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Technical Report

Combination of Probe Electrospray Ionization Mass Spectrometry and Mass Spectrometry Imaging to Analyze Plant Alkaloids in *Narcissus tazetta*

Kaoru Nakagawa[1], Tetsuo Iida[1], Manami Kobayashi[1], and Shuichi Shimma[*,2,3]

[1]*Solutions COE, Analytical & Measuring Instruments Division, Shimadzu Corporation, Kyoto, 1 Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604–8511, Japan*
[2]*Department of Biotechnology, Graduate School of Engineering, Osaka University, 2–1 Yamadaoka, Suita, Osaka 565–0871, Japan*
[3]*Institute for Open and Transdisciplinary Research Initiatives, Osaka University, 2–1 Yamadaoka, Suita, Osaka 565–0871, Japan*

Plant alkaloids are used in a variety of medicines, such as anti-cancer drugs and analgesics. Among these plant alkaloids, galanthamine is an Amaryllidaceae type alkaloid with acetylcholinesterase inhibition activity used in the treatment of neurological diseases such as Alzheimer's disease. Although the chemical synthesis of galanthamine has been successfully achieved, *Narcissus tazetta* is the main source of its production. Research shows that the galanthamine content varies not only with the type of daffodil but also with the stage of development and the part of the plant. Pharmaceutical companies seek plant species with higher galanthamine content to increase pharmaceutical availability. In this study, we were able to rapidly confirm the presence of alkaloids, such as galanthamine, lycorine, and tazettine in the *N. tazetta* sample using the probe electrospray ionization coupled with quadrupole time-of-flight type mass spectrometry. After confirmation of the components, we then visualized the distribution of the alkaloids by mass spectrometry imaging using the atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometer. In our method, we can provide qualitative data and visualized data immediately.

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INTRODUCTION

Plant secondary metabolites are a group of compounds that are not directly essential for survival or growth. However, they play a significant role in environmental adaptation and interactions between organisms.[1)] These metabolites are known to have diverse functions, including plant defense mechanisms, stress responses, pollinator attraction, and pharmacological activity for humans.[2)] The presence of these secondary metabolites confers a number of advantages upon plants, including enhanced resistance to pathogens and predators, greater resilience to environmental stress, and an improved ability to compete with other plants.[3)] For humans, these secondary metabolites have a long history of use in a variety of applications, including pharmaceuticals, fragrances, dyes, and insecticides. For example, morphine is obtained from the opium poppy, quinine is extracted from quince, taxol is found in yew bark,[4)] and galantamine is derived from *Narcissus tazetta*, which has been used for Alzheimer's treatment.[5–7)] Many other important pharmaceuticals are derived from plant secondary metabolites.

The majority of studies conducted thus far on plant secondary metabolites have employed a methodology based on extraction, isolation, and identification. This approach involves the use of techniques such as chromatography, nuclear magnetic resonance spectroscopy, and mass spectrometry to determine the structure and quantitative analysis of individual compounds.[8)] However, the application of these techniques has proven to be challenging in terms of accurately determining the precise localization and dynamics

*Correspondence to: Shuichi Shimma, *Department of Biotechnology, Graduate School of Engineering, Osaka University, 2–1 Yamadaoka, Suita, Osaka 565–0871, Japan*, e-mail: sshimma@bio.eng.osaka-u.ac.jp

of metabolites within plants, largely due to the loss of spatial information that occurs during the sample preparation process.

The localization of plant secondary metabolites to specific tissues or cell types is a common phenomenon, and their spatial distribution may be closely related to their physiological functions.[9] For instance, many defense-related compounds are concentrated in epidermal cells and hairy bodies, protecting the plant from foreign enemies.[10] Therefore, understanding the spatial distribution of these compounds is crucial to elucidate their physiological roles and biosynthetic pathways.

Mass spectrometry imaging (MSI) has emerged as a promising technique that offers a novel approach to this challenge.[11] MSI enables the visualization of molecule distribution on a sample surface while maintaining its spatial integrity.[12] This technique allows for the high-resolution observation of the localization of secondary metabolites in plant tissues, thereby providing a novel perspective on their physiological roles and biosynthetic pathways.[13] The fundamental premise of MSI is to detect ionized molecules at each location on the sample surface with a mass spectrometer and record their *m/z* and intensity values, along with the corresponding spatial coordinates.[14] The data obtained are then represented as a map, wherein the signal intensity corresponding to a specific *m/z* value is depicted by a color shade. This enables the simultaneous and quantitative evaluation of the distribution of multiple compounds.[15]

The latest developments in MSI technology have facilitated the conduct of more precise and detailed analyses. In particular, matrix-assisted laser desorption/ionization (MALDI) and desorption electrospray ionization are widely employed for the analysis of plant samples.[16,17] The application of these techniques has enabled the revelation of tissue-specific distribution patterns of a range of secondary metabolites, including alkaloids, flavonoids, and terpenoids.[18,19] Moreover, MSI can also capture dynamic changes in metabolites, thus enabling the tracking of alterations in metabolite distribution across different plant developmental stages and in response to environmental stimuli.[20]

In this study, we employed probe electrospray ionization (PESI) mass spectrometry,[21] which is combined with the quadrupole time-of-flight (QTOF) mass spectrometer. When performing MSI, having the peak information in advance can facilitate the analysis. Therefore, the combination of PESI mass spectrometry and MALDI-MSI will become an excellent combination in metabolites analysis, especially plant alkaloids.

The objective of this study was to propose the efficient measurement of the plant's secondary metabolites. PESI mass spectrometry is particularly suited to the rapid provision of mass spectra on extracts from the sample. By applying this advanced technology of PESI and MSI to plant science, this project represents an attempt to understand plant secondary metabolism from a new perspective not available with conventional methods. This will facilitate the elucidation of the distribution of alkaloids at various sites in the plant body.

EXPERIMENTAL

Chemicals and reagents

Ethanol (99.5) for LC/MS used PESI was purchased from FUJIFILM Wako Chemical Industries, Ltd. (Osaka, Japan).

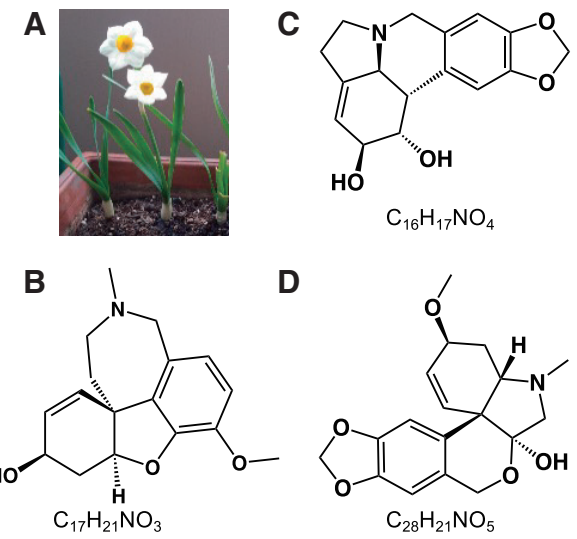

Fig. 1. Sample information. (A) Picture of *Narcissus tazetta*, structure and chemical formula of (B) galanthamine, (C) lycorine, and (D) tazettine.

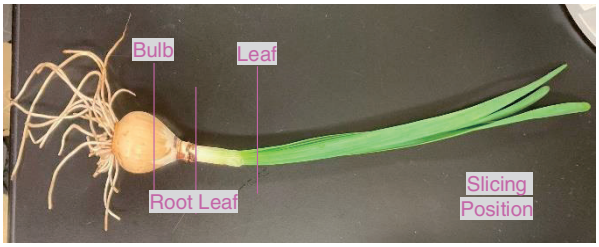

Fig. 2. Locations of *Narcissus tazetta* sections. *N. tazetta* was grown in soil culture with leaves growing to approximately 15 cm. Three types of sections were prepared: bulb, root leaf, and leaf cross-section.

α-Cyano-4-hydroxysilicic acid (CHCA, 99%), which was used as a matrix in the MALDI, was purchased from Merck (Darmstadt, Germany).

Sample preparation for PESI

In this study, we used *N. tazetta* with leaves growing to about 15 cm in length (Fig. 1A). Galanthamine (Fig. 1B), lycorine (Fig. 1C), and tazettine (Fig. 1D) were extracted with 50% ethanol from freeze-dried leaves. Approximately 200 mg of freeze-dried leaves was combined with 100 μL of 50% ethanol and subjected to 5 min of mixing. Subsequently, centrifugation was performed using a DISKBOY FB-4000 (KURABO, Osaka, Japan) at 12,000 rpm for 5 min.

Sample preparation for MSI

We collected the bulb, root leaf, and leaf samples, as shown in Fig. 2. After dissection, the samples were immediately frozen in liquid nitrogen for 3 min. After freezing, frozen sections of 20 μm thickness were prepared from the frozen samples using a cryomicrotome (CM1950, Leica Biosystems, Nussloch, Germany) at −20°C. Frozen sections were collected directly onto indium tin oxide (ITO)-coated glass (Matsunami, Osaka, Japan). The glass was then dried in a 50 mL centrifuge tube containing silica gel for 10 min. After dehydration, the samples were coated with CHCA via vapor

Table 1. Analytical settings of PESI.

System	DPiMS QT + LCMS-9030
Polarity	Positive
Desolvation line temperature	250°C
Heat block temperature	50°C
Interface voltage	3.5 kV
m/z range	50–2000
Measurement time	0.5 min

PESI, probe electrospray ionization.

Table 2. Analytical settings of MSI.

System	iMScope QT + LCMS-9030
Polarity	Positive
Desolvation line temperature	250°C
Heat block temperature	450°C
m/z range	280–335
Spatial resolution (pixel size)	10 or 25 µm
Laser diameter setting (arb unit)	1 or 2
Laser intensity (arb unit)	50 or 60
Laser repetition frequency	20 kHz

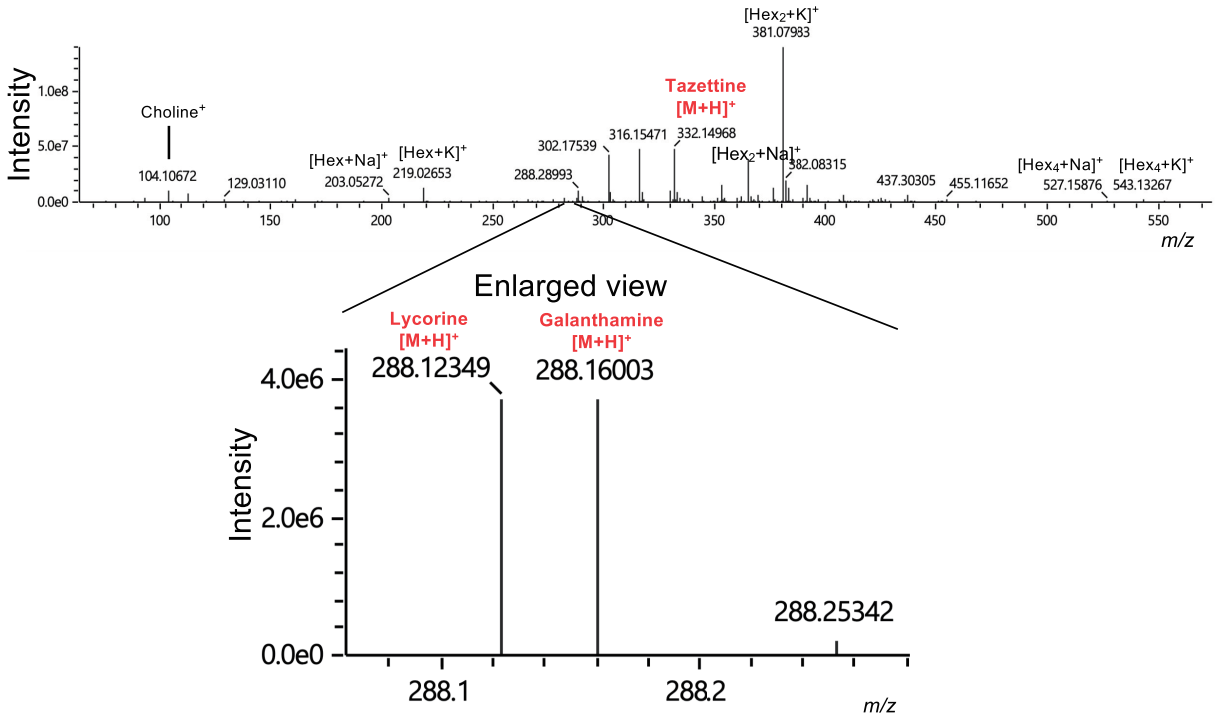

Fig. 3. Mass spectra obtained from the extracted solution of *Narcissus tazetta* leaves using the PESI QTOF system. PESI, probe electrospray ionization; QTOF, quadrupole time-of-flight.

deposition using iMLayer (Shimadzu, Kyoto, Japan) at a thickness of 0.7 µm.

Analysis conditions for PESI and MSI

An amount of 10 µL of the extracted solution was mounted on a sample plate and analyzed by a PESI QTOF mass spectrometer (DPiMS QT, Shimadzu). The analytical settings of the PESI are shown in Table 1. Data analysis was performed using LabSolutions Insight Explore software (Shimadzu). Adjusted tissue sections were analyzed by iMScope QT (Shimadzu). The analytical settings of MSI are shown in Table 2. Data analysis was performed using IMAGEREVEAL MS (Shimadzu).

RESULTS

Qualitative screening of galanthamine in *N. tazetta* by PESI mass spectrometry

The mass spectra obtained rapidly in a few tens of seconds from the extracted solution of freeze-dried *N. tazettta* leaves in positive mode are shown in Fig. 3.

Table 3. Mass accuracy of alkaloids detected in *Narcissus tazetta*.

Compounds	Ion species	Theoretical *m/z*	Measured *m/z*	Errors (mDa)
Galanthamine	Protonated	288.1594	288.1600	0.6
Lycorine	Protonated	288.1230	288.1235	0.5
Tazettine	Protonated	332.1493	332.1496	0.3

Accurate mass analysis using analytical software confirmed the presence of galanthamine, and other plant alkaloids lycorine and tazettine (shown in red), choline and hexose sugars were also detected. The high-resolution QTOF MS system enabled the baseline separation of lycorine and galanthamine, which were close in *m/z* values. The mass resolution of 30,000 full width at half maximum (FWHM) provided sufficient resolving power to distinguish these structurally related alkaloids. The mass accuracy obtained is shown in Table 3. As shown in Table 3, all alkaloids were detected as protonated ions. The mass accuracies were about 0.3 to 0.6 mDa. This high mass accuracy

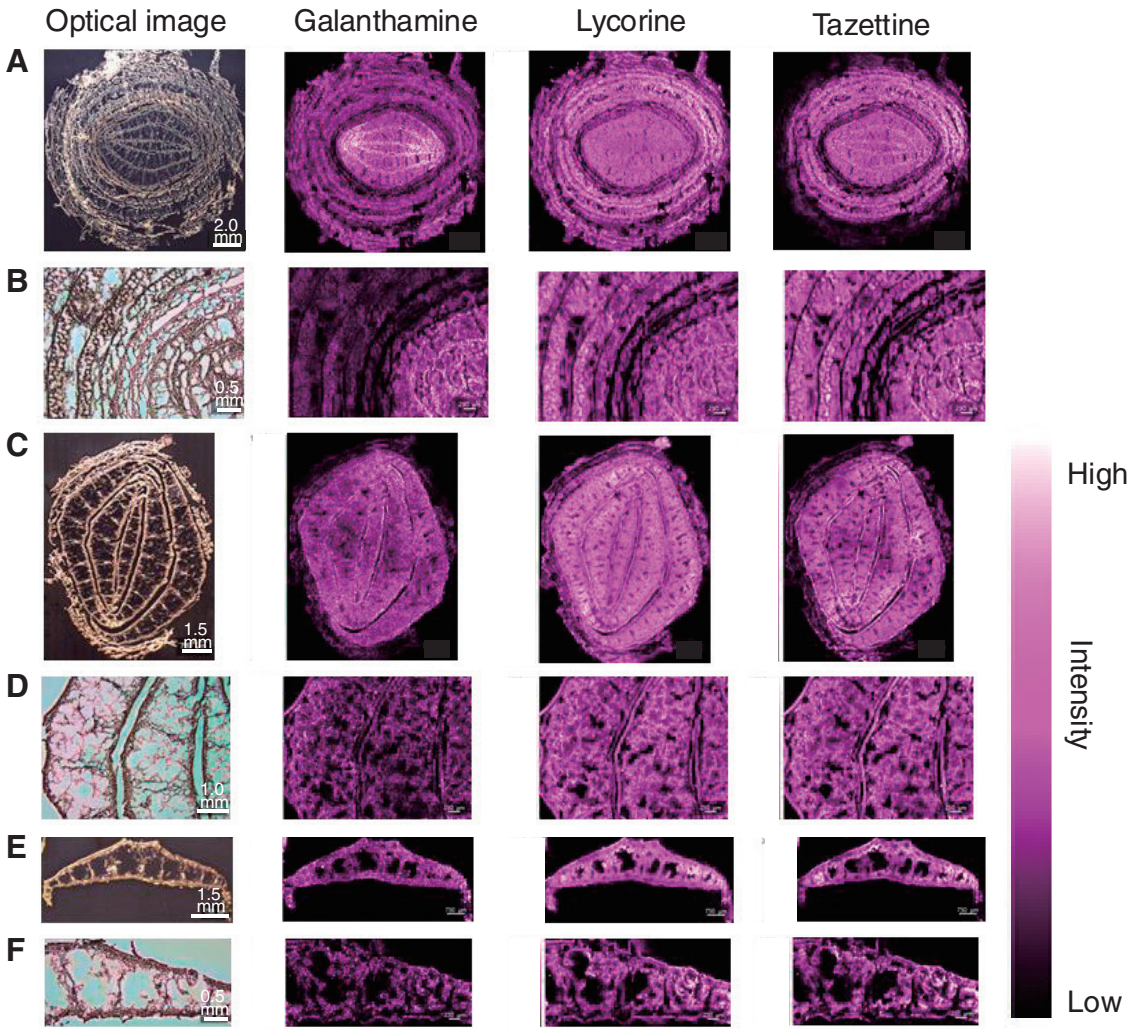

Fig. 4. Mass spectrometry imaging results from different parts of *Narcissus tazetta*. (A) Bulb part (spatial resolution: 25 μm, scale bar: 2 mm), (B) high spatial resolution image of bulb part (spatial resolution: 10 μm, scale bar: 0.5 mm), (C) root leaf part (spatial resolution: 25 μm, scale bar: 1.5 mm), (D) high spatial resolution image of root leaf part (spatial resolution: 10 μm, scale bar: 0.5 mm), (E) leaf part (spatial resolution: 25 μm, scale bar: 1.5 mm), (F) high spatial resolution image of leaf part (spatial resolution: 10 μm, scale bar: 0.5 mm). In these images, lighter color means high ion intensity, and darker color means low ion intensity.

was important to annotate the peaks. Since the PESI mass spectrometer provided high mass resolution mass spectra at high speed, we could easily confirm the component information within the samples.

Distribution analysis of galanthamine in *N. tazetta* by MSI

The MALDI-MSI results of the entire section at 25 μm spatial resolution and the area observed with an optical microscope at 5× objective lens at 10 μm spatial resolution are shown in Fig. 4.

This study, which employs MALDI-MSI, offers intriguing insights into the spatial distribution of galanthamine, lycorine, and tazettine within the bulbs and leaves of *N. tazetta*. Of particular interest is the distribution pattern of galanthamine within the bulb. It was observed that galanthamine is mainly concentrated in future leaves, which suggests that this alkaloid may play an important role in plant growth and developmental processes. For example, this alkaloid may promote the formation and growth of new leaves, protect shoots from pathogens and pests, or be involved in the mobilization and transport of nutrients.[22)]

Conversely, it is noteworthy that lycorine and tazettine did not exhibit a discernible distribution pattern within the bulb. This indicates that these alkaloids may be distributed uniformly throughout the bulb or may possess functions distinct from those of galanthamine. This discrepancy may be indicative of disparate physiological roles and evolutionary origins for each alkaloid.

No significant differences in distribution were observed among the 3 alkaloids in the leaf cross-sections. This indicates that these alkaloids may perform analogous functions in leaves. Such functions may include the protection of photosynthetic tissues, the maintenance of leaf structure, and the defense against pathogens and predators. Nevertheless, further investigation is required to ascertain the actual physiological significance of the uniform distribution of these alkaloids in leaves.

DISCUSSION

The results of this study indicate that alkaloid distribution patterns within bulbs may differ among alkaloids. This is a particularly intriguing finding from an evolutionary

perspective. This interspecific variation may be attributed to adaptations to specific ecological niches, differences in defense strategies against particular predators, pathogens, or evolutionary divergence in metabolic pathways. The existence of such interspecific differences may provide crucial insights into the evolution and adaptive strategies of Amaryllidaceae.

From a methodological standpoint, the MSI technique employed in this study effectively visualizes alkaloid distribution in plant tissues at high resolution (25 μm and 10 μm). The primary advantages of this technique are its capacity to observe metabolite distribution while preserving tissue structure, its ability to analyze multiple alkaloids simultaneously, and its capability to detect differences in distribution with microscopic spatial resolution. These advantages enable the acquisition of detailed spatial information that would otherwise be unattainable through conventional biochemical methods.

The findings of this study have prompted the formulation of several new research questions. For example, further research is required to determine the physiological significance of the specific distribution of galanthamine in bulbs, the functional significance of the uniform distribution of lycorine and tazettine, and the alkaloid distribution patterns among different Amaryllidaceae plant species. Additionally, future research should investigate dynamic changes in alkaloid distribution in response to plant developmental stages and environmental stresses, as well as the relationship between alkaloid biosynthetic pathways and distribution patterns.

CONCLUSION

In conclusion, both the rapid determination of the components using extracts and visualization using MSI have been successfully achieved in *N. tazetta* at high resolution. The MSI analysis using high spatial resolution revealed, in particular, the specific distribution of galanthamine in the bulb. This finding deepens our understanding of plant alkaloid function and evolution and provides new insights into plant defense strategies and metabolic regulation. It is anticipated that future applications of this approach to other plant species and metabolites will contribute to a more comprehensive understanding of plant secondary metabolism. Furthermore, a more detailed investigation of the relationship between alkaloid distribution patterns and their physiological functions and ecological roles in plants may provide new insights that will contribute to the advancement of plant science.

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