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Determination and Imaging of Lactones in Beef by Girard's Reagent T Derivatization Technique

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Derivatization methods improve ionization efficiency and chemical properties such as molecular weight, polarity, and boiling point. Nonderivatized standard lactones evaporate over time due to their volatility. Thus, optimal lactone detection should be performed without delay. In this study, Girard's reagent T-labeled-γ-nonalactone (*m*/*z* 270.2), γ-decalactone (*m*/*z* 284.2), and γ-undecalactone (*m*/*z* 298.3) were stably detected by matrix-assisted laser desorption/ionization mass spectrometry regardless of analysis delay. In a mass spectrometry imaging experiment, we analyzed the localization of lactones in beef meat (Japanese Black Wagyu) using the derivatization method and identified a border between lactones and fatty acids such as free oleic acid.

1. Introduction

Derivatization methods have been used to improve volatility, thermal stability, and ionization efficiency in liquid and gas chromatography as well as mass spectrometry (MS). An example of a well-known derivatization reagent that is commonly used is (hydrazinocarbonylmethyl) trimethylammonium chloride, also called Girard's Reagent T (GirT).[(1)] GirT is the same as conventional carbonyl reagents such as hydrazines and semicarbazides, and forms a Schiff-base-type aqueous condensate (hydrazone inductor) with various aldehydes and ketones in an acetic acid alcohol solution. After derivatization of the target molecule by GirT, the GirT-labeled (GirT-) target molecule gains a hydrophilic property and quaternary ammonium group. The quaternary ammonium group imparts a cationic property. Thus, in MS measurements, we can easily detect the target molecule even if the original chemical structure is difficult to ionize. In addition,

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another derivatization advantage of GirT is that volatile molecules such as flavor compounds become stable due to an increase in their boiling point.(2)

Japanese Black (Wagyu) cattle account for more than 90% of the beef cattle raised in Japan and are characterized by a high degree of marbling. Intramuscular fat is the fat deposited between and within muscle cells and is also called Marbling or Sashi.

Recently, Wagyu beef has become increasingly popular in Japan and throughout the world, where it is prized for its tenderness, juiciness and Wagyu aroma, three of its most desirable qualities. Wagyu flavor is characterized by a sweet and fatty aroma that is produced when the beef is heated and is considered to be unique to Wagyu beef. Lactones are the main components considered to be responsible for the distinctive peach or coconut-like sweet aroma unique to Wagyu beef.(3,4)

Although breeding and fattening methods are being actively studied to produce high-quality Wagyu beef,(5) the location and number of lactones in the meat have not been investigated in detail. Therefore, we aimed to elucidate the localization of lactones using imaging mass spectrometry. However, normal lactones evaporate at atmospheric pressure as well as under vacuum conditions. Thus, it was necessary to identify a derivatization method that permits detection while avoiding evaporation under vacuum. Lactones have ketone groups in their chemical structures that react with GirT, resulting in an increase in their boiling point following derivatization. Derivatization methods are commonly used for chromatography analysis to change the physical characteristics of target molecules, namely, altering their volatility temperature and thermal stability, and to avoid column adsorption in gas chromatography (GC),(6) as well as to lower the detection limit in liquid chromatography.(7) In addition, GirT can also yield ions that are detectable in positive mode due to the positive ions in its structure.

Mass spectrometry imaging (MSI) is an analytical method that can clarify the localization of substances in tissue sections by performing matrix-assisted laser desorption/ionization (MALDI)-MS in two dimensions,(8,9) and it has been attracting increasing attention in agricultural food-related fields in recent years.(10,11) In addition, the fatty acid composition is an indicator of beef quality and is one of the most important parameters of Wagyu beef. We have already reported a fatty acid analysis with a near-infrared fiber-optic detection method.(12)

In this study, we aimed to derivatize lactones on beef sections using GirT and visualize the derivatized lactones using MALDI-MSI. Since oleic acid (OA) content is particularly important, localization analysis of OA was also conducted using MSI.

2. Data, Materials, and Methods

One Japanese Black steer (31.6 months of age) obtained from the Japanese meat market was used in this study. After slaughter, the *longissimus thoracis* muscle was collected and cooled for 14 days before being used as a sample for aging.(13) The beef samples were embedded into super cryo embedding medium (Section Lab Co., Ltd., Hiroshima, Japan), flash frozen in liquid N_2, and stored at −80 °C until use.

2.1 MALDI-MS and MSI analysis of lactone standards

The following GirT-target solutions were prepared: 10 mM γ-nonalactone (γ-NL), γ-decalactone (γ-DL), and γ-undecalactone (γ-UdL) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in methanol. A 100 μL aliquot of each sample solution was mixed with 100 μL of 100 mM GirT (Tokyo Chemical Industry Co., Ltd.) in 20% acetic acid in a sealed 1.5 mL tube. The mixture was allowed to react at room temperature for 10 min. After the reaction, each sample was prepared at 5 nM, 50 nM, 500 nM, 5 μM, and 50 μM in methanol. For comparison, individual nonderivatized target solutions were prepared. A 1.0 μL aliquot of GirT-lactones and nonderivatized target solutions were placed on a target plate using a pipette. A 15 mg/mL solution of the matrix 2,5-dihydroxybenzoic acid (DHB) was suspended in 6 mL of 90% acetonitrile and sprayed onto each spot using an automated pneumatic sprayer (TM-Sprayer; HTX Tech., Chapel Hill, NC, USA). A total of 14 passes were sprayed using the following conditions: flow rate of 130 μL/min, air flow of 10 psi, and nozzle speed of 800 mm/min. Ionization of the GirT-lactones and nonderivatized targets was confirmed by MALDI-TOF-MS (rapifleX; Bruker Daltonics GmbH, Bremen, Germany). Signals between *m*/*z* 150 and 700 were corrected in positive ion detection mode. The laser power was optimized to minimize the in-source decay of targets. The same measurements were also taken 60 min after introduction to the mass spectrometer to check for changes over time. To examine the detection efficiency of GirT-lactones and nonderivatized lactones, spectral data from 20 locations were randomly selected from each 50 μM sample spot, and the signal-to-noise ratio was calculated. We calculated the limit of detection (LOD) and coefficient of determination (R^2) from semilog plots of the signal intensity versus amount to compare the sensitivity between GirT-lactones. LOD is calculated as 3.3 × (s/a), where s is the standard deviation of the signal intensity and a is the slope of the calibration curve.

2.2 MSI of beef sections

2.2.1 Preparation of beef sections for MSI detection of lactones

Beef samples were cut into serial sections (10 μm) using a cryostat (NX70; Thermo Fisher Scientific, Waltham, MA, USA). The beef sections were transferred by thaw mounting onto indium tin oxide glass slides as the conductive transparent electrode (Bruker Daltonics GmbH) for MSI. In the same manner, serial sections were prepared on glass slides for hematoxylin and eosin (H&E) staining. H&E staining was performed according to a standard protocol.

For MSI, a 60 mM GirT solution in 20% acetic acid was sprayed onto the beef sections using an artistic airbrush (Procon Boy FWA Platinum 0.2 mm caliber airbrush; Mr. Hobby, Tokyo, Japan). To enhance the reaction efficiency of GirT on sections, the GirT sprayed sections were allowed to react at room temperature for 10 min. After derivatization, DHB was sprayed onto the sections using an automated pneumatic sprayer under the same condition as above. For the measurement of OA, we used serial sections. A 6.7 mg/mL solution of the matrix 9-aminoacridine (9AA) was suspended in 6 mL of 70% ethanol and sprayed onto each spot using an automated

pneumatic sprayer. A total of 8 passes were sprayed using the following conditions: flow rate of 150 μL/min, air flow of 10 psi, and nozzle speed of 1000 mm/min.

Ionization and imaging of the GirT-lactones and OA were confirmed by MALDI-TOF-MS (rapifleX; Bruker Daltonik GmbH). To detect the laser spot areas, the sections were scanned, and the laser spot areas (200 shots) were detected with a spot-to-spot center distance of 80 μm in each direction of the beef sections. Signals between *m/z* 200 and 1000 were corrected. The section surface was irradiated with YAG laser shots in the positive ion detection mode for lactones and negative ion mode for OA. The laser power was optimized to minimize the in-source decay of targets. The obtained MS spectra were reconstructed as MS images with a mass bin width of m/z 0.1 from the exact mass using Flex Imaging 5.1 software (Bruker Daltonics GmbH). Optical images of beef sections were obtained using a scanner (Nanozoomer, Hamamatsu Photonics, Hamamatsu, Japan), followed by MALDI-TOF-MSI of the sections.

3. Results and Discussion

3.1 Measurement of GirT-lactones by MALDI-TOF-MS and MS/MS analysis

The detected masses of GirT-standards, γ-NL (*m/z* 270.2), γ-DL (*m/z* 284.2), and γ-UdL (*m/z* 298.3) increased by 114.1 Da compared with the original masses (MW 156.1, 170.1, and 184.2, respectively) (Fig. 1). Tandem MS detected a characteristic signal representing a GirT-derived fragment from GirT-lactones on both the standards and sections (Table 1). Representative MS

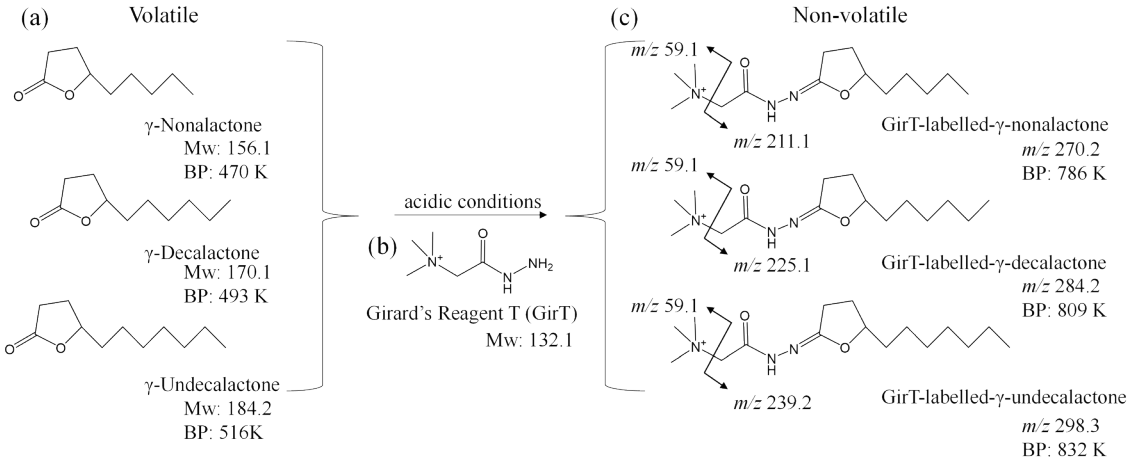

Fig. 1. Schematic of (a) targeted lactones, (b) GirT, and (c) GirT-lactones and product ions. BP: boiling point

Table 1
Precursor ions and product ions for GirT-targets by tandem MS analysis

Target	Precursor ion (*m/z*)	Product ion (*m/z*)
GirT-γ-nonalactone (standard)	270.2	211.1
GirT-γ-nonalactone (on section)	270.2	211.1
GirT-γ-decalactone (standard)	284.2	225.1
GirT-γ-decalactone (on section)	284.2	225.1
GirT-γ-undecalactone (standard)	298.3	239.2
GirT-γ-undecalactone (on section)	298.3	239.2

spectra of GirT-γ-NL, GirT-γ-DL, and GirT-γ-UdL, are shown in Fig. 2. Unlabeled γ-NL was marginally detected with high noise spectra (data not shown). Non-GirT-γ-DL and γ-UdL were not detected. Product ions of GirT-γ-NL (*m*/*z* 211.1), GirT-γ-DL(*m*/*z* 225.1), and GirT-γ-UdL (*m*/*z* 239.2) were correlated with the minus chlorine region (Δ59.1), indicating that GirT successfully reacted with the target molecules on the standards and sample sections.

Next, the detection efficiencies of GirT-lactones and nonderivatized lactones were calculated from the signal-to-noise (S/N) ratio (Table 2). The results showed that both GirT-γ-NL and nonderivatized γ-NL were detected, and the S/N ratio was 2.5-fold higher for GirT-γ-NL than for the nonderivatized γ-NL. While GirT-γ-DL and GirT-γ-UdL were detected, nonderivatized γ-DL and γ-UdL were not detected. This result suggests that the ionization efficiencies of γ-DL and γ-UdL are quite low. Although the S/N ratios for GirT-γ-DL and -γ-UdL were 8.2- and 2.9-fold lower, respectively, than that for GirT-γ-NL, GirT derivatization appears to enable the detection of lactone family members with long carbon chains.

The detection efficiency over time was also examined. GirT-γ-NL showed no change in S/N ratio after 60 min compared with that at 0 min, whereas the S/N ratio for nonderivatized γ-NL decreased by about 60%. For GirT-γ-DL and GirT-γ-UdL, no change in S/N ratio was observed over time (Table 2).

Organic compounds with a boiling point (BP) below 523 K are defined as volatile organic compounds. Thus, nonderivatized γ-NL, γ-DL, and γ-UdL (BP: 470, 493, and 516 K) were not present or their concentration decreased due to volatilization during measurement. In contrast, the detection of GirT-lactones was stable over time. Using chemical information software

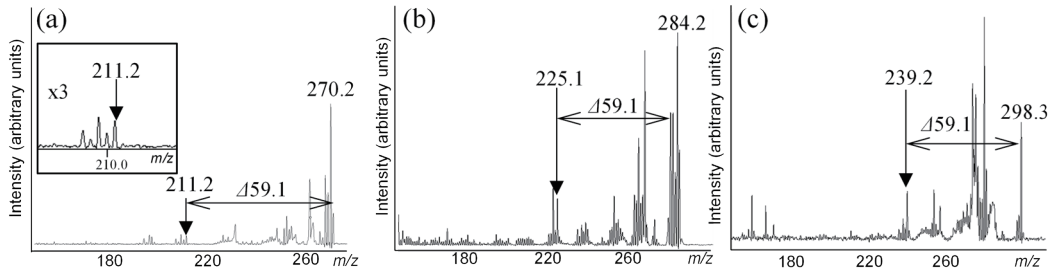

Fig. 2. MS spectra of (a) GirT-γ-NL, (b) GirT-γ-DL, and (c) GirT-γ-UdL on section.

Table 2
Comparison of detectable time between GirT-lactones and unlabeled lactones.

	S/*N* ratios for derivatized standard lactones			
	0 min.		60 min	
Target	Nonderivatization	GirT	Nonderivatization	GirT
γ-Nonalactone	123 ± 61	305 ± 121	51 ± 32	322 ± 155
γ-Decalactone	N.D.	37 ± 18	N.D.	75 ± 31
γ-Undecalactone	N.D.	103 ± 41	N.D.	88 ± 39

(ChemDraw Prime 19, Hulinks, Tokyo, Japan), we estimated the theoretical boiling points of GirT-γ-NL, GirT-γ-DL, and GirT-γ-UdL to be 786, 809, and 832 K, respectively, indicating that the GirT-lactones did not volatilize during measurement.

The LOD for the GirT-lactones was 355-768 fmol (Table 3). Additionally, the coefficient of determination (R^2) obtained from semilog plots of the signal intensity versus quantity indicated high precision. These results indicate that the sensitivity is higher for GirT-lactones than for the nonderivatized lactones. Therefore, we investigated the localization of lactones on beef sections using GirT-derivatized MSI.

3.2 MSI of beef sections

GirT-γ-NL, GirT-γ-DL, GirT-γ-UdL, and OA were detected on beef sections and imaged at *m/z* 270.2, 284.2, 298.3 and 281.2, respectively. H&E staining and MSI images of GirT-lactones and OA in beef sections are shown in Fig. 2. In this research, we imaged a small section to investigate whether there is a correlation between the production of lactones and adipose tissue. The muscle and intramuscular fat regions were observed from the H&E images [Fig. 3(a)]. All lactones were imaged in fat regions [Figs. 3(b)–3(d)]. On the other hand, free OA was mainly localized in muscle regions [Fig. 3(e)]. OA is a precursor molecule of lactones and is considered to be converted to lactones through aging and cooking;[14] however, the evidence supporting this remains unclear.[15] If the OA present in fat tissues is used in the synthesis of lactones, the observed OA intensity should be lower in fat tissues versus muscle regions. In fact, lactones imaged at adipose region although OA marginally imaged at adipose region. The MSI data suggests that unsaturated fatty acids, such as OA stored in intramuscular fat as neutral fat, are converted to the lactones that are detected in the adipose regions on the sections. Moreover, most fatty acids in muscle are incorporated in triacylglycerols and not as free fatty acids, although some fatty acids are also present as phospholipids and free fatty acids. In addition, the plasma-

Table 3
GirT-lactones for correlation coefficients and detection limits.

Lactone	LOD (fmol)	Linearity (R^2)
GirT-γ-nonalactone	442	0.998
GirT-γ-decalactone	355	0.999
GirT-γ-undecalactone	768	0.997

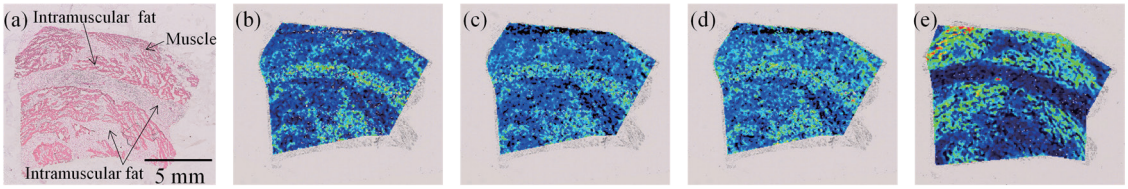

Fig. 3. (Color online) GirT-based imaging mass spectrometry of lactones and oleic acid. (a) H&E-stained image of beef section. Distributions of (b) γ-NL, (c) γ-DL, (d) γ-UdL, and (e) OA.

borne long-chain free fatty acids readily enter skeletal muscle cells, after which they are oxidized or esterified and a fraction remains free (non-esterified). Hence, free fatty acids in muscle are preferentially used for life-sustaining energy production.[16]

4. Conclusions

To the best of our knowledge, this is the first study on the direct and simultaneous visualization of several major aroma molecules in beef. This approach demonstrates the utility of derivatization in MS measurements. Although quantification by MALDI-MSI can be challenging because the signal intensity depends on many factors (e.g., ionization efficiency, extraction efficiency from tissues, and sample preparation), the GirT reagent prevents the volatilization of molecules such as lactones. MSI is a powerful tool for the direct visualization of biomolecules in biological tissues. Our imaging results for the localization of lactones in fat tissues suggest that OA is a precursor molecule of lactones, whereas free OA is mainly localized in muscle. We anticipate that the clarification of the localization of lactones and OA, both of which are important in Wagyu beef, in beef tissues will contribute to improvements in breeding and fattening methods.

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