

# Selective improvement of peptides imaging on tissue by supercritical fluid wash of lipids for matrix-assisted laser desorption/ionization mass spectrometry

メタデータ	言語: eng 出版者: Springer Berlin Heidelberg 公開日: 2018-03-06 キーワード (Ja): キーワード (En): 作成者: Matsushita, Shoko メールアドレス: 所属:
URL	<a href="http://hdl.handle.net/10271/3222">http://hdl.handle.net/10271/3222</a>

**Selective improvement of peptides imaging on tissue by supercritical fluid wash of lipids for matrix-assisted laser desorption/ionization mass spectrometry**

Shoko Matsushita<sup>1-3</sup>, Noritaka Masaki<sup>1,2</sup>, Kohei Sato<sup>4</sup>, Takahiro Hayasaka<sup>5</sup>, Eiji Sugiyama<sup>1,2</sup>, Shu-Ping Hui<sup>5</sup>, Hitoshi Chiba<sup>5</sup>, Nobuyuki Mase<sup>4</sup>, and Mitsutoshi Setou<sup>1,2,6-9,\*</sup>

<sup>1</sup>Department of Cellular & Molecular Anatomy, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan

<sup>2</sup>International Mass Imaging Center, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan

<sup>3</sup>Research Fellow of Japan Society for the Promotion of Science, Kojimachi Business Center Building, 5-3-1 Kojimachi, Chiyoda-ku, Tokyo 102-0083, Japan

<sup>4</sup>Department of Applied Chemistry and Biochemical Engineering, Faculty of Engineering, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan

<sup>5</sup>Health Innovation and Technology Center, Faculty of Health Sciences, Hokkaido University, Kita 12, Nishi 5, Kita-ku, Sapporo, Hokkaido 060-0812, Japan

<sup>6</sup>Preeminent Medical Photonics Education & Research Center, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan

<sup>7</sup>Department of Anatomy, The University of Hong Kong, 6/F, William MW Mong Block 21 Sassoon Road, Pokfulam, Hong Kong SAR, China

<sup>8</sup>Riken Center for Molecular Imaging Science, 6-7-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan

<sup>9</sup>Division of Neural Systematics, National Institute for Physiological Sciences, 38 Nishigonaka Myodaiji, Okazaki, Aichi 444-8585, Japan

**\*Corresponding author:** Prof. Mitsutoshi Setou,

Tel: +81-53-434-5282; Fax: +81-53-435-2468.

E-mail: [setou@hama-med.ac.jp](mailto:setou@hama-med.ac.jp)

**Short title:** Improvement of peptide imaging by scCO<sub>2</sub> wash

## ABSTRACT

There is a high analytical demand for improving the detection sensitivity for various peptides in matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS), because exhaustive distribution analyses of various peptides could help to reveal the function of peptides in vivo. To improve the sensitivity of peptide detection, we used supercritical fluid of CO<sub>2</sub> (scCO<sub>2</sub>) as washing solvent for a pretreatment to remove lipids. We evaluated whether our wash method using scCO<sub>2</sub> with an entrainer improved the detection of peptides and suppressed lipid detection in MALDI-IMS. Our analysis revealed that the signal intensities of peptides such as  $m/z$  3339.8, 3530.9, 4233.3, 4936.7, and 4963.7 were increased in scCO<sub>2</sub>-washed samples. The greatest improvement in the signal-to-noise ratio (S/N) was found at  $m/z$  4963.7, which was identified as thymosin  $\beta$ 4, with the S/N reaching almost 190-fold higher than the control. Additionally, all of the improved signals were associated with the morphological structure. Our method allows us to analyze the distribution of molecules, especially in the region of  $m/z$  3000-5200. For these improvements, the polarity difference between scCO<sub>2</sub> and the matrix solution used was considered as a key. A wider variety of molecules can be analyzed in the future due to this improvement of the detection sensitivity by optimizing the polarity of scCO<sub>2</sub> with various entrainers.

**Key words:** imaging mass spectrometry, supercritical fluid of CO<sub>2</sub>, peptides, pretreatment, matrix-assisted laser desorption/ionization

## **Introduction**

Distribution analyses of peptides are performed using positron emission tomography (PET) by isotope-labeled peptides [1], immunohistochemistry [2], imaging mass spectrometry (IMS) [3], and other techniques. Each of these methods has distinct advantages and disadvantages. The advantage of PET is that it allows non-invasive distribution analyses with pharmacokinetics of an injected peptide [4], and the advantage of immunohistochemistry is that it provides detailed information at sub-cellular spatial resolution [5]. The disadvantage of both PET and immunohistochemistry is the limitation in the number of peptide species that are simultaneously detected. To evaluate the relationships and the functions of peptides associated with morphological structure in the body, comprehensive distribution analyses are needed [3]. IMS is a powerful tool that satisfies the requirement of simultaneous analysis of various molecules without labeling [3]. Matrix-assisted laser desorption/ionization (MALDI)-IMS in particular provides direct information about the localized peptides since MALDI-IMS analyses enables the simultaneous detection of various intact molecules without fragmentation [6]. However, the peptide analysis that MALDI-IMS provides is not satisfactory, due to the low signal intensities of peptides. Improving the sensitivity of peptide detection by MALDI-IMS is thus a major analytical demand.

Several research groups have attempted to improve the detection of peptides by reducing the ion suppression caused by lipids and salts, which are abundant in tissue [7]. For reducing the level of lipids in the tissue, many types of organic solvent [8, 9] have been used in MALDI-IMS analyses. On the other hand, supercritical fluids have been used for the extraction or separation of various lipids in chromatographic analyses [10].

A supercritical fluid can easily infiltrate deeply into tissue and is expected to clear specific molecules completely, since supercritical fluids have low viscosity (e.g. under  $8 \times 10^{-5}$  Pa•s for CO<sub>2</sub>) [11] and high diffusivity (e.g. under  $10^{-4}$  cm<sup>2</sup>/s for CO<sub>2</sub>) [12]. In addition, a supercritical fluid can be coupled with other types of organic solvent (called "entrainer") to effectively remove other molecules such as phospholipids [12]. Among the various types of supercritical fluid, the supercritical fluid of CO<sub>2</sub> (scCO<sub>2</sub>) is widely used, since it can be used under relatively mild conditions (over 7.38 MPa and 31.1°C). In addition, CO<sub>2</sub> is inexpensive and nontoxic [13]. We hypothesized that the use of scCO<sub>2</sub> for removing lipids could maintain the spatial distributions of peptides and enhance their signals.

In this study, we used scCO<sub>2</sub> as a wash solvent in a pretreatment for a MALDI-IMS analysis, and we evaluated whether our method could improve the detection of peptide molecules. We used methanol (MeOH) as the entrainer to remove phospholipids more effectively [12].

## **Materials and Methods**

### *Chemicals*

We purchased 1,5-diaminonaphthalene (DAN) from Tokyo Chemical Industry Co. (Tokyo) and obtained 2,5-dihydroxybenzoic acid (DHB), peptide calibration standard, and protein calibration standard I from Bruker Daltonics (Billerica, MA, USA). Cesium iodide (CsI), bradykinin fragment 1–7, and angiotensin II were purchased from Sigma-Aldrich (St. Louis, MO). Liquid chromatography/mass spectrometry-grade ultrapure water, MeOH for the spray in the matrix application, and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

High-performance liquid chromatography (HPLC)-grade MeOH for the scCO<sub>2</sub> pretreatment was purchased from Kanto Chemicals (Tokyo). Thymosin β<sub>4</sub> was purchased from Tocris Bioscience (Bristol, UK).

### *Animals*

All experiments were conducted according to protocols approved by Animal Care and Use Committee of Hamamatsu University School of Medicine. C57BL/6J mice Eight-weeks-old female mice were purchased from Japan SLC (Hamamatsu, Japan). Mouse brains were rapidly frozen in powdered dry ice after harvest. The tissue was mounted on a sample holder using O.C.T. compound (Sakura Finetek Japan, Tokyo), and sectioned at 10-μm thickness along the sagittal axis by a cryostat system (CM1950; Leica Biosystems, Wetzlar, Germany) with the temperature of the working area maintained at -16°C. Sections were thaw-mounted on electroconductive glass slides coated with indium-tin-oxide (ITO) (100Ω; Matsunami Glass, Osaka, Japan). Samples were stored at -80°C until use.

### *Pretreatment*

The entire system for scCO<sub>2</sub> washing is shown in Supplementary Figure S1. scCO<sub>2</sub> fluid was pumped by a PU-1586 Intelligent HPLC Pump (JASCO, Tokyo). The sample glass slides kept in the extractor unit (Nikkaki Bios, Tokyo) were soaked in the scCO<sub>2</sub> fluid (flow rate, 10 mL/min) for 1 hr at 40°C under 20 MPa, with MeOH (flow rate, 5 mL/min with SCF-Get pump; JASCO) used as an entrainer. In a previous report, 94.70±8.45% of phospholipids was extracted within 1 hr in a one-dimensional chromatographic extraction with 20% ethanol [14]. We therefore set the extraction time

in the present study to 1 hr, which was sufficient for removal of the phospholipids. The extract in MeOH was collected in glass vials and dried under a vacuum. The dried extract was stored at 4°C.

#### *MALDI-IMS for $m/z$ 1800-6000*

One mL of matrix solution (DAN; 10 mg/mL in 70% MeOH, 0.1% TFA) was sprayed manually for each section on the glass slide, using an air-brush with a 0.2-mm nozzle (Procon Boy FWA platinum; Mr. Hobby, Tokyo). Tissue samples were analyzed using a MALDI-Fourier transformation ion cyclotron resonance (FT-ICR)-type IMS instrument, the solariX XR (Bruker Daltonics) with a 355-nm Nd:YAG laser at a 2000 Hz repetition rate. The laser power was set at 40%, and 500 laser shots were irradiated for each measured point. The raster scan pitch was set at 100  $\mu\text{m}$ , and a medium laser diameter (approx. 70  $\mu\text{m}$ ) was used.

Measured regions were manually set and data were acquired at  $m/z$  1800-6000 in a positive ion mode. External calibration was performed using CsI clusters, the peptide calibration standard, and protein calibration standard I. The following conditions were used for the analysis: plate offset (100 V), deflector plate (200 V), time of flight delay (3 ms), transient length (2.2 s), and resolving power (23615 at  $m/z$  4961.6). Averaged mass spectra and ion images were reconstructed using flexImaging 4.0 data analysis software (Bruker Daltonics). The signal-to-noise ratio (S/N) was calculated with the data exported by the flexImaging 4.0 program.

## **Results**



During the experiments, neither slippage nor deformation of the tissue sections on the glass slide was observed. Figure 1 shows the spectra at  $m/z$  1800-6000 obtained from mouse brain tissue sections washed by  $scCO_2$  with the entrainer (upper, blue) and the control without the wash (lower, red). Some of the signal intensities, such as  $m/z$  3339.8, 3530.9, 4233.3, 4936.7, and 4963.7, were increased by the  $scCO_2$  wash (Fig. 1a,b; black arrow). The greatest improvement in the S/N was found at  $m/z$  4963.7; the ratio was almost 190-fold greater than the control value (Fig. 1c). In contrast, several signals such as those around  $m/z$  2292.8, 3127.4, and 3888.0 were diminished by the wash with  $scCO_2$  and the entrainer.

The region at  $m/z$  700-900, where the signals of phospholipids (especially phosphatidylcholines; PCs) are concentrated [15], showed few signals in the  $scCO_2$ -washed samples, whereas the corresponding  $m/z$  region in the control samples showed abundant signals (Suppl. Fig. S2a,b). PCs were consistently detected from the extract of a concentrated solution used in  $scCO_2$  washing (Suppl. Fig. S3). These results clearly demonstrated that phospholipids were extracted in the  $scCO_2$  from tissue sections by the wash. Supercritical fluid can easily infiltrate deeply into tissue and is expected to clear specific molecules completely since  $scCO_2$  have low viscosity (under  $8 \times 10^{-5}$  Pa·s) [11] and high diffusivity (under  $10^{-4}$  cm<sup>2</sup>/s) [12].

The distributions of the signals improved by the  $scCO_2$  wash are shown in Figure 2. We classified these signals into four groups according to the distributions. The first group was  $m/z$  3339.8, 3477.9, 3633.0, and 4248.3, which were distributed on the callosum, anterior commissure, stria medullaris, optic nerve, midbrain, white matter of the cerebellum, and medulla oblongata (Fig. 2a). The second group was signals at  $m/z$  3417.9, 3503.9, 3976.2, 4105.2, 4233.3, 4919.7, 4947.7, 4963.7, and 4979.7, which

were similarly distributed on the olfactory bulb, callosum, anterior olfactory nucleus, and hippocampus (Fig. 2b). The third group was signals at  $m/z$  3530.9 and 3544.9, which were similarly distributed on the callosum, anterior commissure, midbrain, and white matter of the cerebellum (Fig. 2c). The fourth group has only one signal at  $m/z$  4936.7, which was distributed on the callosum, striatum, and anterior commissure (Fig. 2d). Signals found only in control samples, such as  $m/z$  2292.8, 3127.4, and 3888.0, were distributed in the cerebral cortex and gray matter of the cerebellum (Suppl. Fig. S4). The region of  $m/z$  500-2500, which includes the  $m/z$  values of several bioactive peptides, was also analyzed (Suppl. Fig. S5a,b), and signals at  $m/z$  942.5 and 1834.0 were found to be associated with the morphological structure on scCO<sub>2</sub>-washed samples (Suppl. Fig. S5c,d).

High-spatial-resolution analysis in the hippocampus revealed that the signal of  $m/z$  4963.7 (which was most improved by the scCO<sub>2</sub> wash) was distributed at CA1 and the dentate gyrus, which are subregions of the hippocampus (Suppl. Fig. S6). The signal of  $m/z$  4963.7 showed almost the same distribution as that reported in a study using MALDI-IMS [16], which assigned the signal as thymosin  $\beta$ 4. Consistently, tandem mass spectrometry (MS/MS) using a MALDI-time of flight (TOF)/TOF-type instrument for the signal, showed almost the same pattern of product ions between the thymosin  $\beta$ 4 standard and the signal obtained from tissue (Suppl. Fig. S7).

It was reported that an alcohol-based wash also improved the detection of thymosin  $\beta$ 4 [16]. We compared the wash of 33% MeOH only and that of scCO<sub>2</sub> with an entrainer since 33% MeOH was used as an entrainer in our method. The results showed that the scCO<sub>2</sub> wash increased the signal intensity more than the 33% MeOH wash did (Suppl. Fig. S8a,b). In addition, phospholipid signals had remained only in the section

washed with 33% MeOH (Suppl. Fig. S8c–e). As 33% MeOH only was not enough to improve the detection of the signal or the removal of lipids by itself, it is clear that scCO<sub>2</sub> is essential for increasing the signal intensity.

For our evaluation of the improvement of signal intensity, we compared washing by scCO<sub>2</sub> with entrainer with conventional washing by two types of organic solvent, MeOH and isopropanol [9]. We observed that the scCO<sub>2</sub>-washed samples showed higher signal intensity of thymosin  $\beta$ 4 compared to the samples washed with MeOH or isopropanol (Suppl. Fig. S9a–c). Additionally, the baseline of the signals was decreased in the washes by scCO<sub>2</sub> with an entrainer compared to the wash with isopropanol around  $m/z$  3000-5000.

## Discussion

The intensity of some signals at  $m/z$  3000-5200 was increased by the wash with scCO<sub>2</sub> (Fig. 1). On the other hand, the intensity of signals at  $m/z$  700-900 corresponding to phospholipids decreased on scCO<sub>2</sub>-washed tissue, and the same signals were detected from extract solution (Suppl. Figs. S2, S3). These results indicate that our method removed phospholipids from tissue sections and reduced the ion suppression by phospholipids, enabling the detection of peptide signals at  $m/z$  3000-5200.

All of the improved peaks from the scCO<sub>2</sub>-washed samples corresponded to the molecular weights of the peptides and were associated with the morphological structure. We classified the peaks into four groups according to the patterns of distribution (Fig. 2, Suppl. Fig. S5). The morphological structures were maintained even in the high-spatial-resolution analysis (Suppl. Fig. S6). These results suggested that the

distribution of detected signals considered to represent peptides was not affected by the scCO<sub>2</sub> wash.

Other signals at  $m/z$  3000-5200 were specific to the control sample and were not detected in scCO<sub>2</sub>-washed samples (Suppl. Fig. S4). It appeared that the scCO<sub>2</sub> wash removed the signals specific to the controls in addition to phospholipids, which is the opposite of the increase in the peptide signal intensities in the scCO<sub>2</sub>-washed samples. We therefore propose that our method allows simultaneous distribution analyses of peptides other than phospholipids.

MS/MS analysis for the highest signal at  $m/z$  4963.7 on the scCO<sub>2</sub>-washed samples identified the signal as thymosin  $\beta$ 4 (Suppl. Fig. S7), and thus the marked improvement of the signal intensity offers benefits for both MALDI-IMS and MS/MS analyses on tissue. Unfortunately, most of the signals from scCO<sub>2</sub>-washed samples were not enough for identification by the MS/MS analysis. Among the unidentified signals, we assigned  $m/z$  4936.7 as thymosin  $\beta$ 10 based on a literature search [16]. We expect that the uses of different entrainers will improve these signal intensities enough to enable an MS/MS analysis to identify the rest of the signals.

The use of 33% MeOH only was not enough to increase the sensitivity of the detection of thymosin  $\beta$ 4 or to remove phospholipids signals (Suppl. Fig. S8). This result suggests that scCO<sub>2</sub> was crucial for our method to increase signal intensities at  $m/z$  3000-5200, and this is because characteristics of scCO<sub>2</sub> such as low viscosity and high diffusivity were essential for the removal of phospholipids with the assistance of a low volume of MeOH. The detection of peptide signals including the signal of thymosin  $\beta$ 4 was considered to be improved by the removal of the interference from phospholipids.

Our wash method successfully improved both the signal intensity and the S/N of thymosin  $\beta$ 4, and better results were observed compared to those obtained by conventional methods using an organic solvent (Suppl. Fig. S9). Our findings indicate that our method can completely remove lipids and chemical noise, which are thought to suppress the ionization of peptides. Our method has the advantage of sensitivity for peptide signal intensities compared to conventional wash methods, whereas the conventional methods have the advantage of being easy and quick.

We consider that the improved detection of thymosin  $\beta$ 4 in this study was due to the difference in the polarity of solutions between the scCO<sub>2</sub> wash step and the matrix application step (Suppl. Fig. S10). Polarity is usually estimated as the partition coefficient XlogP3 [17], which is an atomic-based prediction of the logarithmic ratio, (concentration in octanol)/(concentration in water). We therefore considered XlogP3 for each step in our method. The first step was the removal of phospholipids from the tissue to the scCO<sub>2</sub>. We calculated the partition coefficient of scCO<sub>2</sub> by using the XLogP3 value of CO<sub>2</sub>. We used CO<sub>2</sub> with MeOH to remove phospholipids such as PC (32:0), which was likely to be PC (16:0/16:0). The XlogP3 values were 0.9 for CO<sub>2</sub>, -0.5 for MeOH, and 13.5 for PC (16:0/16:0) [18].

The solution used in these experiments consisted of 70% CO<sub>2</sub> and 30% MeOH, and thus the total XlogP3 is calculated as  $0.7 \times (0.9) + 0.3 \times (-0.5) = 0.63 + (-0.15) = 0.48$ , indicating that the solution is hydrophobic and sufficient to dissolve PC (16:0/16:0). The second step was performed in the matrix application from the tissue to the matrix solution. The XlogP3 of MeOH and water is -0.5, and that of thymosin  $\beta$ 4 is -43.5 [18]. The total XlogP3 of the solution containing 70% MeOH and 30% water is calculated as

$0.7 \times (-0.5) + 0.3 \times (-0.5) = -0.35 + (-0.15) = -0.5$ , which represents sufficient hydrophilicity for the extraction of thymosin  $\beta$ 4. The removal of phospholipids makes it easy to extract peptides in place of lipids, and to that end extracted peptides were well-mixed with matrix in the second step.

As thymosin  $\beta$ 4, a major intracellular peptide, is an abundant and highly hydrophilic peptide [19], thymosin  $\beta$ 4 was the most improved signal in the present analysis. We consider that the unidentified signals improved in the scCO<sub>2</sub> extracted sample were also hydrophilic peptides. In the future, our method could be used to detect various peptides related to diseases, such as Alzheimer's disease [20]: as the XlogP3 for amyloid-beta peptide (A $\beta$ ) 40 is estimated as  $-13.1$ , and that for A $\beta$  42 as  $-12.2$ , A $\beta$  40 and A $\beta$  42 are potential targets for MALDI-IMS using our method.

In conclusion, we investigated a new pretreatment method using scCO<sub>2</sub> with an entrainer for distribution analyses by MALDI-IMS. Our results demonstrate the effective removal of phospholipids and the increased detection of peptides at  $m/z$  3000-5200, including unidentified molecules. The pretreatment method using scCO<sub>2</sub> described herein is thus a powerful technique to enhance distribution analyses of peptides. Further improvements for analyses of other molecular specie could also be achieved by optimizing the polarity of the wash conditions, such as by using different supercritical fluids, and adding various entrainers. We propose that our wash method with a supercritical fluid could be used to reveal the distributions of a variety of molecules in tissue samples.

## **Acknowledgments**

We thank S. Yamamoto and Y. Nakaya for their technical support with the scCO<sub>2</sub> instrument. This work was supported by grants from Japan's Ministry of Education, Culture, Sports, Science and Technology (grant nos. 15H05900 and 15H05898B1), a Research Fellowship from the Japan Society for the Promotion of Science (JSPS KAKENHI grant no. JP16J05476), and a grant from Yamada Ryozyo Kikin of the Hamamatsu Foundation for Science and Technology Promotion to M. Setou.

## **Conflict of Interest**

The authors declare that they have no conflict of interest.

## References

1. Morgat C, Mishra AK, Varshney R, Allard M, Fernandez P, Hindié E. Targeting neuropeptide receptors for cancer imaging and therapy: perspectives with bombesin, neurotensin, and neuropeptide-Y receptors. *J Nucl Med.* 2014;55:1650–7. doi:10.2967/jnumed.114.142000
2. Dumont Y, Bastianetto S, Duranton A, Breton L, Quirion R. Immunohistochemical distribution of neuropeptide Y, peptide YY, pancreatic polypeptide-like immunoreactivity and their receptors in the epidermal skin of healthy women. *Peptides.* 2015;70:7–16. doi:10.1016/j.peptides.2015.05.002
3. OuYang C, Liang Z, Li L. Mass spectrometric analysis of spatio-temporal dynamics of crustacean neuropeptides. *Biochim Biophys Acta.* 2015;1854:798–811. doi:10.1016/j.bbapap.2014.10.023
4. Kamei N, Morishita M, Kanayama Y, Hasegawa K, Nishimura M, Hayashinaka E, Wada Y, Watanabe Y, Takayama K. Molecular imaging analysis of intestinal insulin absorption boosted by cell-penetrating peptides by using positron emission tomography. *J Control Release.* 2010;146:16–22. doi:10.1016/j.jconrel.2010.05.004
5. Gerfen CR, Sawchenko PE. An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: immunohistochemical localization of an axonally transported plant lectin, *Phaseolus vulgaris*-leucoagglutinin (PHA-L). *Brain Res.* 2016;1645:42–5. doi:10.1016/j.brainres.2015.12.040

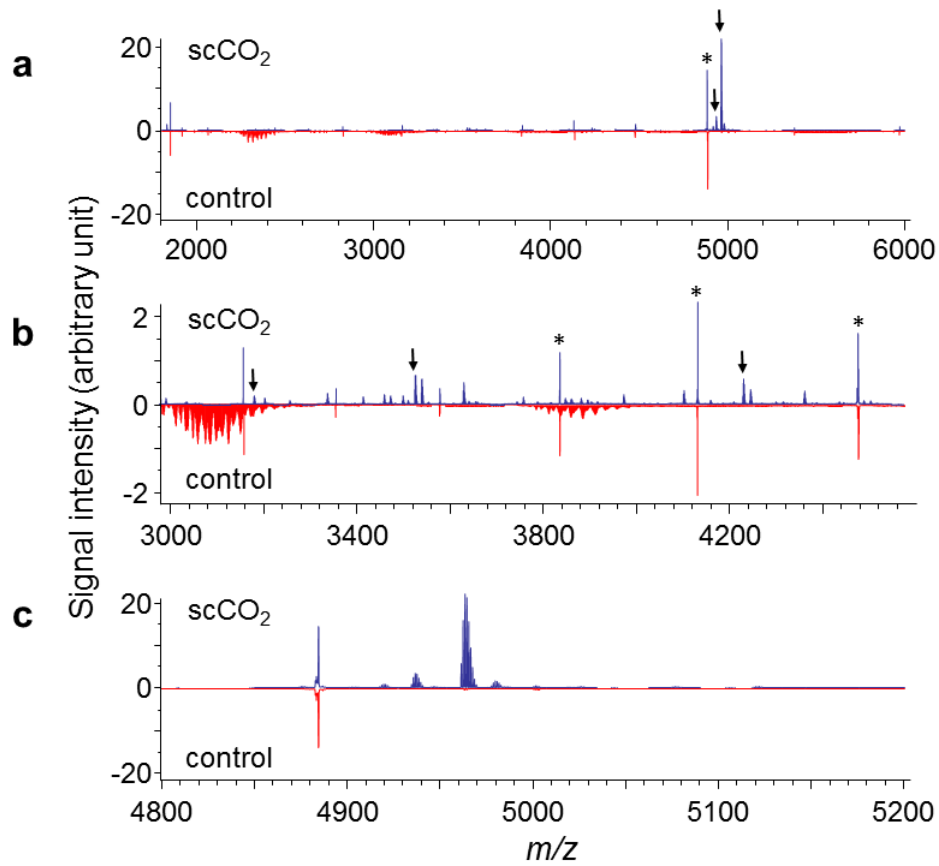


6. Hanrieder J, Malmberg P, Ewing AG. Spatial neuroproteomics using imaging mass spectrometry. *BBA – Proteins Proteomics*. 2015;1854:718–31.  
doi:10.1016/j.bbapap.2014.12.026
7. Goodwin RJA, Pennington SR, Pitt AR. Protein and peptides in pictures: Imaging with MALDI mass spectrometry. *Proteomics*. 2008;8:3785–800.  
doi:10.1002/pmic.200800320
8. Lemaire R, Wisztorski M, Desmons A, Tabet JC, Day R, Salzet M, Fournier I. MALDI-MS direct tissue analysis of proteins: Improving signal sensitivity using organic treatments. *Anal Chem*. 2006;78:7145–53. doi:10.1021/ac060565z
9. Seeley EH, Oppenheimer SR, Mi D, Chaurand P, Caprioli RM. Enhancement of protein sensitivity for MALDI imaging mass spectrometry after chemical treatment of tissue sections. *J Am Soc Mass Spectrom*. 2008;19:1069–77.  
doi:10.1016/j.jasms.2008.03.016
10. Lída M, Holčapek M. High-Throughput and Comprehensive Lipidomic Analysis Using Ultrahigh-Performance Supercritical Fluid Chromatography–Mass Spectrometry. *Anal Chem*. 2015;87:7187–95. doi:10.1021/acs.analchem.5b01054
11. Heidaryan E, Hatami T, Rahimi M, Moghadasi J. Viscosity of pure carbon dioxide at supercritical region: Measurement and correlation approach. *J Supercrit Fluids*. 2011;56:144–51. doi:10.1016/j.supflu.2010.12.006
12. Sahena F, Zaidul ISM, Jinap S, Karim AA, Abbas KA, Norulaini NAN, Omar AKM. Application of supercritical CO<sub>2</sub> in lipid extraction – A review. *J Food Eng*. 2009;95:240–53. doi:10.1016/j.jfoodeng.2009.06.026
13. Huang Z, Shi XH, Jiang WJ. Theoretical models for supercritical fluid extraction. *J Chromatogr A*. 2012;1250:2–26. doi:10.1016/j.chroma.2012.04.032

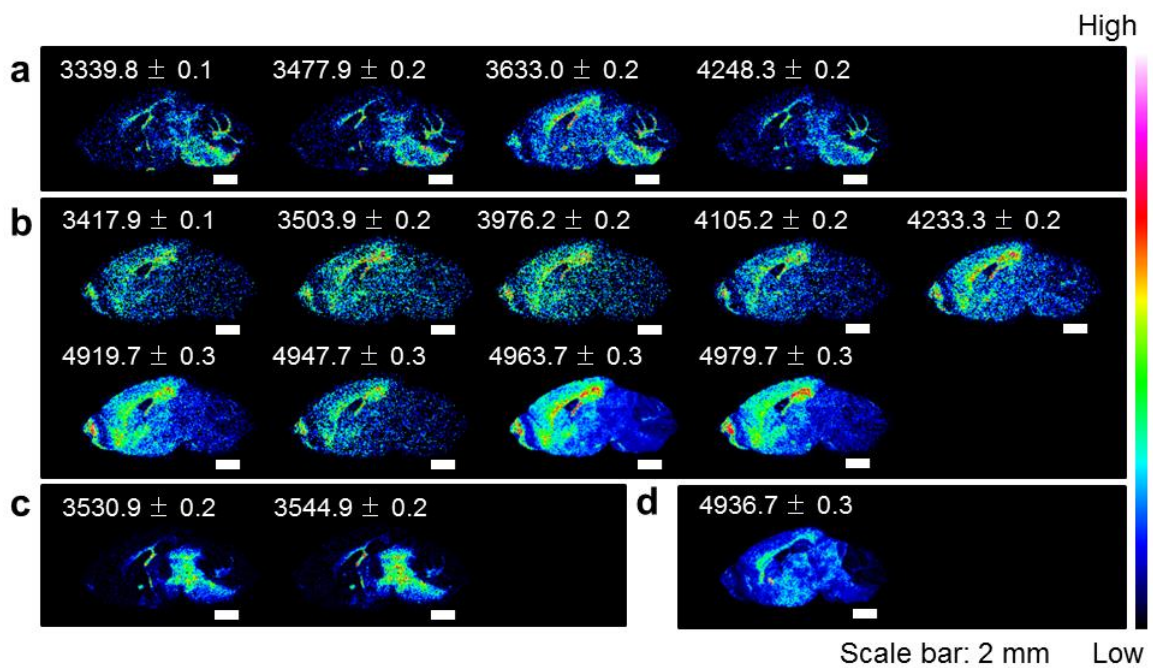
14. Tanaka Y, Sakaki I, Ohkubo T. Extraction of phospholipids from salmon roe with supercritical carbon dioxide and an entrainer. *J Oleo Sci.* 2004;53:417–24. doi:10.5650/jos.53.417
15. Sugiura Y, Konishi Y, Zaima N, Kajihara S, Nakanishi H, Taguchi R, Setou M. Visualization of the cell-selective distribution of PUFA-containing phosphatidylcholines in mouse brain by imaging mass spectrometry. *J Lipid Res.* 2009;50:1776–88. doi:10.1194/jlr.M900047-JLR200
16. Spraggins JM, Rizzo DG, Moore JL, Rose KL, Hammer ND, Skaar EP, Caprioli RM. MALDI FTICR IMS of intact proteins: Using mass accuracy to link protein images with proteomics data. *J Am Soc Mass Spectrom.* 2015;26:974–85. doi:10.1007/s13361-015-1147-5
17. Cheng T, Zhao Y, Li X, Lin F, Xu Y, Zhang X, Li Y, Wang R, Lai L. Computation of octanol-water partition coefficients by guiding an additive model with knowledge. *J Chem Inf Model.* 2007;47:2140–8. doi:10.1021/ci700257y
18. Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, Han L, He J, He S, Shoemaker BA, Wang J, Yu B, Zhang J, Bryant SH. PubChem Substance and Compound databases. *Nucleic Acids Res.* 2016;44:D1202–13. doi:10.1093/nar/gkv951
19. Fricker DL. Analysis of mouse brain peptides using mass spectrometry-based peptidomics: Implications for novel functions ranging from non-classical neuropeptides to microproteins. *Mol Biosyst.* 2010;6:1355–65. doi:10.1039/c003317k

20. Stoeckli M, Staab D, Staufenbiel M, Wiederhold K, Signor L. Molecular imaging of amyloid  $\beta$  peptides in mouse brain sections using mass spectrometry. *Anal Biochem.* 2002;311:33–9. doi:10.1016/S0003-2697(02)00386-X

## Figures



**Fig. 1.** Spectra obtained from a scCO<sub>2</sub>-washed tissue (upper, blue) and a control tissue (lower, red) sample. **(a)** Whole spectra of  $m/z$  1800-6000. **(b)** Enlarged spectra of  $m/z$  3000-4400. **(c)** Enlarged spectra of  $m/z$  4800-5200. \*an artifact signal.



**Fig. 2.** Ion distribution of the signals specifically detected from scCO<sub>2</sub>-washed samples. We grouped several peaks according to their distributions. **(a)** Signals distributed on white matter of the cerebellum and medulla oblongata. **(b)** Signals distributed in the hippocampus. **(c)** Signals distributed in the midbrain. **(d)** Signal distributed in the callosum.