precursor ions and the product ions were obtained using collision-induced dissociation MS/MS mode.

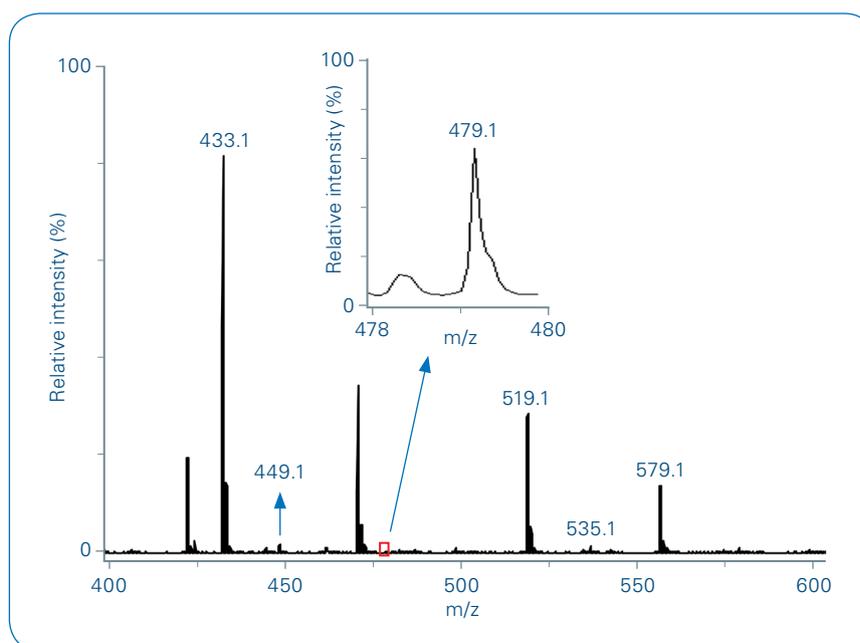


Figure 1: Mass spectrum obtained from crude strawberry extract using MALDI-MS

### Sample Preparation of Strawberry Sections for MSI

Fresh mature fruits were immersed in 2% CMC (carboxymethyl cellulose sodium salt, Wako Chemicals) and flash frozen in liquid nitrogen. Subsequently, longitudinal and cross sections, 80  $\mu\text{m}$  thick, were consecutively prepared using a CM 1860 cryostat (Leica Microsystems, Wetzlar, Germany). Sections including achenes, the small shells enclosing

the seeds on the surface of the fruit, were likewise prepared. The sections were then mounted onto ITO-coated glass slides, which were dried in a vacuum desiccator for 20 minutes. The mounted sections were placed in 50 mL conical centrifuge tubes containing silica gel to dry and were stored (within these tubes) at  $-80^\circ\text{C}$  until analysis.

MALDI matrix was applied to the strawberry sections using a

TM-Sprayer system (HTX Technologies LLC, Carrboro, NC, USA). A DHB solution, 30 mg/mL in 50% aqueous methanol containing 0.1% FA, was used. The solvent flow rate, nozzle speed, number of passes, and temperature were set at 0.2 mL/min, 1200 mm/min, 81 and 85  $^\circ\text{C}$ , respectively.

MSI was performed according to a previous study [5] with some modifications. Data was acquired on the Bruker ultrafleXtreme MALDI time-of-flight (TOF)/TOF MS with a step size of 200  $\mu\text{m}$ . The laser diameter was set to medium size. Other measurement conditions were as previously described.

### Data Analysis

For the profiling of the strawberry fruit extracts, the obtained MS and MS/MS spectra were analyzed using flexAnalysis 3.4 software (Bruker Daltonics). The sugar moieties of anthocyanin species were identified from their neutral losses and previous reports [6].

For the MSI studies, the acquired mass spectra were normalized by total ion current (TIC) using flexImaging 4.0 software (Bruker Daltonics). The software was also used to create two-dimensional ion-density maps.

## Results and Discussion

The mass spectrum of the ripe strawberry extract is shown in Figure 1. The anthocyanins in the crude extract were identified by MALDI-MS/MS, with six unique species clearly detected. Based on previously analyses [6], a number of anthocyanin precursor ions were expected, including both anthocyanidin and sugar moieties. The detected anthocyanins are shown in Table 1, including delphinidin-3-O-glycoside, identified in strawberries for the first

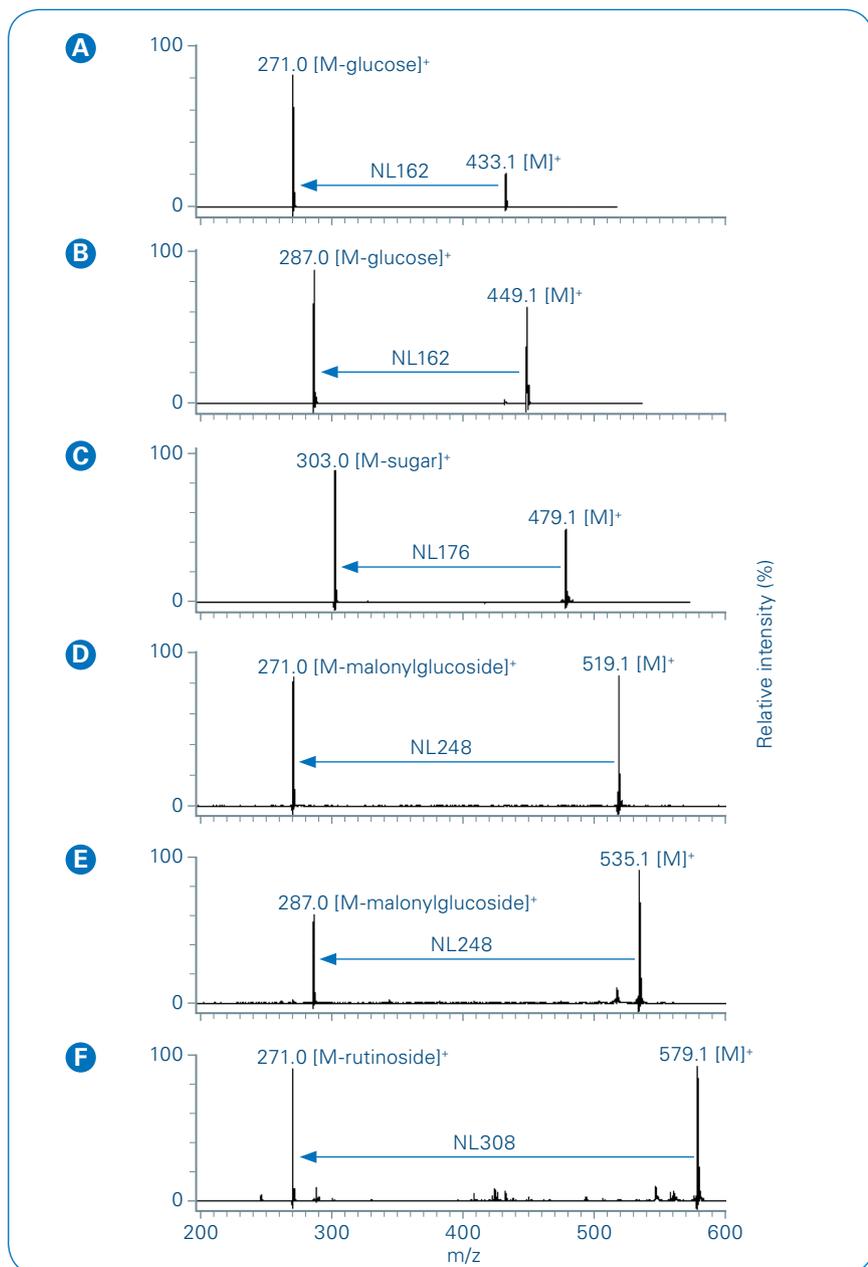


Figure 2: MS/MS spectra of identified anthocyanins in strawberry crude extract using MALDI-MS/MS. MS/MS spectra of precursor ions at  $m/z$  **A** 433.1, **B** 449.1, **C** 479.1, **D** 519.1, **E** 535.1, and **F** 579.1

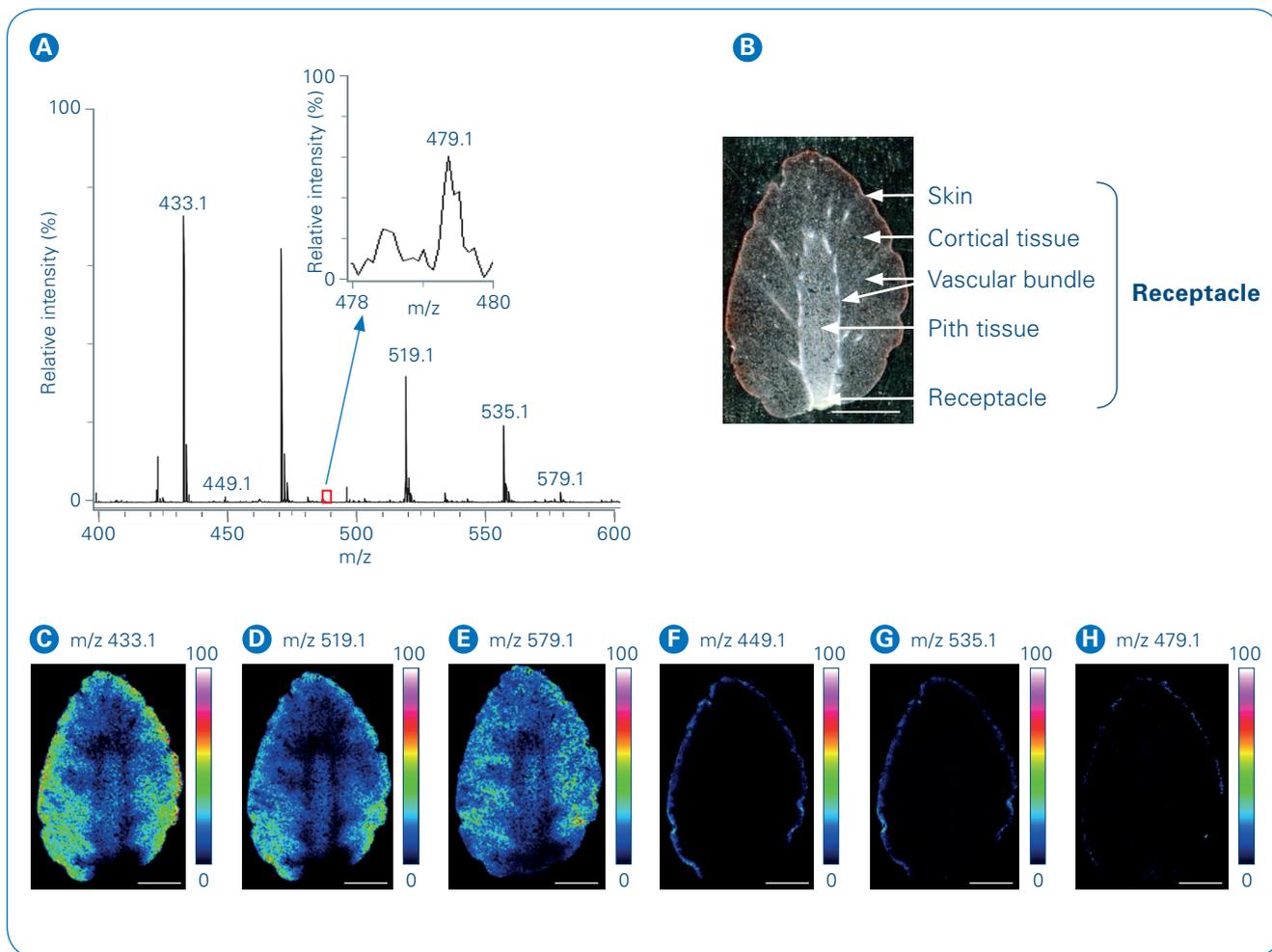


Figure 3: MALDI-MSI analysis of anthocyanins in strawberry sections. **A** Mass spectrum obtained from a section. **B** Optical image of a strawberry section. **C** - **H** Representative ion images of  $m/z$  433.1, 519.1, 579.1, 449.1, 535.1, and 479.1. Scale bar = 5 mm

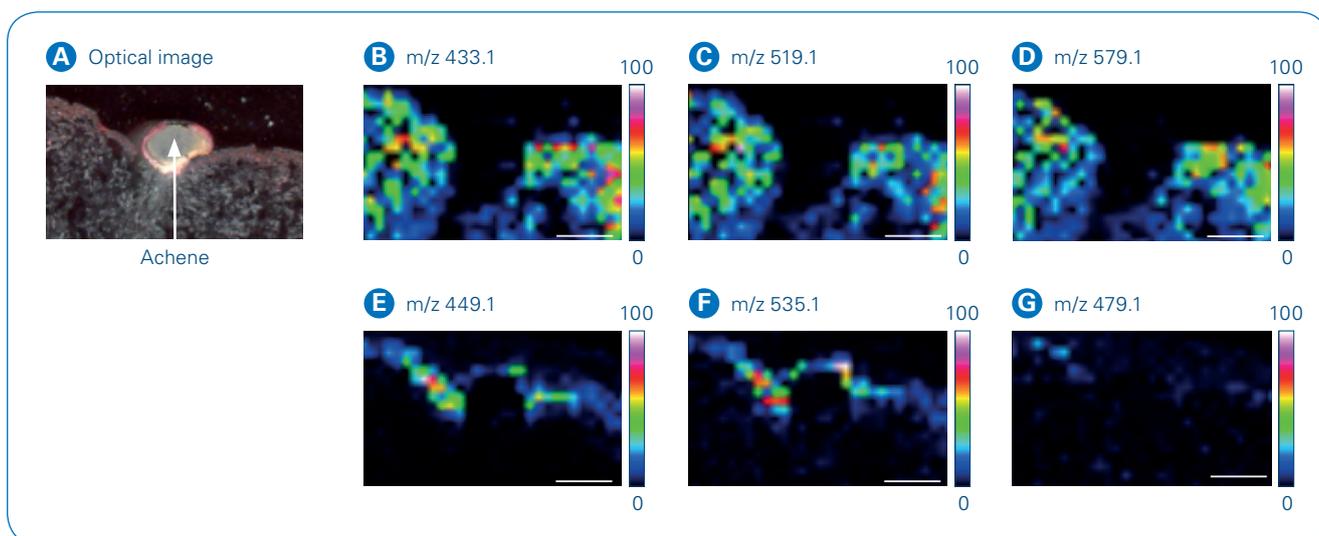


Figure 4: MALDI-MSI analysis of anthocyanins in an achene. **A** Optical image of an achene section. **B** - **G** Representative ion images of  $m/z$  433.1, 519.1, 579.1, 449.1, 535.1, and 479.1. Scale bar = 1 mm

time. The MS/MS spectra, shown in Figure 2, indicate the neutral loss of sugars in each of the anthocyanins by the detection of their aglycone forms.

All peaks corresponding to the anthocyanins identified in the crude extract (Figure 1) were also observed in the spectra from the MSI data collected on strawberry slices (Figure 3A). An optical image of a longitudinal cross section of a strawberry is shown in Figure 3B. Red pigment is present in the skin, cortical, and pith tissues

of the fruit, indicating the presence of anthocyanins, and the detected anthocyanin species showed different distribution patterns within the sections. Pelargonidin species were distributed in the skin, cortical, and pith tissues (Figure 3C-E), whereas cyanidin and delphinidin species were localized in the skin (Figure 3F-H). Their aglycones showed similar distribution patterns to these glycosides (data not shown).

An optical image of an achene section is shown in Figure 4A. As

shown in Figure 4B-F, pelargonidin glycosides and cyanidin glycosides were detected in the (surface) fruit skin, while delphinidin glycoside was only slightly detected (Figure 4G). Cyanidin species were also notably detected on the outer surface of the achene, which is of interest given previous studies indicating that the antioxidant capacity of the achene is considerably higher than that of the strawberry receptacle flesh [7].

## Conclusion

- In this study, anthocyanin compounds were detected and localized within "Tochiotome" strawberry fruits slices via MALDI-TOF MSI on a Bruker ultraflextreme MS system. Embedding the fresh fruit in CMC prior to freezing and slicing maintained the form of the delicate flesh. Matrix application using the HTX Technologies TM-Sprayer provided a uniform and reproducible distribution of the DHB matrix solution, and the use of elevated temperature (85°C) promoted extraction of targeted analytes from the strawberry slices. A preliminary study of extracts prepared from the strawberries confirmed the presence and identities of the anthocyanins. These anthocyanins include delphinidin-3-O-glycoside, identified in strawberries for the first time.
- MSI techniques, such as those made by MALDI-TOF MS, can rapidly provide a unique depth and breadth of molecular spatial distribution information within a sample. This analytical approach can provide significant detail on target compound production during fruit maturation, under different growth conditions, and in varietal selection screens. Knowledge of the expression and localization of these compounds of interest may direct the development of "healthier" fruits for direct consumer consumption and to improve yields in the preparation of anthocyanin-rich extracts. Further, this analytical approach may be applied to help assess the absorption, distribution, and metabolization of anthocyanin compounds – with both topical and oral administration – as their potential benefits for health and well-being are better understood.



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