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Detection of changes in the structure and distribution map of triacylglycerol in fatty liver model by MALDI-SpiralTOF

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ABSTRACT

Matrix-assisted laser desorption/ionisation spiral orbit-type time-of-flight mass spectrometry (MALDI-SpiralTOF) can analyse lipid profiles and characterise lipid structure. Imaging mass spectrometry (IMS) also provides distribution maps of selected *m/z* values. Here, we investigated triacylglycerol (TG) structure and distribution using these technologies to estimate mouse fatty liver. The distribution and intensity of the most intense mass spectrum ion was indicated by IMS at *m/z* 881.7 (52:2). Analysis using MS/MS showed a structural change between liver TG and dietary TG. These findings suggest that MALDI-SpiralTOF is a powerful tool for clinical screening and estimating fatty liver.

1. Introduction

Lipids are important biological substances that are involved in signalling roles and energy storage, and include a diverse class of molecules including structural isomers. Among them triacylglycerol (TG) is a major component of food and animal fats that is closely associated with lifestyle-related diseases, such as type 2 diabetes and coronary heart disease. Hamaguchi et al. reported that non-alcoholic fatty liver disease (NAFLD) is likely to occur in patients with metabolic syndrome [1]. Because visceral fat significantly affects human health, we analysed TG in fatty liver.

TG comprises a diverse class of molecules, and this has caused difficulties with analysing the structure and/or identifying TG species in foods and animal fats. However, contemporary analytical equipment now permits the screening of many molecular species of lipids. Mass spectrometric approaches, including NMR, have been applied to lipidomic analyses [2] and TG has been structurally characterised by Cheng et al. [3] using fast atom bombardment (FAB) and tandem magnetic sector mass spectrometry with high-energy collision-induced dissociation (CID) fragmentation of $[M + Na]^+$ species. Pittenauer et al. [4] recently showed that tandem time-of-flight (TOF/TOF) mass spectrometry can provide the same complete structural information as tandem magnetic sector mass spectrometry. Kubo et al. [5] recently used matrix-assisted laser desorption/ ionisation spiral orbit-type time-of-flight (MALDI-SpiralTOF) mass spectrometry featuring high precursor ion selectivity to structurally analyse TG in olive oil. Monoisotopic precursor selection allows the generation of mass spectra without interference from species that differ by only a single double bond. Kubo et al. structurally determined all TG including structural isomers by interpreting the charge-remote fragmentation resulting from high-energy CID of sodiated TGs. In addition to structural analyses, MALDI-SpiralTOF allows imaging mass spectrometry (IMS) of lipids via distribution maps of selected specific *m/z* values in biological specimens. Satoh et al. [6] used IMS to analyse

Abbreviations: CID, collision-induced dissociation; HE, haematoxylin and eosin; HF, high-fat diet; HF + Res, high-fat diet supplemented with 0.2% resveratrol; IMS, imaging mass spectrometry; MALDI-SpiralTOF, matrix-assisted laser desorption/ionisation spiral orbit-type time-of-flight mass spectrometry; TG, triacylglycerol; TOF/TOF, tandem time-of-flight

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Table 1
Composition of experimental diets and food intake of mice in the three groups.

Composition of experimental diets	Control diet*	High-fat (HF) diet	HF + Res diet
Carbohydrates (%)	78.5	35	35
Fat (%)	3.8	50	50
Protein (%)	17.7	15	15
Resveratrol (%)	0	0	0.2
Mean food intake (g/mouse/week)	22.0	18.2	18.6
Mean Caloric intake (kcal/mouse/week)	75.3	86.6	88.5

* CLEA rodent diet CE-7 was used for the control diet (Clea Japan, Ltd., Tokyo, Japan).

phosphatidylcholine in rat brain tissues. They could separate doublet peaks differing by <0.1 μ, which would otherwise be problematic when using low-resolution reflectron-type time-of flight mass spectrometry. We used these technologies to analyse lipid profiles and structures in fatty liver.

The severity of fatty liver, including NAFLD, is clinically estimated by histological assessment under microscopy. However, such estimations of severity depend on medical evaluator skills. The application of mass spectrometry technology to estimating fatty liver would likely yield objective and informative data that could also be useful in screening for this condition. This technology also has the potential to provide new information regarding the mechanism of fatty liver. To our knowledge, fatty liver has not been assessed using MALDI-SpiralTOF. Although TG and fatty acid have often been analysed using LC/MS/MS [7] this modality cannot generate information about the position of a fatty acid on the glycerol backbone or distribution maps of a target TG in tissues. From this perspective, MALDI-SpiralTOF is highly suitable for evaluating fatty liver and other related lipids.

Here, we investigated whether TG could be analysed in mouse models of fatty liver using two MALDI-SpiralTOF technologies. One was a structural analysis of TG using TOF/TOF with monoisotopic ion selection as described by Kubo et al. [5], and the other was IMS analysis according to Satoh et al. [6]. We compared groups of mice with different severities of fatty liver to assess whether these technologies could distinguish them. One group had severe fatty liver that was generated by feeding with a high-fat diet, and the other had ameliorated fatty liver that was generated by feeding with a high-fat diet supplemented with 0.2% resveratrol, which is a polyphenol extracted from red grapes that improves metabolic disease and lipid metabolism [8,9] as well as the fatty acid content of the liver [10].

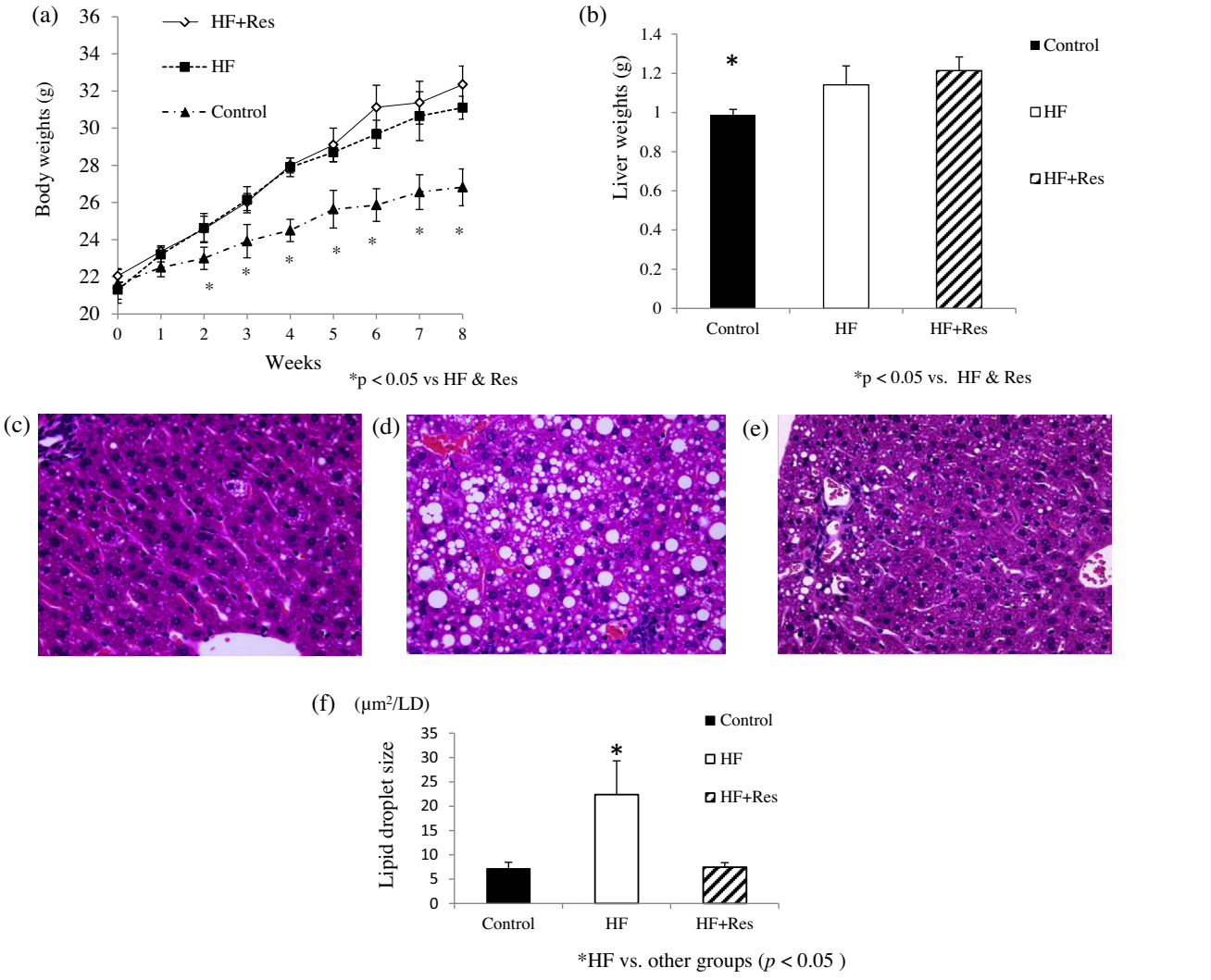

Fig. 1. Body and liver weights, and liver histology in mice fed with high-fat (HF) diet, high-fat diet with 0.2% resveratrol (HF + Res) or a control diet. Body weight (a), and liver weight (b). Liver histology of haematoxylin and eosin stained liver sections from representative mice in each group. Control diet (c), HF diet (d) and HF + Res diet (e). Sizes of lipid droplets in livers were evaluated using WinROOF software ver. 6.5 (f). Original magnification, (a, b, and c) 100×.

The results of the MALDI-SpiralTOF analysis showed structural differences in TG extracted from the liver and the diet, specific intense peaks, and distribution maps. The data suggested that MALDI-SpiralTOF analysis could be a powerful tool for lipid analysis in patients with fatty liver.

2. Materials and methods

2.1. Animals and diets

All protocols proceeded in accordance with the Animal Experimentation Guidelines of the National Defence Medical College (No. 12067). Six-to-seven-week-old male C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) were maintained in a temperature-controlled facility with fixed 12/12-h light/dark cycles. The mice were given access to water and a 342.7 kcal/100 g Rodent Diet CE-7 control diet (Clea Japan Ltd., Tokyo, Japan), high-fat (HF) diet (475.96 kcal/100 g), or a high-fat diet supplemented with 0.2% resveratrol (HF + Res; 475.96 kcal/100 g) for 8 weeks (Table 1). The mice were then anaesthetised with sodium pentobarbital and blood was withdrawn from the heart using a heparinised needle and syringe. Livers were collected and stored at −80 °C.

2.2. Histological assessment and estimation of lipid droplet size

Mice fed with the HF, HF + Res or control diets (n = 8 per group) were euthanised after 8 weeks. Liver samples were fixed in 20% formalin for two days, embedded in paraffin, cut into 4-μm sections and stained with haematoxylin and eosin (HE). Images of HE-stained slides were captured using WinROOF software ver. 6.5 (Mitani Co., Tokyo, Japan). The sizes of lipid droplets (n = 6 per group) were estimated according to the manufacturer's instructions.

2.3. Extraction of lipids from the liver and determination of TG structure by MALDI-SpiralTOF-TOF (MS/MS)

Lipids were extracted from liver samples according to the Folch method [11]. Briefly, liver samples were homogenised in 10 mL of chloroform:methanol (2:1) per 0.5 g of tissue and incubated overnight at 4 °C. The liquid phase of homogenates recovered by centrifugation (1000g) was filtered, washed with water, separated by centrifugation again (1000g) and the upper phase of the mixture was removed. The chloroform phase was evaporated under a stream of nitrogen gas to a volume of 1 mL, and the extracted lipid was analysed. The matrix compound, 2,5-dihydroxybenzoic acid (DHB; Sigma Aldrich, St. Louis, MO, USA), was dissolved in tetrahydrofuran (THF; Wako Ltd., Osaka, Japan) at a concentration of 20 mg/mL. Lipid samples were mixed with the DHB solution at a ratio of 1:1 (v/v) and then 1 μL of the mixture was deposited on a stainless steel MALDI sample plate using the dried-droplet method. A JMS-S3000 MALDI-SpiralTOF-TOF (JEOL Ltd., Akishima, Japan) instrument was used for MS/MS measurements.

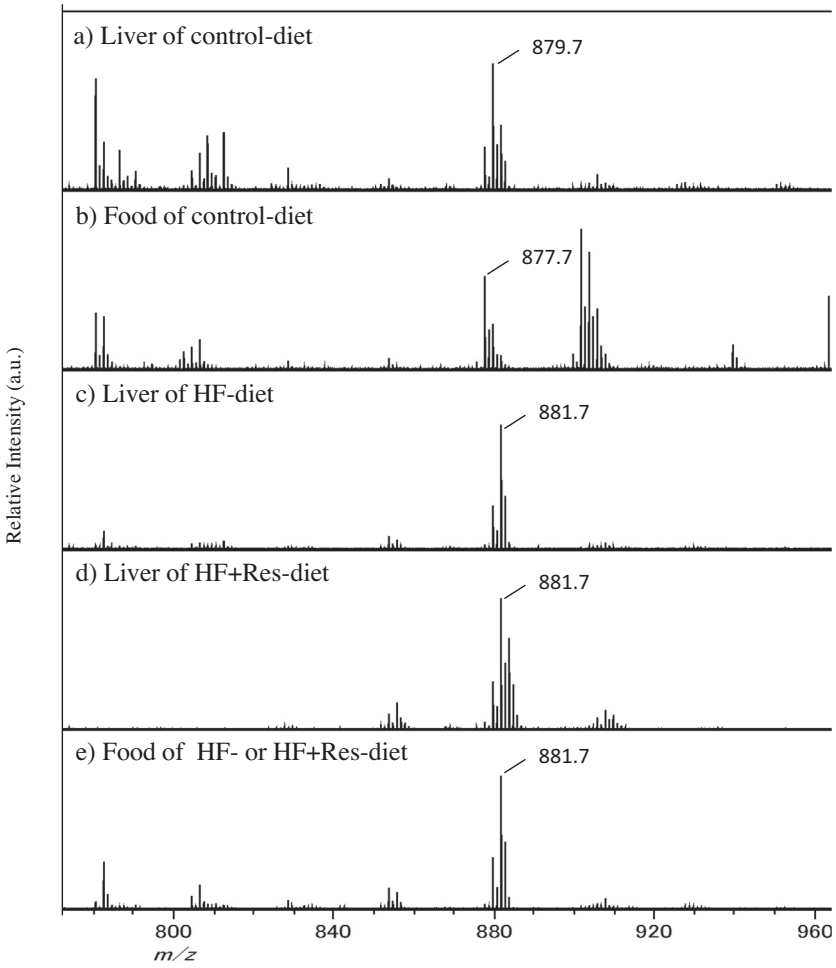

Fig. 2. Representative mass spectra of lipid extracts from liver or food. Lipid extracts from livers of mice fed with control diet (a) and from extracts of control food (b). Lipid extracts from livers of mice fed with HF (c) and HF + Res (d) diets. Mass spectral profiles of lipids extracted from HF and HF + Res diets ranged from m/z 800-960; thus representative data are shown (e).

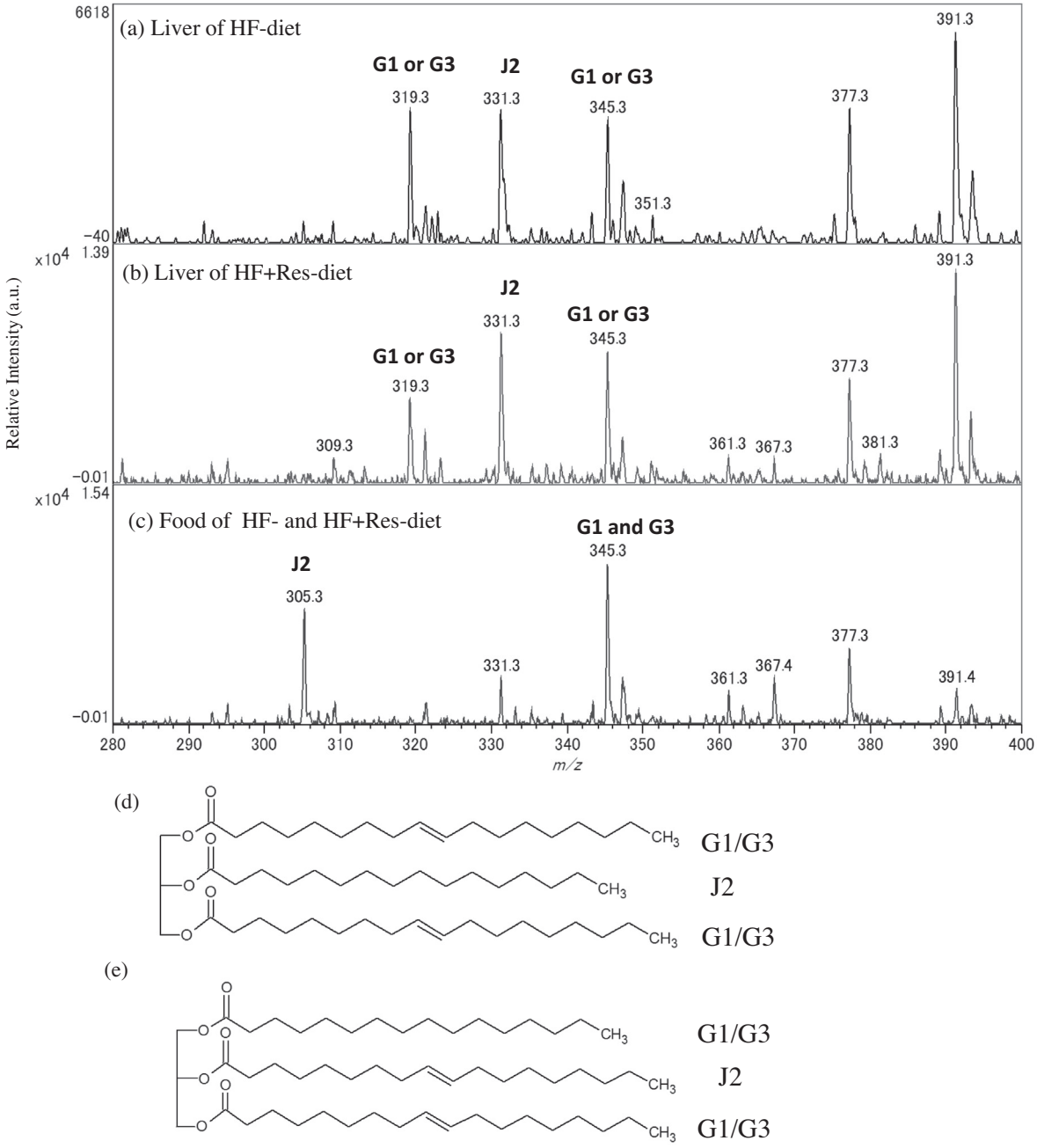

Fig. 3. Product ion spectra of the ions for precursor at *m/z* 881.7 from liver or food extracts. Product ion spectrum from livers of mice fed with HF (a) and HF + Res (b) diets from extracts of HF or HF + Res diets (c). Structure of 1-oleoyl-2-palmitoyl-3-oleoyl-glycerol rac-glycerol in TG extracted from HF and HF + Res diets (d). Structure of 1-palmitoyl-2-oleoyl-3-oleoyl-rac-glycerol in TG extracted from livers of mice fed with HF and HF + Res diets (e).

2.4. IMS by MALDI-SpiralTOF

The mice were anesthetised, sacrificed and dissected after 8 weeks of feeding with the experimental diets. Liver blocks were immediately frozen in liquid nitrogen and stored at −80 °C to minimise degradation. Tissues were sliced into 10-μm-thick sections using a cryostat, and thaw-mounted on indium-tin oxide glass sample plates (HST Inc., Newark, NJ, USA). The DHB matrix was dissolved in methanol (Kanto Chemical. Co. Inc., Tokyo Japan) to a concentration of 30 mg/mL and a total of 2 mL was manually sprayed using a Prokon Boy WA double action platinum airbrush (0.2 mm injection nozzle) onto tissue sections. A MALDI-SpiralTOF instrument was equipped with a 349-nm Nd:YLF laser for MALDI and the laser repetition rate was 250 Hz for IMS. Mass spectra were acquired for IMS in 2.0 × 2.0-mm regions and divided into 0.04-mm squares per pixel. The mass range of *m/z* 500–1000 was measured over a period of 4.5 h. Raw data from the JMS-S3000 were converted to the imzML format and analysed using Biomap 3.8.0.4 software (http://www.maldi-msi.org/index.php?option=com_content&view=article&id=14&Itemid=32, Dornach, Switzerland).

3. Results and discussion

3.1. Body and liver weight

The mice on HF, HF + Res and control diets consumed 18.2 (86.6 calories), 18.6 (88.5 calories) and 22.0 (75.3 calories) g/mouse/week, respectively (Table 1). The mean body weights of the mice

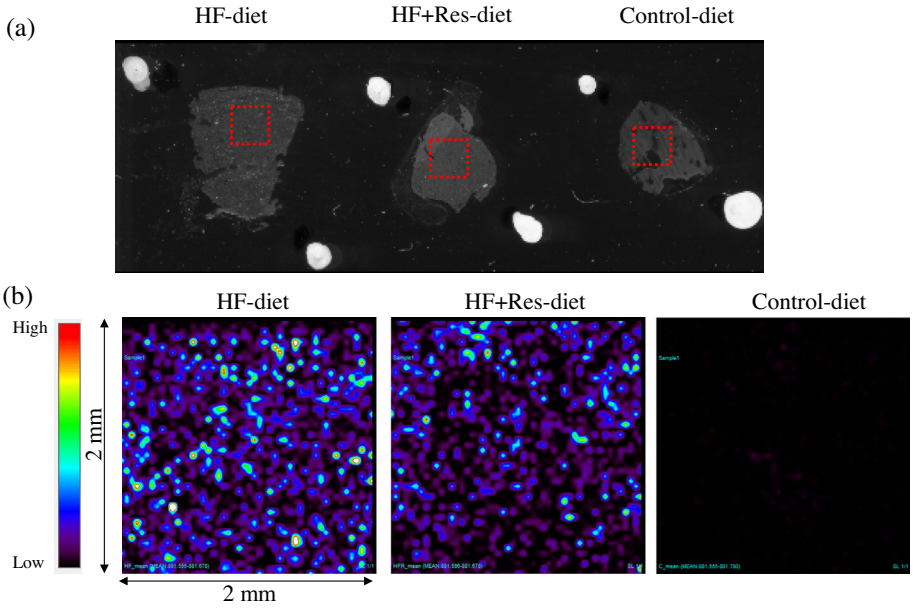

Fig. 4. Distribution map of monoisotopic ion at *m/z* 881.7 in livers of mice fed with three diets generated by imaging mass spectrometry (IMS). Preparation of liver specimens after the three diets. Region surrounded by red dotted line was analysed by IMS (a). Distribution map was captured at *m/z* 881.7 in each specimen (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fed with the HF, HF + Res and control diets at 8 weeks were 32.2, 33.4 and 26.2 g, respectively (Fig. 1a). The mice fed with both HF diets were significantly heavier than those fed with the control diets after 2 weeks. Likewise, the livers were significantly heavier from mice fed with the HF and HF + Res diets than with the control diet for 8 weeks (Fig. 1b).

3.2. Liver histology and lipid droplet size

Fig. 1c–e shows liver sections stained with haematoxylin and eosin (HE). Lipid droplets were larger and more concentrated throughout the livers of mice fed with the HF diets (Fig. 1d). The sizes of lipid droplets in the liver were obviously reduced in the mice fed with the HF + Res, compared with the HF diet (Fig. 1e). Lipid droplets were small and scant in liver samples from mice fed with the control diet (Fig. 1c). The lipid droplets on HE-stained slides evaluated using the WinROOF software were significantly larger from mice fed with the HF than the other diets (Fig. 1f). These results indicated that resveratrol helped to ameliorate fatty liver as described (8–10). The HF, HF + Res and control diets generated severe, mild and absent fatty liver, respectively.

3.3. Mass spectral profiles from food and liver TG

Fig. 2a–e shows mass spectral profiles from *m/z* 800 to 925 in the three groups of mice. The range of ions at *m/z* 800–925 was regarded as being TGs [5]. The most intense peak identified in liver and food extracts from both HF and HF + Res diet samples was located at *m/z* 881.7 (Fig. 2c–e). The HF diet and HF + Res diets themselves were analysed, and the mass spectral profiles were in the range of *m/z* 800–925. Fig. 2e shows representative data for TGs from the HF and HF + Res diets. The most intense peaks in liver extracts from mice fed with the control diet and in extracts from the control diet were located at *m/z* 879.7 (Fig. 2a) and *m/z* 877.7 (Fig. 2b). These differences were caused by the sources of the dietary lipid. Most lipids in the HF diet and HF + Res diet were derived from lard, whereas those in the control diet were from soybean oil. These findings suggest that the source of lipid directly affects the type of TG that accumulates in the liver. To confirm the structure of the most intense TG peak in the HF and HF + Res diets, we determined the structure using MALDI-SpiralTOF-TOF.

3.4. Determination of TG structure of the peak at m/z 881.7 by MALDI-SpiralTOF-TOF

Fig. 3a–c compares the product-ion mass spectra for the precursor at *m/z* 881.7 (52:2) from the livers of mice fed with the HF (Fig. 3a), HF + Res (Fig. 3b) diets and food extracted from these diets (Fig. 3c). Peaks characteristic of fatty acid fragmentation are predicted as G- and J-type ions, using the described nomenclature [3]. G1 or G3 in the glycerol backbone was expected to be exterior and J2 was located in the centre. The substituents at *sn*-1 and *sn*-3 (sites that determine stereochemistry) are indistinguishable by mass spectrometry because it cannot identify the steric structure of TG. Therefore, we labelled the fatty acid substituents at positions *sn*-1 and *sn*-3 (exterior sites on the glycerol backbone) as G1/G3. Mass profiling determined the dietary TG structure of as 1-oleoyl-2-palmitoyl-3-oleoyl-rac-glycerol (Fig. 3d), and that in the liver as 1-palmitoyl-2-oleoyl-3-oleoyl-rac-glycerol (or 1-oleoyl-2-oleoyl-3-palmitoyl-rac-glycerol) (Fig. 3e).

These results revealed structural differences between food and liver TG, despite apparently having the same precursor at *m/z* 881.7. Palmitic acid (16:0) was located at either of the exterior G1 or G3 sites on the glycerol backbone in liver TG, but at J2, the central site in dietary TG. These results suggest that dietary TG affects liver TG, but the structures differ between them. Dietary TG is digested and hydrolysed after intake, and then the hydrolysed TG is re-synthesised before it accumulates in the liver. Palmitic acid might be linked to a different site on the glycerol backbone from the original food-derived TG at J2 to the liver TG at G1 or G3 during such re-synthesis.

3.5. Distribution map of TG at m/z 881.7 in livers by MALDI-SpiralTOF

The distribution of TG at *m/z* 881.7 (±0.1 μ tolerance) was determined in thin sections of liver specimens from mice fed with the three diets. Fig. 4a shows the preparation of liver specimens and Fig. 4b shows the distribution map of *m/z* 881.7. The TG peak

was spread throughout the liver specimens in mice fed with the HF diet. The peak intensity diminished in specimens from mice fed with the HF + Res diet and was very low (black-purple circles) in specimens from mice fed with the control diet (Fig. 4b). The average mass spectra of the three different liver specimens were shown in the supplemental Fig. S1. Although the signal at *m/z* 881.7 from both HF and HF + Res diet mice was evidently confirmed (Fig. S1a and b), the signal from control diet mice showed a very weak intensity, so we could recognise only in the enlarged view of mass spectral profiles (Fig. S1c). These results are consistent with the liver histology determined by HE staining (Fig. 1c–e). Peak intensity also reflected the amount of TG in lipid droplets in the liver specimens. Accordingly, abundant TG species in fatty liver can be detected by IMS and the results provided a reliable distribution map of the TG peaks. Compared with histological analyses, IMS provided better semi-quantitative visualisation of preferred TG peaks in liver specimens and would thus serve as a useful tool for clinical and other estimations of fatty liver.

4. Conclusions

Structural differences in dietary and liver TG were distinguished by MALDI-SpiralTOF with IMS which can also provide distribution maps of selected intense mass peaks in liver specimens. Thus, MALDI-SpiralTOF with IMS is a powerful tool with which to study lipids, including evaluations of fatty liver.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.02.005.

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