Research Paper

DESI-IMS coupled with triple quadrupole mass spectrometry demonstrated the capability of NMN imaging in *Shelfordella lateralis* tissue

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Abstract Imaging mass spectrometry (IMS) has become an indispensable analytical tool for visualizing molecular distributions in biological tissues. However, the detection of many important biomolecules, such as nicotinamide mononucleotide (NMN) in tissue by IMS is still challenging due to their lower abundance. Although the sensitivity of widely used IMS instruments, desorption electrospray ionization (DESI) utilizing a time-of-flight (TOF) mass analyzer, is not sufficient to detect NMN in tissues, the recently developed DESI coupled with a triple-quadrupole (TQ) provides about ten times higher sensitivity than the DESI-Q-TOF. In this study, we first screened six insect species for NMN content using ultra-performance liquid-chromatography-tandem mass spectrometry (UPLC-MS/MS). Interestingly, *Shelfordella lateralis* showed significantly higher NMN content (15.5 mg/100 g of dry weight) than five other insect species used in this study. After screening, we tried to observe the distribution of NMN in *S. lateralis* using both DESI-Q-TOF and DESI-TQ, and only the DESI-TQ successfully detected the NMN in *S. lateralis.* DESI-TQ revealed prominent distributions of NMN in the abdomen of *S. lateralis* in selective reaction monitoring mode. To the best of our knowledge, this is the first report of NMN imaging in animal tissue applying IMS. Our study will contribute to the extended application of IMS for NMN imaging in mammalian tissues in the future. Furthermore, NMN imaging may help researchers to study nicotinamide adenine dinucleotide (NAD^+) metabolism in living organisms, which has significant implications for medical research and diagnosis.

Key words: IMS, NMN, DESI-TQ, DESI-Q-TOF, UPLC-MS/MS, *Shelfordella lateralis*

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Received: August 16, 2023. Accepted: January 11, 2024.
Epub April 19, 2024.
DOI: 10.24508/mms.2024.06.001

Introduction

Imaging mass spectrometry (IMS) has been increasingly used to visualize biomolecules due to its high sensitivity and label-free nature. This technology has made it possible to reveal the identity and distribution of the specific molecule and the localization of the molecule in organs[1)]. Nowadays, imaging with desorption electrospray ionization (DESI), in combination with quadrupole-time-of-flight

(Q-TOF) mass spectrometry, has been gaining much popularity for visualizing molecular distribution on a sample surface under ambient conditions[2]. DESI-Q-TOF has shown the capability of visualizing spatial distributions of molecules with diverse chemical natures, such as small molecules[3], free fatty acids[4–6], phospholipids[7], lipid biomarker[8], active compounds[9], and other metabolites[10]. One of the significant advantages of DESI-IMS is that it requires a minimal sample preparation than traditional mass spectrometry, such as matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)[11]. Recently, DESI combined with triple quadrupoles (TQ) mass spectrometry has emerged and offers advantages over DESI-Q-TOF. The TQ mass analyzer consists of three quadrupole mass filters, while DESI-Q-TOF consists of a single quadrupole mass filter. These three quadrupole mass filters make DESI-TQ more specific for precursor-to-product ion transitions of the targeted compound analysis[12]. This allows DESI-TQ to exhibit higher specificity and excellent sensitivity in selective reaction monitoring (SRM) mode than other high-resolution mass analyzers[13].

Despite the significant improvements in the sample preparation and instrumentations of IMS, detecting and imaging of many biologically important molecules in tissue is still challenging. For example, nicotinamide mononucleotide (NMN), a well-known vital precursor of nicotinamide adenine dinucleotide (NAD^+), has been found difficult to be detected by IMS. The analytical detection of NMN is challenging due to its physicochemical characteristics[14]. NMN is a nucleotide containing a pyridine base. It is an acid- and water-soluble nucleotide found in all living cells as a coenzyme. Increasing NAD^+ levels improves insulin sensitivity, reverses mitochondrial dysfunction, and enhances lifespan[15]. When taken orally, NMN is quickly absorbed and converted into NAD^+[16]. NAD^+ availability is essential for slowing the aging process[17]. Oral nicotinamide supplementation within the daily acceptable upper threshold is an efficient strategy to raise NAD^+ levels and influence blood lipid composition[18]. Thus, NMN has important applications in medicine and healthcare[19], but the chemical synthesis of NMN is complex[20]. Therefore, it is worth exploring a rich source of natural NMN and its imaging in medical research.

Unlike other natural products, insects have received comparatively little attention in imaging studies as sources of modern medicines[21]. Insects can provide proteins for human use, and people in many parts of the world consume insects as a potential source of calories and protein[22]. In traditional folk medicine and Chinese medicine, the whole body extracts of several bees, wasps, flies, butterflies, moths, cockroaches, beetles, and other insects act as antiviral, antibacterial, and anti-cancer agents[21]. While insects are consumed as food in various cultures around the world and are known to be rich in protein and nutrients, there is not adequate knowledge or research about their NMN content.

Recently, our group has explored high NMN content in *Cinnamomum verum J. Presl* bark, a medicinal plant, using liquid chromatography mass spectrometry (LC-MS)[23]. Applying the same method, we first screened NMN content in six insect species in this study, namely, the Turkestan cockroach *Shelfordella lateralis*, the field cricket *Gryllus bimaculatus*, the damp-wood termite *Hodotermopsis sjostedti*, the black soldier fly *Hermetia illucens*, the Japanese subterranean termite *Reticulitermes speratus*, and the giant mealworm *Zophobas atratus*. Finally, we employed both DESI-Q-TOF and DESI-TQ to observe the distribution of NMN in *S. lateralis* bodies, which showed the highest NMN content among the other species under this study.

Materials and Methods

Chemicals and reagents

We purchased the standard β-nicotinamide mononucleotide (β-NMN) from Tokyo Chemical Industry Co. (Tokyo, Japan). LC/MS-grade formic acid (99%), acetonitrile (ACN), methanol, ethanol, and ultra-pure water were obtained from Fujifilm Wako Pure Chemical Corporation, Ltd. (Osaka, Japan). Ammonium format solution was purchased from Kanto Chemical Co., INC. (Tokyo, Japan). Sodium formate was obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA) and, leucine enkephalin from Waters Corporation (Milford, MA, USA).

Sample preparation for the quantification of NMN

Two types of termite species (*H. sjostedti*, *and R. speratus*) were used in this study. *H. sjostedti* were collected initially from logs from brown-rotted Luchu pine wood (*Pinus luchuensis*) in the forest of Amami Oshima Island in Japan. And the colonies of *R. speratus* were collected from logs from brown-rotted cedar wood (*Cryptomeria japonica*) in the forest in Kyoto, Japan. The termite colonies were maintained at the Laboratory of Insect Ecology, Kyoto University, at about 25°C under dark. The Individuals of *H. sjost-*

edti were randomly extracted from each of the six colonies (colony code: KM022, KM024, KM025, KM103, KM109, TMK091). And the individuals of *R. speratus* were randomly extracted from each of the three colonies (colony code: MT616D, MT617E, and MT619G). We bought the cockroach *S. lateralis* from Rakuten Group, Inc., Tokyo, Japan, and the freeze-dried *G. bimaculatus* from Leaf Corporation, Gunma, Japan. The two other insect species, dried *H. illucens*, and dried *Z. atratus*, were purchased from GEX Corporation, Osaka, Japan, and Sanko-Tokai Co., Ltd., Osaka, Japan, respectively.

All the samples were kept in a −80°C freezer until freeze-dried treatment. The frozen insect samples were freeze-dried (Freeze Dryer FD-1000, EYELA, Bunkyo, Tokyo, Japan) and ground (Wonder Crusher WC-3 L, Osaka Chemical Co. Ltd., Kita, Osaka, Japan) to form a powder. The resulting insect powder (each about 5 g) was respectively vacuum-sealed (Impulse Vacuum Sealer V301G-10WK, FUJIIMPULSE Co. Ltd., Toyonaka, Osaka, Japan) and kept in −20°C freezer until use for analyses.

Standard sample preparation

For standard sample preparation, an analytical balance (AUY220, SHIMADZU, Kyoto, Japan) was used to weight the NMN standards. Then, 25% ethanol was used to dissolve the NMN standard. The prepared NMN standard was then serially diluted to control solutions at concentrations of 500, 200, 100, 50, and 20 ng/mL for calibration curve preparation.

Insect extract preparation for UPLC-MS/MS

For each sample, approximately 50 mg of powder was weighed three times. Then 25% ethanol was added (40 μL/mg of the sample) and extracted for one minute on ice with a mechanical homogenizer (HK-1, AS ONE, Osaka, Japan) at 9000 rpm. The homogenized insect extracts were then centrifuged for 10 minutes at 3000 rpm in 4°C. Then the supernatant was filtered through a 0.42 μm filter, and the cleared sample was transferred to LC-MS vials.

UPLC-MS/MS analysis

ACQUITY (UPLC H-Class PLUS, Waters) coupled with a triple quadrupole mass spectrometer (Xevo TQ-XS, Waters) was used to analyze the insect extracts. An Intrada Amino Acid column (3 μm, 3.0×100 mm, Imtakt) was carried out for chromatographic separation, which was kept at 40°C throughout the measurement. The electrospray ionization (ESI) source was used on the mass spectrometer, and the analysis was performed in positive ion mode. The capillary voltage was set to 3.5 kV, the cone voltage to 22 V and the source temperature was 150°C. The desolvation temperature was fixed at 500°C and the desolvation gas flow rate was 600 L/h. The cone and nebulizer gas flows were set to 150 L/h and 7.0 Bar, respectively. Mobile phase A consisted of 0.3% formic acid in ACN, while mobile phase B was acetonitrile/100 mM ammonium formate (20:80, v/v). The flow rate used for elution was 0.6 mL/min. The following setup was used to perform the gradient elution procedure: 0 to 0.5 min, 20% B; 0.5 to 2.5 min, 20% to 100% B; 2.5 to 4.5 min, 100% B; 4.5 to 4.6 min, 100% to 20% B; 4.6 to 7.0 min, 20% B. The whole duration of the run time was 7 min. An aliquot of 5 μL of the prepared sample was injected into the system while the autosampler was kept at a constant temperature of 10°C. The SRM transition from *m/z* 335.1 to *m/z* 123.0 using a collision energy of 10.0 eV was used for NMN quantification.

A MassLynx software (Waters, version 4.1) was used to acquire and analyze the UPLC-MS/MS data, and, TargetLynx software (Waters, version 4.1) was used for the quantitative measurement. The statistical analysis was carried out using GraphPad Prism (version 8.42).

Sample preparation for DESI-IMS analysis

After collecting the samples, the whole body of the cockroach was embedded overnight by a super cryo-embedding medium in the middle of a cryomold and frozen at −20°C. The frozen cockroach samples were then mounted on a sample holder using the optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan). The tissues were sectioned sagittally at 20 μm thickness at −20°C by a cryostat system (CM1950; Leica Biosystems, Wetzlar, Germany). Then the sections were mounted on glass slides (Matsunami, Osaka, Japan) and stored at −80°C. Before DESI-IMS analysis, the slices were air-dried.

DESI-Q-TOF analysis

DESI-IMS analysis was performed in positive ion mode using DESI source attached to a Q-TOF mass analyzer (Xevo G2-XS Q-TOF, Waters). Before the measurements, sodium formate solution (500 μM) in 2-propanol: water (90:10, v/v) was used to calibrate the mass spectra externally, and leucine enkephalin solution (500 μM) was used

to set up the detector. A solvent pump (ACQUITY UPLC Binary Solvent Manager, Waters) was used to deliver the spray solvent (98:2 methanol/water, v/v) at a flow rate of 2 μL/min. A 2D sampling stage was equipped with a DESI source to scan the designated area with a scan rate of 200 μm/sec, and the pixel size was 100 μm × 100 μm. The DESI source conditions were optimized using the following parameters: a nebulizing nitrogen gas pressure of 0.4 MPa, a capillary voltage of 4.0 kV, a spray impact angle of 70°, a sampling cone of 50, and an inlet temperature of 120°C. The setup for mass resolution, mass window, and collision energy were at 20000, 0.02 Da, and 4.00 V. The mass spectra were collected using a mass range of *m/z* 100 to 1000 of the 1000 peaks with the highest intensities. The data were then further processed at the range of *m/z* 300 to 400 to detect the ion images of lower intensities.

DESI-Q-TOF data were acquired and processed using the MassLynx software (Waters, version 4.1). And, HDI Imaging software (Waters, version 1.4) was used to analyze the ion images.

DESI-TQ analysis

A Xevo TQ-XS equipped with a DESI source (Waters) has been employed for imaging in SRM mode. A syringe pump delivered the DESI-IMS solvent ($MeOH/H_2O$, 98/2, v/v) at a flow rate of 2 μL /min. For NMN imaging, the DESI source conditions were optimized using the following parameters: N_2 nebulizing gas pressure of 0.8 Mpa; capillary voltage of 0.85 kV (Positive); MS source temperature of 150°C; sampling cone voltage of 20 V. The distances between the emitter tip and the surface, the mass inlet and the surface, and the mass inlet were roughly 2, 6, and 0.5 mm, respectively. The identified areas on the glass slide were scanned at a scan rate of 10 scan/sec. The pixel size was set as 50 μm × 50 μm for tissue and 100 μm × 100 μm for standard. A collision energy of 20 eV was used for NMN transition (*m/z* 335.1 to *m/z* 123.0). The SRM dwell time was set as 0.0016 sec/pixel. At least two serial sections of the cockroach sample were analyzed under the same experimental conditions. Following the measurement, tissue sections were stained with hematoxylin and eosin (H&E) for histological analysis.

The ion image of DESI-TQ data was acquired and processed using the updated version of MassLynx (Waters, version 4.2) software and HDI Imaging (Waters, version 1.6) software.

Results

In this study, we first conducted a quantification study of NMN in six different insect species *S. lateralis*, *G. bimaculatus H. sjostedti*, *H. illucens*, *R. speratus*, and *Z. atratus* by LC/MS (Fig. 1b). As seen in Fig. 1a, a linear calibration curve (Y=9.3056X−154.914, R=0.99) of NMN standards was observed in the range of 20−500 ng/mL. Chromatograms showed the clear detection of NMN standard at each point (Fig. S1a−e).

A Chromatogram of NMN (SRM transition at *m/z* 335.1 to *m/z* 123.0) was observed with high intensities in *S. lateralis*, *G. bimaculatus* (Fig. 1b, Fig. S2a−b), *H. sjostedti*, *H. illucens* (Fig. 1b, Fig. S3c−d), and *R. speratus* (Fig. 1b, Fig. S4e). On the other hand, NMN was not detected in the species of *Z. atratus* (Fig. 1b, Fig. S4f). The average concentration of NMN in *S. lateralis*, *G. bimaculatus*, *H. sjostedti*, *H. illucens*, and *R. speratus* were 15.5, 6.93, 3.01, 0.143, and 0.13 mg/100 g (dry weight) respectively. *S. lateralis* showed significantly higher NMN content than all other species in this study ($p<0.0001$) (Fig. 2). *G. bimaculatus* showed the second highest content of NMN, which was significantly higher than *H. illucens*, *R. speratus*, and *Z. atratus* ($p<0.005$) (Fig. 2).

For the imaging study, we first performed DESI-Q-TOF analysis in the whole body of *S. lateralis*. But we could not detect NMN in the entire body of *S. lateralis* (Fig. S5a), we could only detect NMN at *m/z* 335.1 $[M+H]^+$ in NMN standard (20 μg/mL) (Fig. S5a). In this analysis, NMN was not detected with other adducts in both NMN standard and *S. lateralis* (Fig. S5b).

Next, we performed DESI-TQ in the SRM mode to see the distributions of NMN in the sagittal sections of whole-body *S. lateralis*. NMN (SRM transition of *m/z* 335.1 to *m/z* 123.0) was successfully detected in almost entire regions of the *S. lateralis* section (Fig. 3b). To observe the region-specific distribution of this molecule in *S. lateralis*, the histological images were overlaid on the ion image (Fig. 3c). Interestingly, NMN was notably higher in some specific parts of the *S. lateralis* abdomen, possibly in the midgut. For further confirmation of the NMN assignment, pure NMN solution (1 μg/mL) and extract from dry *S. lateralis* were spotted on the same slide and measured subsequently. As expected, the ion images at the same NMN transition were clearly detected for pure NMN (Fig. 3d) and the *S. lateralis* extract (Fig. 3e).

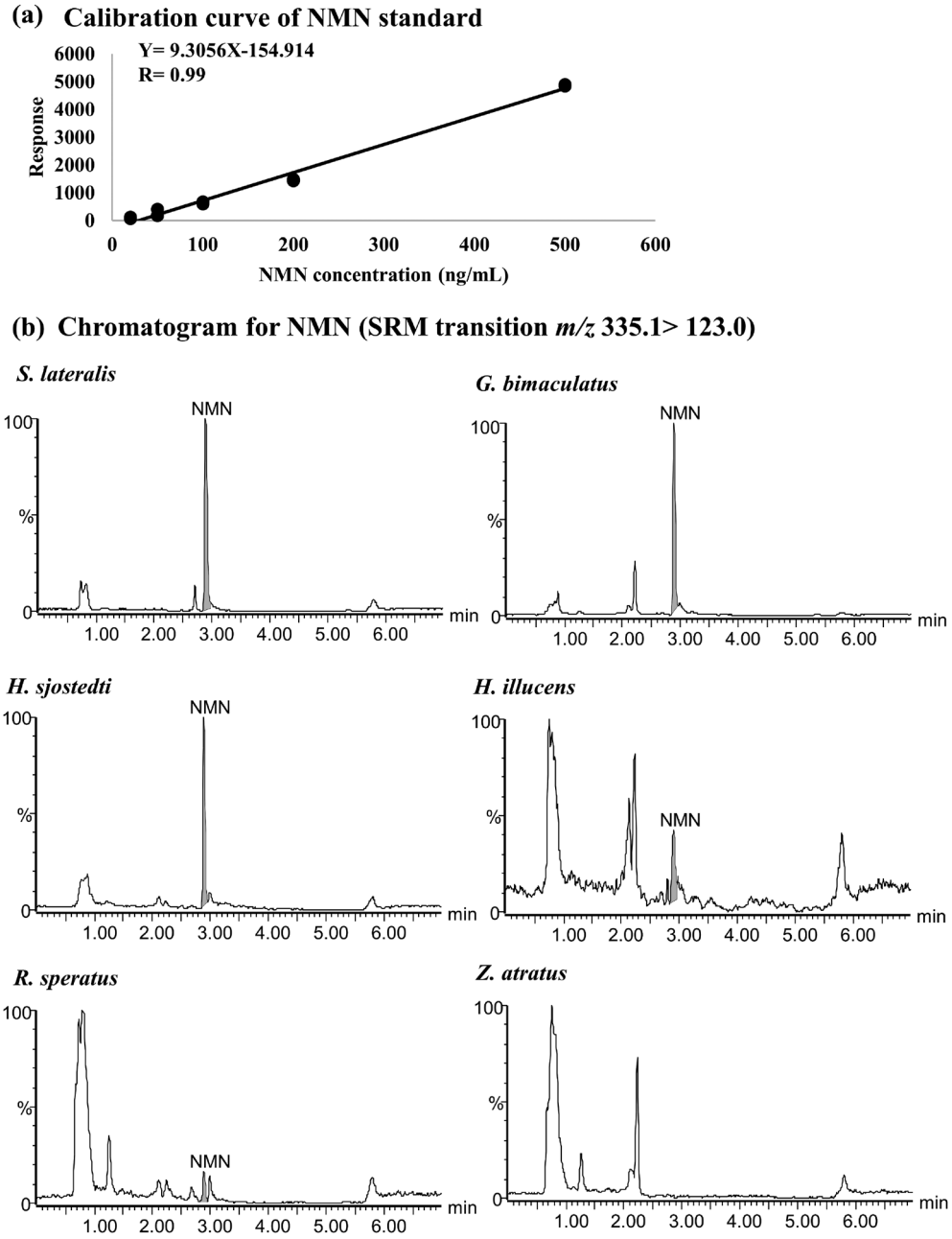

Fig. 1. Calibration curve of NMN standards (a) and representative chromatogram of NMN obtained from six different species of insects (b).

Discussion

For the first time, in this study, we demonstrated the NMN imaging in biological tissue employing DESI-TQ in SRM mode that showed higher sensitivity and excellent selectivity than DESI-Q-TOF. Detection of NMN in tissue is challenging by IMS since this molecule is very labile and readily undergoes in-source fragmentation under standard electrospray ionization settings[24]. DESI-Q-TOF is typically used for untargeted analysis where the target ion is mixed with myriads of other ions and could not be detected. In our experience, DESI-TQ provides about ten to a thousand times higher sensitivity than the DESI-Q-TOF. In this study, DESI-TQ successfully detected NMN in *S. lateralis* (Fig. 3), which could be attributed to its improved sensitivity. It is to be noted that the sensitivity of DESI-TQ is influenced by various factors, including dwell time. Dwell time refers to the duration the mass spectrometer spends analyzing a particular ion in a particular spot on the sample surface. Increasing dwell time leads to higher sensitivity as the instrument has more time to accumulate ions from the sample. However, longer dwell times can compromise spatial resolution. This is because the instrument spends more time

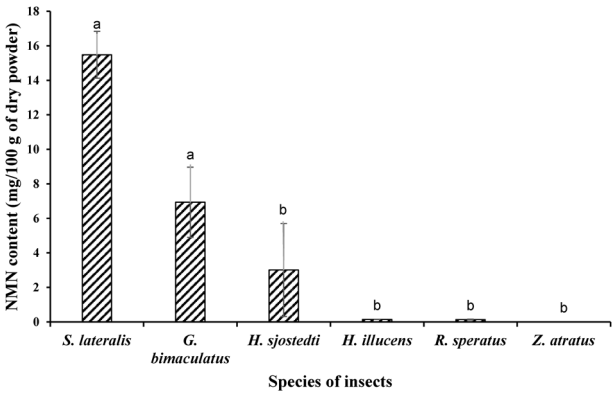

Fig. 2. Comparison of NMN content among different species of insects.
Different letters indicate a significant difference ($p < 0.01$) among different species. One-way ANOVA was performed followed by Tukey's multiple comparison tests among different species of insects. All the data are represented as mean±SD. The same insect samples were prepared in triplicate.

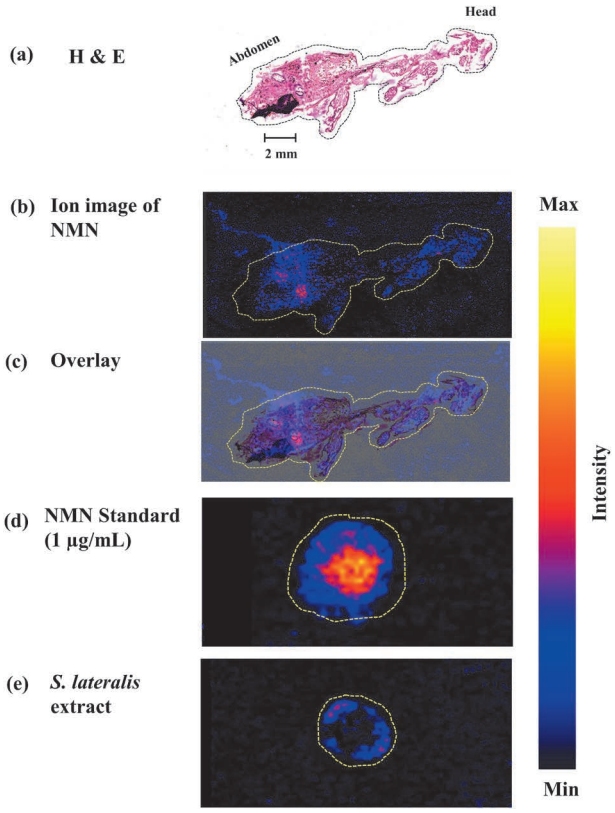

Fig. 3. Distribution of NMN in the whole body of *S. lateralis by* DESI-TQ.
H&E images of *S. lateralis* section (a). Representative DESI-IMS ion image for NMN (SRM transition at *m/z* 335.1 to *m/z* 123.0) in *S. lateralis* section (b), NMN standard (d), and *S. lateralis* extract (e). Transparent overlaid images between H&E and ion images for NMN in *S. lateralis* (c).

at each spot, resulting in a larger area being sampled, which may reduce the ability to discriminate features at a finer spatial scale. Therefore, finding the optimal balance between dwell time, sensitivity, and spatial resolution is recommended.

The analysis of insects, such as *S. lateralis*, by IMS presents inherent challenges. Ideally, freezing the sample immediately after sacrificing the animal or insect is crucial to prevent post-mortem degradation of certain analytes. However, the delicate anatomical structure of *S. lateralis* poses difficulties in cryo-sectioning fresh-frozen samples without using an embedding medium. To address this issue, we employed a super cryo-embedding medium for the entire body of *S. lateralis*. This approach facilitates cryo-sectioning without compromising the mass spectral quality, thanks to the medium's monomeric property. It is important to note that freezing samples using cryo-embedding medium requires a longer time compared to direct freezing, this procedure may impact the imaging of certain biomolecules susceptible to rapid post-mortem degradation. Fortunately, there is no reported literature on the post-mortem degradation of NMN, supporting the compatibility of NMN imaging in frozen and embedded samples.

One of the challenges in NMN imaging is the potential delocalization during sample preparation. In our experience, NMN delocalization was observed when tissue section was stored in the freezer before analysis. This is because NMN is highly soluble in water. In our study, we dried the sample immediately after cryo-sectioning to minimize the NMN delocalization. Previously MALDI-IMS was used for peptide imaging in formalin-fixed and paraffin-embedded (FFPE) *S. lateralis*[25]. However, FFPE samples are not suitable for NMN imaging since they require deparaffinization and extensive washing procedure.

Our group recently developed a rapid SRM method (7 min run per injection) to screen and quantify NMN in plant extract using UPLC-MS/MS[23]. SRM has been a gold standard for quantification studies. We employed the same method in our current study and found a significantly higher content of NMN in *S. lateralis* compared to other species of insects. Cockroaches are one of the most successful and diverse insects in traditional and Chinese medicine[26]. Previously Miles et al. investigated NMN content in various natural raw foods, including edamame, broccoli, cucumber seed, cucumber peel, cabbage, avocado, tomato, mushroom, beef, and shrimp. Among these, edamame

showed the highest content of NMN (0.47−1.88) mg/100 g[27]. Considering the moisture content (60−65%)[28], the NMN content in edamame should be approximately 1.18−4.7 mg/100 g dry weight. In contrast, cockroaches have an NMN content of 15.5 mg/100 g dry weight (Fig. 2), about 3 to 13 times higher than edamame. This is the first study that revealed the surprisingly higher content of NMN in insects. Cockroach extracts have been used for centuries to treat aches, pains, inflammation, and even chronic heart failure[29]. The gut microbial metabolites of cockroach is a valuable source of new bioactive compounds with therapeutic promise for human health[26]. Our study also indicated the promising use of this insect, particularly in pharmaceuticals. A recent study found that milk from cockroach species had a higher potential for nutrition than milk from typical mammals[30]. As a result, it is thought of as a potential new superfood that might be made accessible for ingestion shortly. It is to be noted that cockroaches carry food-borne pathogens that may transmit diseases to humans[31]. Therefore, more research is required to focus on the sanitation and safety of cockroaches.

In our study, the second higher amount of NMN was present in *G. bimaculatus*. This insect species is used as food and feed[32]. A previous study showed that the extract of *G. bimaculatus* can treat aging in the WKY mice model by dramatically lowering creatinine phosphokinase levels in the serum[33]. We also found a significant amount of NMN in *G. bimaculatus*, suggesting it might have a potential role in anti-aging. We have analyzed two species of termites, *R. speratus*, and *H. sjostedti*. In our investigation, the third higher amount of NMN was present in *H. sjostedti*. Termites are eusocial insects used as model organisms in aging research[34]. Recently, DESI-IMS analysis of ^{13}C-labeled termites revealed that the royal diet contained phosphatidylinositol and acetyl-L-carnitine, which are known to be anti-aging agents[35] and this may explain termite royals' remarkable longevity in eusocial insects[36]. This fact strongly suggests that termites have anti-aging agents in their bodies, and therefore, NMN may also be involved in the longevity of *H. sjostedti*.

Our study has some limitations. We could not assign the anatomical region of *S. lateralis* unambiguously where NMN exhibited high distribution. The current data did not explore the potential post-mortem degradation of NMN within the *S. lateralis*.

In conclusion, the current study demonstrated the NMN imaging in tissue by DESI-TQ and revealed *S. lateralis* as a rich source of NMN, specifically in the abdomen. We believe that, our method will help to develop other animal imaging in the field of biomedical research. In the future, NMN imaging could aid in understanding age-related changes in NAD^+ metabolism and its potential role in human health.

Acknowledgment

This work was supported by the Cabinet Office, Government of Japan, Moonshot Research and Development Program for Agriculture, Forestry and Fisheries (funding agency: Bio-oriented Technology Research Advancement Institution) Grant number JPJ009237 (to K.M.), JSPS KAKENHI (Grant Number JP18H05268 to K.M.), and JSPS KAKENHI (Grant Number JP23H69332 to K.M.).

Conflicts of Interest

The authors declare no conflict of interest.

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