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Application Note

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Plants

Visualization of Flavonoids in Viola Flower Petals Using Mass Spectrometry Imaging (MSI)

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Keywords: Mass spectrometry imaging, IMScope *TRIO*, flavonoids, plants, petals, viola

Plants

Abstract

Mass spectrometry imaging (MSI) has attracted considerable interest as a technology for visualizing the localization of compounds in biospecimens. MSI enables label-free analysis of the localization of multiple analytes from the same section, and also has the potential for discovery of compounds which exist in a section even in trace amounts if the compounds have accumulated locally. For these reasons, major breakthroughs are expected by efficiently searching for functional substances from biospecimens using localization information as indicators (biomarkers).

As advantages of the Shimadzu iMScope *TRIO* imaging mass microscope, the laser irradiation position can be determined accurately from the optical image obtained by microscopic observation, and the effect of fine irregularities of the sample surface, like those on flower petals, is minimal due to the ion trap feature. The ion trap also enables accurate selection of the precursor ion in MS/MS analysis.

This Application Note introduces an example of MSI of the flavonoids that coexist locally with the known plant pigment anthocyanin, using viola flower petals as samples.

1. Introduction

The color of flowers is expressed by the accumulation of pigments in the cells of the flower petals. The color also changes greatly depending on the type and amount of pigments and the environment in which the pigment components are placed. Anthocyanin is known as a plant pigment which is found in roses and other flowers, and displays a red color under acidic environments and a blue color under alkali environments. However, it is also known that the pH in the petal cells of many blue flowers is weakly acidic, suggesting that a special mechanism which supports the blue color development of anthocyanin exists in the cells of blue flowers. Against this backdrop, the blue coloring of petals associated with anthocyanin has long been a subject of research.

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With progress in biochemical techniques and instrumental analysis technology in recent years, it has become possible to elucidate the mechanism of the blue color development of flower petals by anthocyanin using various approaches. For example, research has shown that the indigo plant (Fig. 1, left) has a blue hue because anthocyanin and metals form complexes in the petals [1].

Nevertheless, the reason for the blue color of the viola (Fig. 1, right; a type of violet), which is the subject of the present research, was not understood in detail. Therefore, the authors undertook research on the mechanism responsible for the blue coloring of the viola. As this research progressed, it became clear that the viola develops a blue color due to the coexistence of anthocyanin and flavonoids, but because this flower has a pattern, the connection between the blue pattern and the substances will be lost if an entire petal is extracted and its contents are analyzed. To solve this problem, an iMScope *TRIO* imaging mass microscope (Fig. 2) was used to search for flavonoids that show colocalization with anthocyanin on the flower petals.

Indigo plant | Formation of anthocyanin-metal complex | Viola (one type of violet)

Fig. 1 Examples of Mechanism of Blue Coloring in Flower Petal

2. Experiment

2-1. MSI of Flavonoids

As shown in Fig. 3, viola petals were fixed on an ITO slide glass via electrically conductive double-sided adhesive tape and then mounted in the dedicated sample holder as an analysis sample. The sample was observed with the built-in optical microscope of the iMScope *TRIO*, and optical images were acquired by the analytical software (Imaging MS Solution™). The sample holder was then removed once, and the petals were spray-coated with 500 µL/petal of a 2,5-dihydroxybenzoic acid (DHB) adjusted to a concentration of 50 mg/mL in methanol/water = 70/30 (v/v). This DHB matrix was deposited by using an airbrush for crafted models.

Since Imaging MS Solution is equipped with a position-matching function for overlaying mass spectrometry (MS) images on the optical images acquired with the microscope, the measurement can be started immediately after setting the sample without setting parameters for sample height adjustment or positional alignment. The analysis region was specified on the image acquired using Imaging MS Solution, and MSI was conducted under atmospheric pressure using the analysis conditions shown in Table 1.

With iMScope, the acquisition region can be set arbitrarily based on the optical microscope image, and the analysis can be carried out at a rate of 170 ms per 1 pixel. Thus, assuming this sample (4355 pixels) is to be analyzed with spatial resolution of 150 µm, the analysis time is only 12 min approximately. The purpose of this experiment was to detect glycosides of quercetin and myricetin, and also violanin, which is an anthocyanin pigment, by measurement in the negative ion mode.

Fig. 2 iMScope *TRIO* Used in Analysis

Fig. 3 Samples Mounted in Dedicated Holder (ITO Slide Glass)

Table 1 Conditions of MSI Analysis

Acquisition pitch (spatial resolution)	: 150 µm
Number of pixels	: 4355 (65 × 67) pixels
Ion type	: Negative ion mode
MS range	: *m/z* 500 - 1000
Measurement time	: 12 min (approx.)

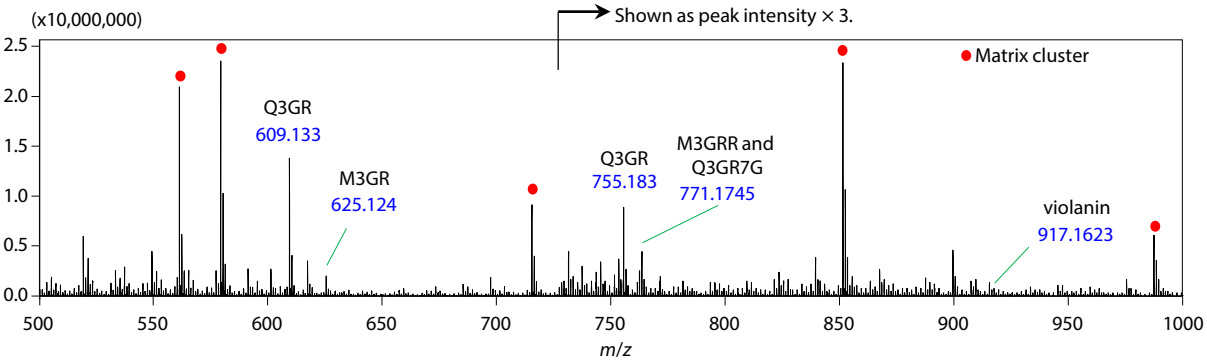


Fig. 4 Average Mass Spectrum of Analysis Region

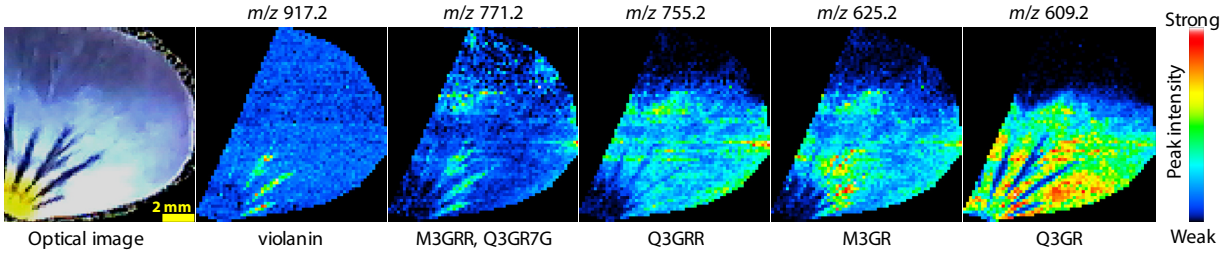


Fig. 5 MS Image of Flavonoids Contained in Viola Petal (Bar: 2 mm)

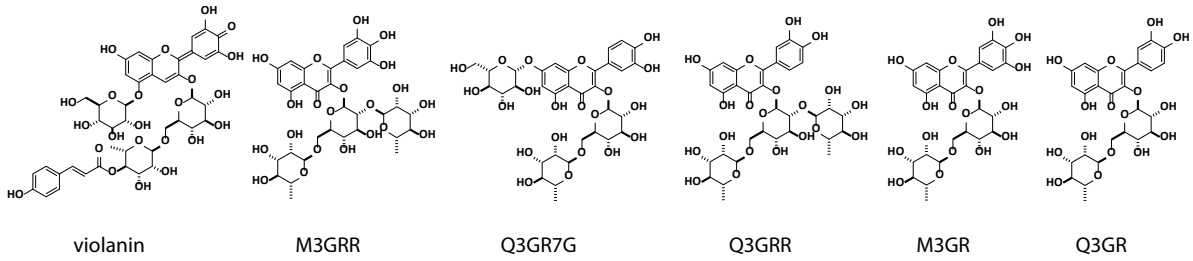


Fig. 6 Structures of Flavonoids Contained in Viola [2]
(Q: quercetin, M: myricetin, G: *O*-*β*-D-glucopyranose, R: *O*-*α*-L-rhamnopyranose, 3: 3-*O*-glycoside, 7: 7-*O*-glycoside)

Fig. 4 shows the average mass spectrum of the analysis region. Fig. 5 show the MS images for the peaks at *m/z* 917.2, 771.2, 755.2, 625.2, and 609.2, which correspond to the main flavonoids contained in the viola flower. Fig. 6 shows the abbreviations and structures of the flavonoids used in Fig. 4 and Fig. 5.

It was found that the types of flavonoids contained in the petals differ corresponding to the petal color, as in the MS images in Fig. 5. In the optical image on the left, whisker-shaped dark blue areas called nectar guides can be seen near the base of the petal, and the anthocyanin pigment violanin was detected in these areas. It is thought that M3GR and a substance corresponding to *m/z* 771.2 are colocalized in areas where violanin exists. On the other hand, the quercetin glycosides called Q3GR and Q3GRR were heavily distributed in the light-colored parts of the petal. As this example shows, MSI is extremely effective in establishing the relationship between the petal pattern and the substances contained in a petal.

2-2. Confirmation of Molecules by MS^2 and MS^3

Excluding the matrix cluster, the peak with the highest intensity was *m/z* 609.2. Because this peak was identified as Q3GR (Quercetin-3-(*β*-D-glucopyranoside-6″-*O*-*α*-L-rhamnopyranosyl)) shown in Fig. 6, the areas that were expected to have large contents of Q3GR from the MS image for *m/z* 609.2 were measured by MS^2 and MS^3. As the precursor ions, *m/z* 609.2 was selected for MS^2 and *m/z* 301.0 was selected for MS^3. Fig. 7 and Fig. 8 show the mass spectra corresponding to MS^2 and MS^3, respectively. The assignment of *m/z* 609.2 to Q3GR was confirmed by the measured fragment peaks as shown in Fig. 9.

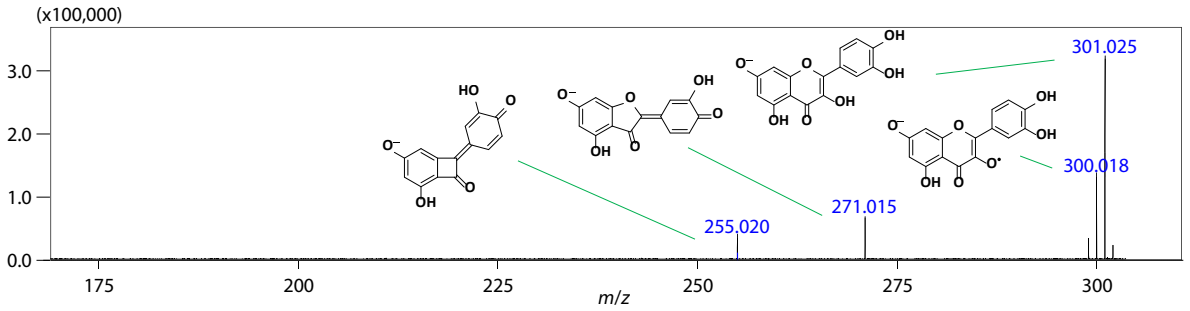


Fig. 7 Mass Spectrum for MS^2 (Precursor Ion; *m/z* 609.2)

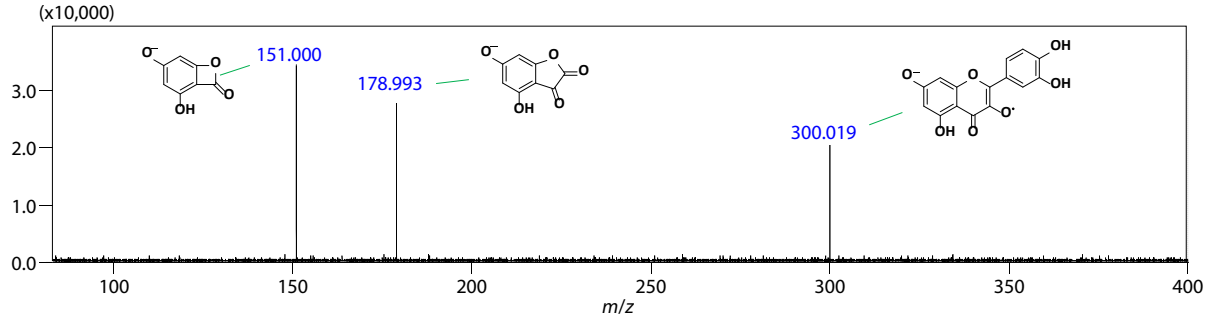


Fig. 8 Mass Spectrum for MS^3 (Precursor Ion; *m/z* 301.0 < 609.2)

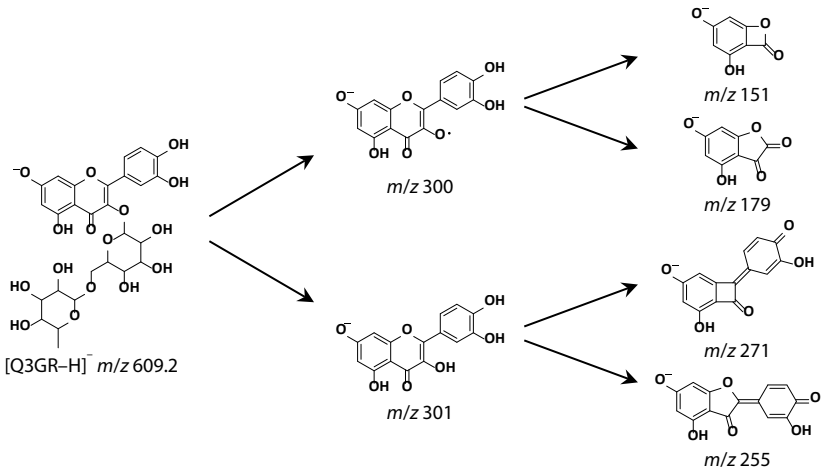


Fig. 9 Predicted Structures of Product Ions Formed from Q3GR [3]

Next, in an MS^2 measurement using the *m/z* 771.2 observed at the nectar guide portion (Fig. 5, optical image) as the precursor ion, product ions originating from at least two kinds of compounds were observed at *m/z* 301 and *m/z* 316, as shown in Fig. 10. Therefore, the molecular structures of these compounds were analyzed.

The peaks at *m/z* 609 and *m/z* 301 in Fig. 10 were assigned respectively to $[Q3GR\text{-}H]^-$ and $[Quercetin\text{-}H]^-$, in which sugar is detached from $[Q3GR\text{-}H]^-$, as shown in Fig. 11. Based on this information, one of the molecules corresponding to *m/z* 771.2 was considered to be Q3GR7G.

On the other hand, it was thought that the peak of *m/z* 316 is $[myricetin\text{-}H]^-$, which is formed by detachment of one hexose molecule and two deoxyhexose molecules from the compound corresponding to *m/z* 771.2, and the two peaks at *m/z* 287 and *m/z* 271 which were observed at the same time are ions formed by further detachment from $[myricetin\text{-}H]^-$. These peaks were actually observed by MS^3 measurement using *m/z* 316.0 as the precursor ion (Figs. 12, 13). Based on these results, it was found that the two compounds Q3GR7G and M3GRR exist as flavonoids corresponding to the *m/z* 771.2 peak of the nectar guides of the viola petal.

Here, focusing on the intensity ratio of *m/z* 301 and *m/z* 316, which are the product ions formed from Q3GR7G and M3GRR in Fig. 10, it can be understood that the peak originating from M3GRR is larger than that originating from Q3GR7G. Assuming that the ionization efficiency of these two compounds is equal, it can be thought that the blue portion of the petal has a large amount of M3GRR. In other words, M3GRR and violanin, which are the anthocyanin pigments, are colocalized in the blue portion of the petal. Accordingly, it was suggested that the coexistence of M3GRR with violanin assists the blue color development in the petals.

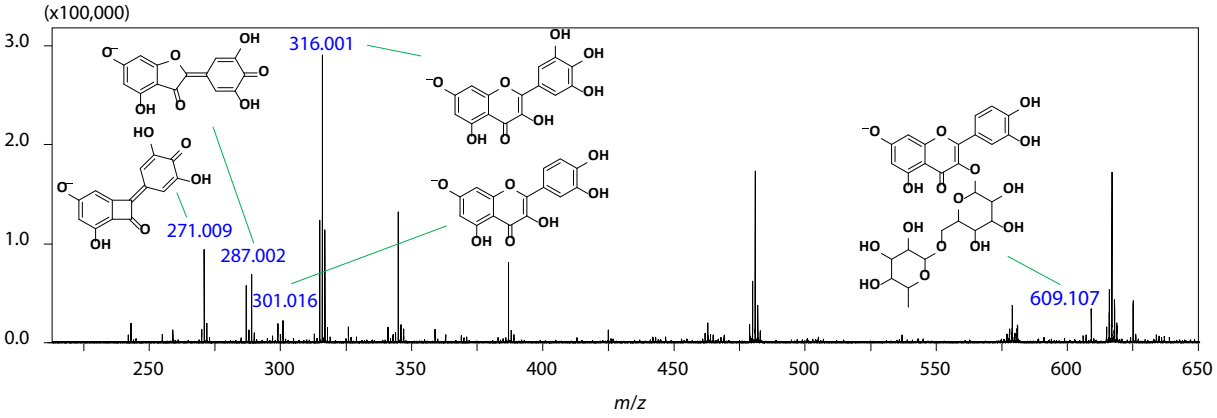


Fig. 10 Mass Spectrum for MS^2 (Precursor Ion; *m/z* 771.2)

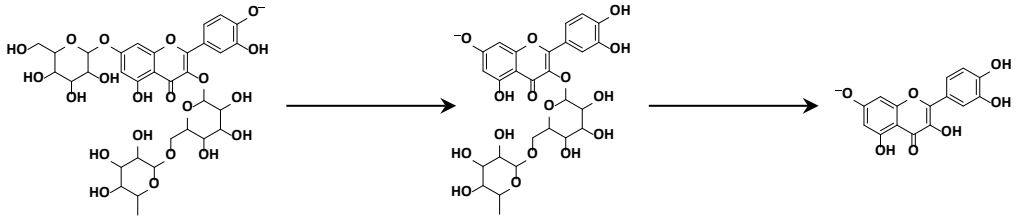


Fig. 11 Predicted Structures of Formed Product Ions by MS^2 Measurement for Q3GR7G

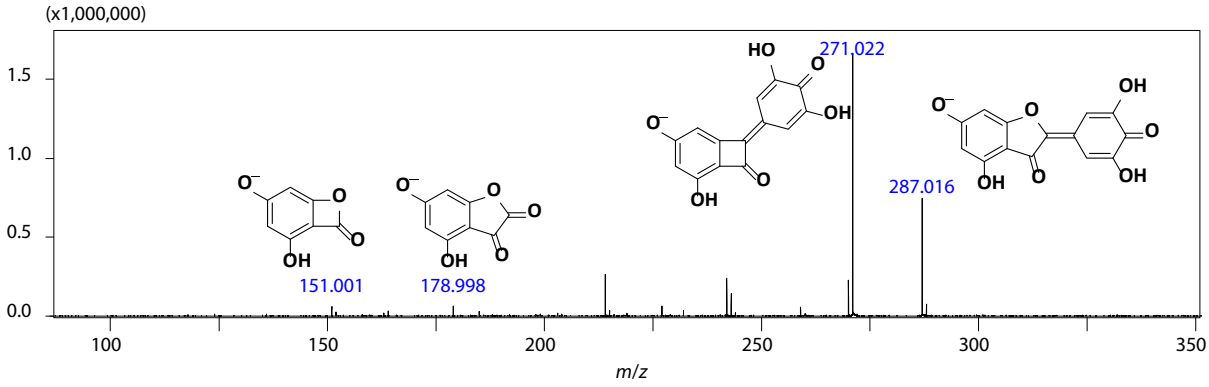


Fig. 12 Mass Spectrum for MS³ (Precursor Ion; *m/z* 316.0 < 771.2)

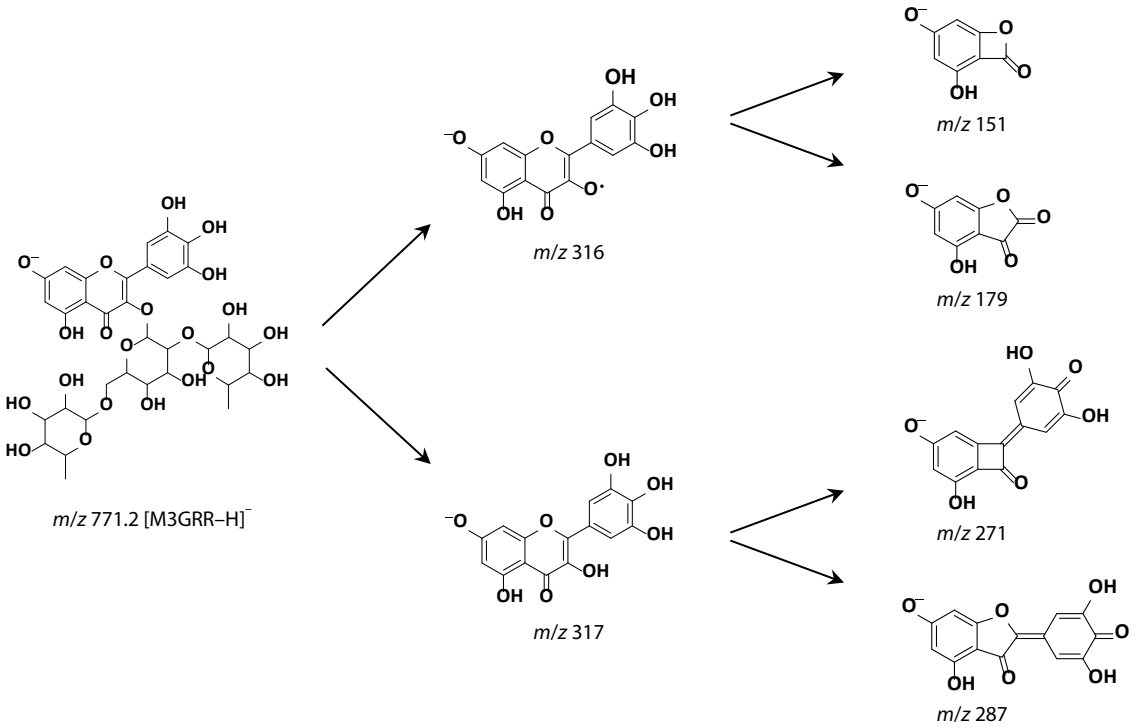


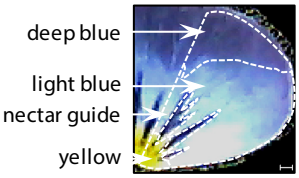
Fig. 13 Predicted Structures of Product Ions Formed from M3GRR

2-3. Quantitative MSI Experiment Combining Quantitative Analysis by MSI and LCMS

The distribution of compounds obtained by MSI is shown by the MS peak intensities of the substances of interest. However, in functional research of biological materials, quantitative information such as "How much is the substance of interest contained in a designated region" is important. In recent years, methodologies for expressing the MS peak intensity by the amount of a substance have been studied intensively with the aim of bridging this gap. This section presents an example in which the distribution of Q3GR, which is present in large amounts in the viola flower, was calculated quantitatively for regions of the petal with different colors by using a technique combining MSI and LC-MS quantitative analyses [4].

1. Quantitative analysis by LC-MS
Weight of compound in tissue (W_t)

2. MSI
Ion content in total measurement region (I_t)
Ion intensity in region of interest (I_i)



3. Calculation of substance amount (W_i) in region of interest using the following formula:

$$W_i = \frac{I_i}{I_t} W_t$$

Fig. 14 Schematic Explanation of Quantitative MSI [2]

Fig. 14 shows a schematic explanation of the quantitative MSI conducted here. As samples, two sets of petals 2 weeks after the flower had opened were prepared. For one sample, the amount of flavonoids (W_t in Fig. 14-1) contained in half of the petal was obtained by a quantitative LC-MS experiment. Next a MSI experiment was carried out using the other petal sample. In this experiment, four regions of interest (ROI), i.e., deep blue, light blue, nectar guide, and yellow, are defined according to the color of the viola flower petals, and the ion amount of the flavonoids in each ROI and the ion amount of the total measurement region (I_i and I_t, respectively, in Fig. 14-2) are obtained from the MSI data. The amounts of flavonoids contained in the ROIs (W_i) are proportional to the total amount of flavonoids measured by the quantitative LC-MS, as shown by the formula in Fig. 14-3.

In this study, the LC-MS experiment for quantitative analysis of the Q3GR contained in half of the petal was conducted under the conditions shown in Table 2 and Table 3 using an LCMS™-8030plus triple quadrupole mass spectrometer.

Fig. 15 shows the petal and the four ROIs defined for the MSI measurement. The white bar graphs show the amount of Q3GR contained in each ROI. From the MS image in Fig. 5, it was found that the light blue portion of the petal contains a large amount of Q3GR, but use of this information in combination with the quantitative analysis showed that the light blue ROI of 1 petal contains 163 nmol, i.e., 100 µg of Q3GR. While the information in Fig. 5 indicated qualitatively that the Q3GR amount of the nectar guides is small in comparison with that of the light blue region, quantitation revealed that the nectar guides also contain 16 nmol of Q3GR.

We also attempted to evaluate the reliability of the results of quantitative MSI obtained as described above. For this, a quantitative LC-MS analysis was conducted by cutting out portions of fresh flower petals similar to the ROIs defined in the MSI experiment, as shown in Fig. 16, and calculating the Q3GR amount in each ROI (black bar graphs in Fig. 15). A good correlation was found between the quantitative values obtained by the quantitative MSI and the quantitative values of the LC-MS where live tissue parts were cut out. Taking into account the individual differences between petals, these are considered to be satisfactory results.

Table 2 Analysis Conditions of LCMS-8030plus

Column	: Cosmosil 5C18-AR-II 2.0 × 150 mm
Mobile phase A	: 0.1 % Formic acid-Water
Mobile phase B	: 0.1 % Formic acid-Acetonitrile
Flow rate	: 0.15 mL/min
Column temp.	: 40 °C
Injection amount	: 3 µL

Table 3 Gradient Program

Time [min]	B conc [%]
0	10
15	25
20	80
20.1	10
27	Stop

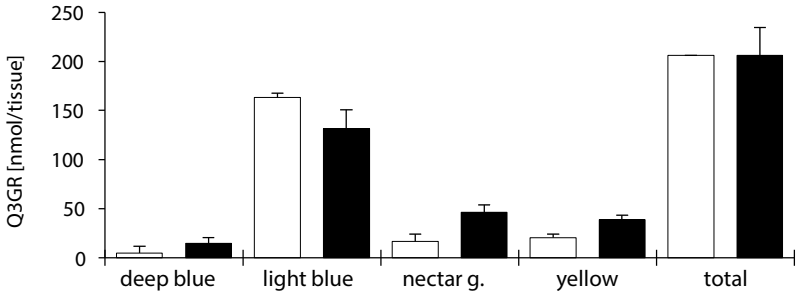


Fig. 15 Amount of Q3GR in ROIs (White bar graphs: amount obtained by quantitative MSI (mean ± SD, N = 3), black bar graph: amount obtained by quantitative LC-MS analysis of samples taken from same ROIs of live flower petal (mean ± SD, N = 5) [2])

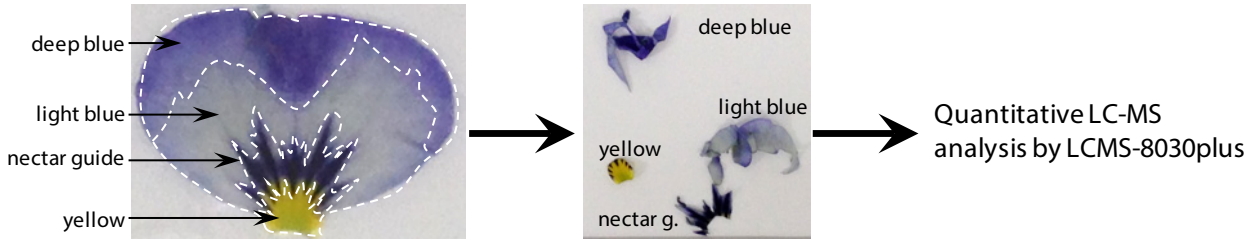


Fig. 16 Preparation of Tissues with Different Colors by Cutting Samples of Live Flower Petal from ROIs Defined in MSI

3. Conclusion

Mass spectrometry imaging (MSI) revealed that violanin, an anthocyanin contained in the petals of the viola flower, is distributed in the deep blue nectar guides of the flower. The results also confirmed that the distributions of the flavonoids contained in the petal, that is, Q3GR, M3GR, and Q3GRR, differ depending on the color of the petal.

By conducting MS^2 and MS^3, it was possible to assign the peak at *m/z* 609.2 to the quercetin glucoside Q3GR, and it was also found that the peak at *m/z* 771.2 originates from the quercetin glucoside Q3GR7G and myricetin glucoside M3GRR.

Quantitative interpretation of the distribution information obtained by MSI was possible by using a combination of MSI and LCMS, as shown in the example of Q3GR. Quantitative analysis of flavonoids which are colocalized with anthocyanin in viola flower petals can be realized by applying this technique.

Use of the ion trap technology of the iMScope *TRIO* imaging mass microscope for MS^n structural analysis in combination with the quantitative MSI technique is expected to lead to the discovery of unknown factors which influence the blue coloring of the viola flower.

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