Review

Metabolic profiling by integrated analysis with LC-MS and MSI

Daisuke Saigusa[1, 2]*

[1]Laboratory of Biomedical and Analytical Sciences, Faculty of Pharma-Science, Teikyo University,
2−11−1 Kaga, Itabashi-ku, Tokyo 173−8605, Japan
[2]Tohoku Medical Megabank Organization, Tohoku University,
2−1 Seiryōmachi, Aoba-ku, Sendai, Miyagi 980−0872, Japan

Abstract The metabolome is a group of small molecules that reflect phenotypic changes; this set of molecules is a potential source of biomarkers to predict the risk of disease development and progression. Therefore, it is necessary to establish a comprehensive and precise method to analyze the metabolome in biological samples. Mass spectrometry (MS) is one of the most suitable technologies to detect those molecules for metabolic profiling. In particular, liquid chromatography (LC)-MS-based methodology has been applied to various disease models and clinical samples, and biomarker candidates have been identified in previous studies. However, it is necessary to clarify their functions by in vivo research, and their molecular mechanisms in cells and/or tissues should be considered before the markers are deemed clinically applicable. MS imaging (MSI) is a promising technology that can visualize molecules on tissue surfaces without labeling and thus can clarify the distribution of biomarker candidates on tissue to evaluate the results by means of LC-MS-based analysis. Here, we describe the outline of the protocols for biomarker discovery by LC-MS and explain the utilization of integrated analyses using LC-MS and MSI to evaluate the function of biomarker candidates.

Key words: metabolome, biomarker, mass spectrometry, LC-MS, MSI

1. Introduction

It is expected that next-generation medicine will provide us with advanced and accurate medical technologies to predict and prevent disease processes for early diagnosis and personalized medicine[1)]. While genetic diagnosis has made remarkable progress in the clinic, the identification of risk factors (biomarkers) to anticipate and prevent disease development remains a challenge because multiple environmental factors, including lifestyle, habits, diet, stress and gut microbiota, are influenced by disease manifestations[2)]. Therefore, the development of novel analytical technology is required to understand the effects of not only genetic but also environmental factors on biological processes.

The metabolome is a group of small molecules that directly reflect the phenotypic changes derived from genetic and environmental factors[3)]. Therefore, metabolic profiling can be utilized to understand the origins and mechanisms of disease processes[4, 5)]. Several technologies have already been established for metabolic profiling in biology[6)], and recent technological improvements in the system sensitivity, molecular coverage and analytical robustness of liquid chromatography-mass spectrometry (LC-MS) have made it possible to detect and annotate a wide variety of metabolomes and have contributed particularly to research on biomarker discovery using disease models and clinical samples derived from humans[7, 8)].

Metabolic profiling for biomarker discovery is generally conducted using biofluids, such as plasma, serum, urine and cerebrospinal fluid[9)]. Although metabolic profiling by LC-MS can obtain quantitative information, the tissue must

*Corresponding author
Daisuke Saigusa
Laboratory of Biomedical and Analytical Sciences, Faculty of Pharma-Science, Teikyo University, 2−11−1 Kaga, Itabashi-ku, Tokyo 173−8605, Japan
Tel: +81−3−3946−8069
E-mail: saigusa.daisuke.vf@teikyo-u.ac.jp
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be homogenized as part of the preparation process before metabolic profiling, and information on the localization of the molecules is lost[10].

Mass spectrometry imaging (MSI) is a promising approach that allows label-free detection and mapping of the metabolome on biological sample surfaces and provides localized information by visualizing molecules on tissue sections[11, 12]. Thus, MSI enables the spatial localization of molecules. This approach has been utilized to facilitate biomarker discovery, and it has contributed greatly to validating molecular functions[10, 13]. Therefore, combining LC-MS and MSI is expected to provide additional reliability and clarify the functions of biomarker candidates[14]. In this review, we first describe the typical protocol, including sample preparation; data acquisition by LC-MS-based metabolic profiling; data processing; and multivariate analysis for biomarker discovery. Then, we illustrate the integrated use of LC-MS and MSI by summarizing previous studies that have used this approach for disease biomarker discovery.

2. Biomarker Discovery by LC-MS

Recent improvements in methodology for sample preparation, analytical methods and data processing have enabled researchers to realize biomarker discovery using disease models and clinical samples[15]. The typical protocol of metabolic profiling for biomarker discovery is shown in Fig. 1.

2.1. Sample preparation

First, since biomarker discovery generally aims to detect a wide range of molecules in the metabolome, small molecules are often extracted from plasma and tissue samples using deproteinization methods with organic solvents, such as methanol and acetonitrile[16, 17]. However, while deproteinization has advantages in extracting hydrophilic molecules, including amino acids, organic acids and sugars, it is less efficient in extracting hydrophobic molecules, such as lipid species. Therefore, it is recommended to select the Folch method or the Bligh and Dyer method[18, 19], both of which are suitable for extracting hydrophobic molecules to target lipid species in biomarker discovery research[20].

Comprehensive analysis of lipid species is known as lipidomics[21, 22], and a number of disease-related lipid mediators have been identified in previous studies[23]. However, it is important to consider targeting lipid species according to their polarity range by means of different extraction methods. Fig. 2(a) illustrates the total ion current/chromatogram (TIC) from lipidomic profiling by LC-MS using methanol deproteinization and the Folch method for extraction from human plasma based on the previously established method.

The most abundant and significantly different molecules indicated by the *S*-plot of orthogonal partial least square discriminant analysis (OPLS-DA) in the deproteinization method are shown in Fig. 2(b). The identified lipid species that were significantly more abundant with the deproteinization method were lysophosphatidylcholines (LPCs), lipid species that are relatively hydrophilic due to a group of phosphates in their molecular structure, whereas they were detected at lower levels with the Folch method. In contrast, the hydrophobic lipid species annotated as tri-

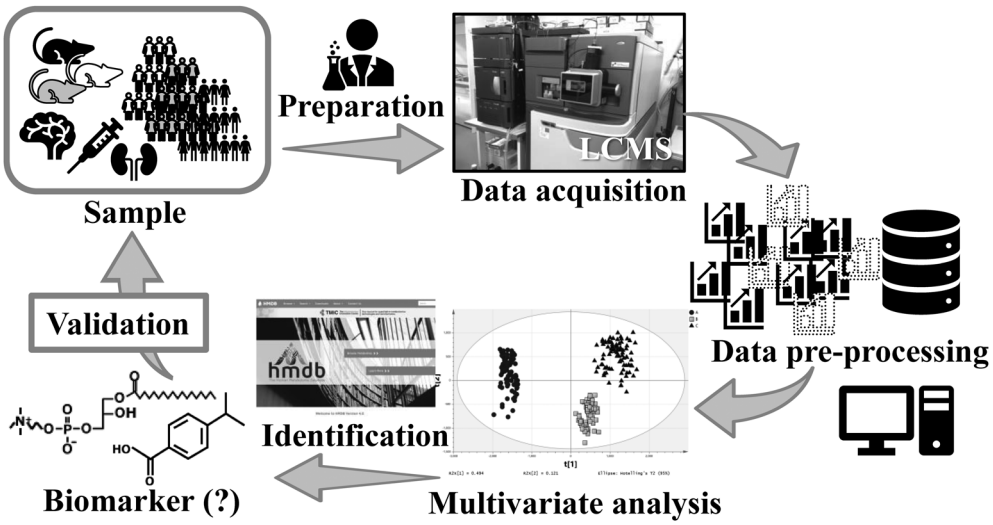


Fig. 1. Typical protocol of metabolic profiling by LC-MS for biomarker discovery.

glycerides (TGs) and cholesteryl esters (CEs) were significantly more abundant with the Folch method because they were extracted with high efficiency by chloroform. In fact, similar results were observed in the tissue lipidomics analysis (data not shown). Thus, appropriate sample preparation methods for biomarker discovery targeting lipid species should be carefully considered.

Recently, kit-based metabolic profiling has become possible with ultrahigh-performance liquid chromatography triple quadrupole mass spectrometry (UHPLC-MS/MS), which is performed in strict accordance with a standard operating procedure involving detailed documentation for sample preparation, instrument setup, system suitability testing, and data analysis; this technique can quantify several hundred metabolites[24–26]. For example, UHPLC-MS/MS can detect representative metabolites such as amino acids, amino acid−related metabolites, bile acids, biogenic amines, cresol, fatty acids, hormones, indole derivatives, nucleobases and vitamins; for this reason, the technique is widely applied in biomarker discovery projects[27, 28]. However, the lipid species detected by the kit (such as acylcarnitines, ceramides, CEs, diacylglycerols, dihydroceramides, and glycerophospholipids including LPCs and phosphatidylcholines (PCs), glycosylceramides, sphingolipids, and TGs) include several isobaric and isomeric compounds that cannot be separated by flow injection analysis (FIA)-MS/MS. These isomers are detected together, and low-abundance species are detected due to ion suppression, which particularly compromises the accurate quantification of lipid species[26]. Therefore, it is necessary to first select the optimal pretreatment method for metabolic profiling.

2.2. *Data acquisition by LC-MS-based metabolic profiling*

In the process of data acquisition, the run order should be considered by metabolic profiling by LC-MS analysis[29, 30]. The quality control (QC) standard was first prepared by mixing all the samples. Then, this QC sample is subjected to LC-MS analysis 5 to 10 times under the appropriate conditions for the step, which depends on the type of analytical column[31]. After the QC injection, the actual samples are injected in randomized order, with the QC being repeated once in an optimized number of injections to correct for the trends of intensity variation among individual molecules or the overall median value, which may be influenced by the run order[31]. Previous studies recommend injecting the QC sample every 2 h[32].

Methods of LC-MS-based metabolic profiling targeting hydrophobic molecules have been developed using C18 columns in previous studies[33]. However, functional metabolites contain many types of hydrophilic molecules, such as amino acids, organic acids, nucleic acids and sugars, and hydrophilic interaction chromatography (HILIC) columns have been utilized to retain those hydrophilic molecules by means of LC-MS analysis[31, 33–35]. Ten injections of the QC as the conditioning column were necessary to maintain stable analysis using the HILIC column until data acquisition was completed[31].

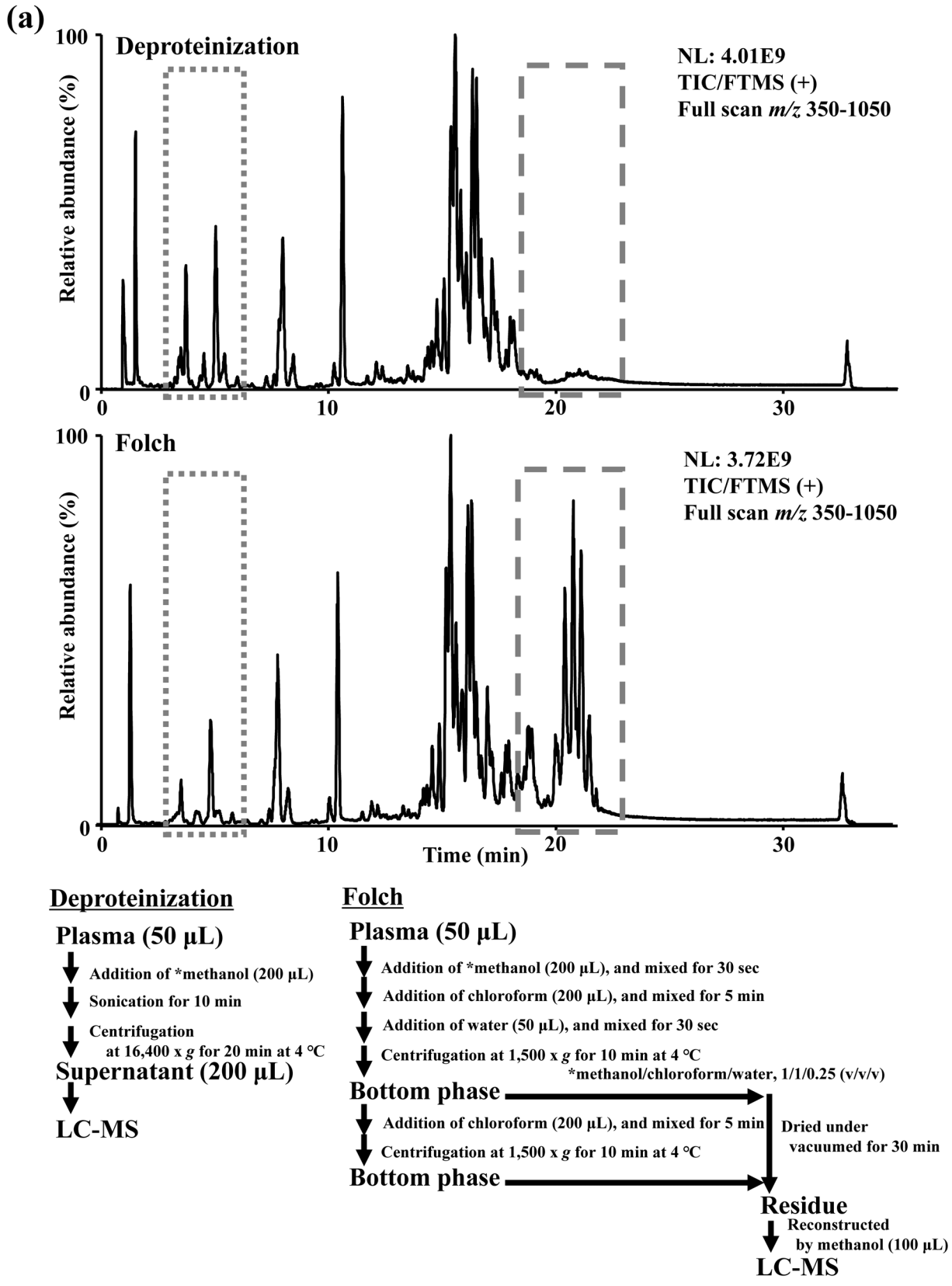
Recently, mixed-mode columns that can retain both hydrophilic and hydrophobic molecules have been developed and demonstrated to be useful for biomarker discovery[36, 37]. Shotgun metabolic profiling by FIA analysis without columns has been conducted for large-scale analysis for high-throughput metabolic profiling[38]. However, there are still disadvantages for the accuracy of quantitative values, and careful evaluation is needed in future studies.

2.3. *Data processing*

Metabolic profiling is generally classified into global metabolomics (G-Met), which consists of untargeted metabolomics for comprehensive analysis of thousands of molecules, and targeted metabolomics (T-Met) or widely targeted metabolomics (WT-Met) for quantified analysis of tens to hundreds of molecular species[7].

High-resolution MS (HRMS), such as time-of-flight (TOF) or Fourier transform (FT) MS, is generally used for G-Met and is suitable for biomarker discovery because it provides a large quantity of spectral data and has the potential to detect unknown compounds[6, 33–35]. However, data analysis of G-Met consumes much time due to complicated data preprocessing, such as chromatogram alignment, peak detection, and data curation to eliminate artifacts[7]. Indeed, expertise in data analysis for MS spectra is often required to identify molecules using databases.

In contrast, T-Met and WT-Met were analyzed with an MS/MS system. The data preprocessing is generally simple because the spectra have been obtained by chemical standards of known metabolites, and these techniques can be used as a beginning step in biomarker discovery[6]. Although there is a possibility that significant unknown biomarkers will be missed, the quantitative information provided by these techniques also provides a major advantage


(a)
Deproteinization
NL: 4.01E9
TIC/FTMS (+)
Full scan m/z 350-1050
Relative abundance (%)
Folch
NL: 3.72E9
TIC/FTMS (+)
Full scan m/z 350-1050
Time (min)
Deproteinization
Plasma (50 µL)
Addition of *methanol (200 µL)
Sonication for 10 min
Centrifugation at 16,400 x g for 20 min at 4 °C
Supernatant (200 µL)
LC-MS
Folch
Plasma (50 µL)
Addition of *methanol (200 µL), and mixed for 30 sec
Addition of chloroform (200 µL), and mixed for 5 min
Addition of water (50 µL), and mixed for 30 sec
Centrifugation at 1,500 x g for 10 min at 4 °C
*methanol/chloroform/water, 1/1/0.25 (v/v/v)
Bottom phase
Addition of chloroform (200 µL), and mixed for 5 min
Centrifugation at 1,500 x g for 10 min at 4 °C
Bottom phase
Dried under vacuumed for 30 min
Residue
Reconstructed by methanol (100 µL)
LC-MS

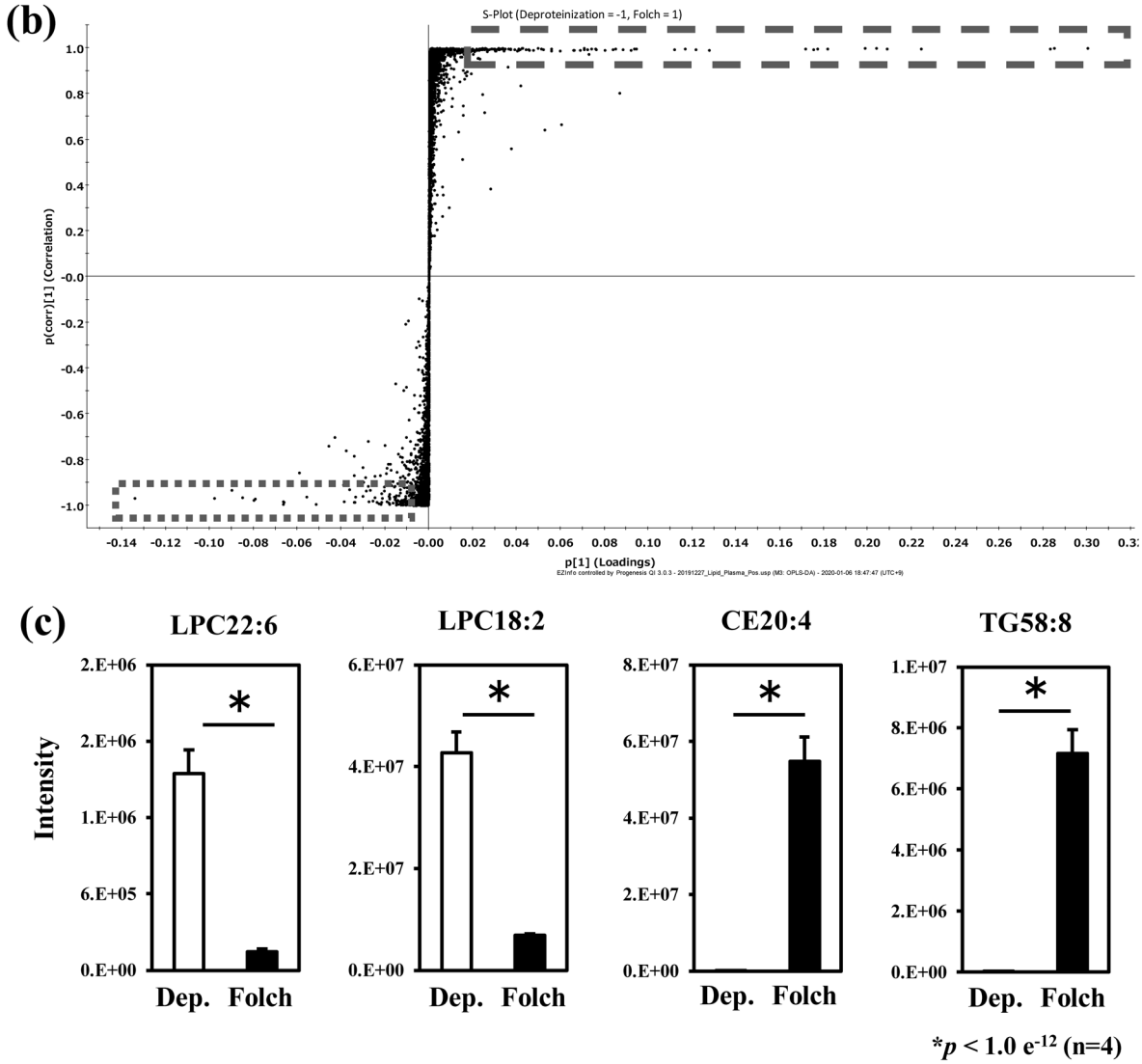


Fig. 2. Example of the difference in detected molecules between the deproteinization and Folch extraction methods by lipidomic profiling by LCMS analysis.

(a) Total ion current/chromatogram (TIC) using methanol deproteinization (Dep.) and the Folch extraction method for human plasma lipidomic profiling. The sample preparation procedures are shown below, and the analytical method was performed by the previously established method (ref. 26). Surrounded by dots square at 5 and 20 min indicates the detection ranges for lysophosphatidylcholines (LPCs) and cholesterylesters (CEs)/triglycerides (TGs), respectively. NL: normalized

(b) *S*-plot of orthogonal partial least square discriminant analysis (OPLS-DA). The left bottom surrounding dotted square shows 36 significantly lower features in Folch with p(corr)[1]＜−0.9, p[1]＜−0.02, p value＜0.05, and fold change＞2.0. The right upper surrounding dotted square shows 91 significantly higher features in Folch with p(corr)[1]＞0.9, p[1]＞0.02, p value＜0.05, and fold change＞2.0.

(c) Histograms of molecules significantly different between the two extraction methods.

in later functional analysis.

2.4. Multivariate analysis for biomarker discovery

Multivariate analysis is widely used for large amounts of metabolome information[39]. Although the analysis of each detected individual metabolite has the potential to be a biomarker, multivariate analysis is often utilized to capture trends as the first step to perform metabolic profiling in such a large-scale data set without bias. In particular, principal component analysis (PCA), in which data obtained in multiple dimensions are represented in fewer new dimensions designated PC1, PC2, PC3, ..., PCn by reducing a data set that originally contained hundreds to thousands of dimensions, is an essential method to understand the trends

in the metabolic profiles of interest[40]. Therefore, PCA is the first option for multivariate analysis in biomarker discovery, as it often yields characteristic distributions on a score plot or PCA.

On the other hand, metabolic profiles of human specimens are often affected by not only environmental factors such as diet and lifestyle but also artifacts such as time of blood collection and storage procedures and periods, making it impossible to observe a clear PC axis that captures trends of data and distributions that show characteristics on a score plot of PCA for extracting disease biomarkers[41]. Discriminant analysis methods such as PLS-DA and OPLS-DA, which add group parameters as a new dimension, can be used to observe the trends of data and extract the contributing factor(s). In fact, volcano plots and *S*-plots provide a variable importance projection score, a criterion for evaluating significance, and are utilized for extracting metabolites as biomarker candidates that contribute to differences between the two selected groups[40, 42].

Although license-based software and programs have already been developed by the producers of MS systems to perform multivariate analysis, the freely available online software MetaboAnalyst (https://www.metaboanalyst.ca/) has become increasingly popular in recent years due to the simple process of loading data prepared in text or csv format, and it can be used for most multivariate analyses[43, 44]. In fact, there are several options for multivariate analysis that contain complicated parameters for metabolic profiling; however, it is essential to consider the variation in metabolic changes in groups or biological specimens to select an optimal multivariate analysis.

An overview of typical protocols for metabolic profiling by LC-MS has been described above, but there are still points to be noted depending on the type of biological specimens. In general, plasma and serum are used for biomarker discovery using metabolomics techniques[7]. However, metabolome analysis in a large-scale cohort revealed that blood-derived metabolites show large variation due to environmental factors, such as age, gender, body mass index, food and lifestyle habits, as well as genetic factors[45, 46]. Therefore, it must be considered that identified molecules take into account the possibility of false positives and/or negatives in blood-based biomarker discovery.

Recently, there have been some studies on metabolic profiling using extracellular vesicles derived from blood biopsy; these studies have reported the possibility of a new approach to biomarker discovery[47, 48]. This method has the potential to identify biomarker candidates indicating the characteristics of disease with higher sensitivity and specificity without being affected by the artifacts described above.

Metabolic profiling has also been conducted with urine, feces, cells and tissues. Although a large variation in molecular concentration could be observed in urine among individuals, it has been found that reliable urine results can be achieved with appropriate dilution according to a preanalysis of creatinine values[49]. In addition, the effect and relationship with gut microbiota can be analyzed by metabolic profiling of feces[50]. On the other hand, some previous studies have identified markers directly related to diseases by using cells and tissues derived from patient specimens[51, 52]; however, it was extremely difficult to obtain control samples because most of the samples were collected from patients in hospitals.

Thus, biological specimens for metabolic profiling are becoming more diverse, and it is necessary to have more bioinformatics knowledge and techniques to treat more complicated and large data sets to extract biomarkers and understand their biological function with a simpler and more precise strategy for metabolic profiling in the future.

3. Analysis of Metabolic Profiles by Combined LC-MS and MS Imaging

3.1. Impact of metabolic profiling in tissue

Although several biomarker candidates have been identified by LC-MS-based T-Met, sensitivity and specificity are not satisfactory for application in the clinical field due to the metabolites, such as amino acids and lipid molecular species, being altered by multiple diseases[53, 54]. In contrast, most metabolites annotated by G-Met are often less known or unknown in relation to disease; however, G-Met sometimes has extremely high potential to identify biomarkers even if the metabolites were unknown at the time of identification[55].

For instance, Kikuchi et al. reported phenyl sulfate (PS), which is derived from the intestinal microbiota and produced via biosynthetic enzymes in the liver, as an unknown molecule that accumulates in plasma from a rat model of diabetic kidney disease by treatment with streptozotocin[55]. They demonstrated that accumulation of PS in the kidney was suppressed in transgenic rats with the excretion transporter SLCO4C1 in proximal tubular cells. They examined

molecular biological techniques to demonstrate the function of PS using animal models and showed that the inhibitor suppressed PS accumulation in the kidney. Furthermore, they quantified PS in hundreds of human samples and revealed that PS could be a prognostic marker for diabetic renal dysfunction after 2 years of correlation with clinical information, indicating that environmental factors have potential as biomarkers for application in clinical practice.

Furthermore, they clarified the distribution and accumulation of PS contained in kidney sections by MSI and showed by visualization that administration of azatyrosine, which is an inhibitor of PS synthetic enzymes, suppressed the accumulation[55]. A previous study showed the importance of validating molecular function using other metabolomics techniques, such as tissue metabolomics, to demonstrate the advancement of candidate disease biomarkers to understand function with a high degree of confidence[10].

3.2. Strategies of metabolic profiling in tissue

The characteristics of strategies for tissue metabolic profiling are shown in Fig. 3. In general, metabolites are extracted by homogenizing a certain amount of tissue with an organic solvent (Fig. 3(a)). Since whole or partial tissue is prepared for biomarker discovery, it is utilized to reveal the differences in metabolic changes associated with the level, presence or absence of disease onset between the disease model and control[56, 57]. However, the spatial distribution of metabolites is completely lost during the sample preparation procedure. Therefore, it is necessary to apply the optimal method to preserve the localized information of metabolites for the tissue, which has a complicated structure such as the brain.

In this case, a cryosection of tissue is often used for distribution analysis[58]. Two typical analytical techniques are shown in this review. One is a laser microdissection (LMD) technique, which can cut the small region of interest for molecular localization. The other is the MSI technique represented by matrix-assisted laser desorption/ionization (MALDI)-MSI, which can visualize the detailed distribution by reconstructing the spectral intensity and spatial

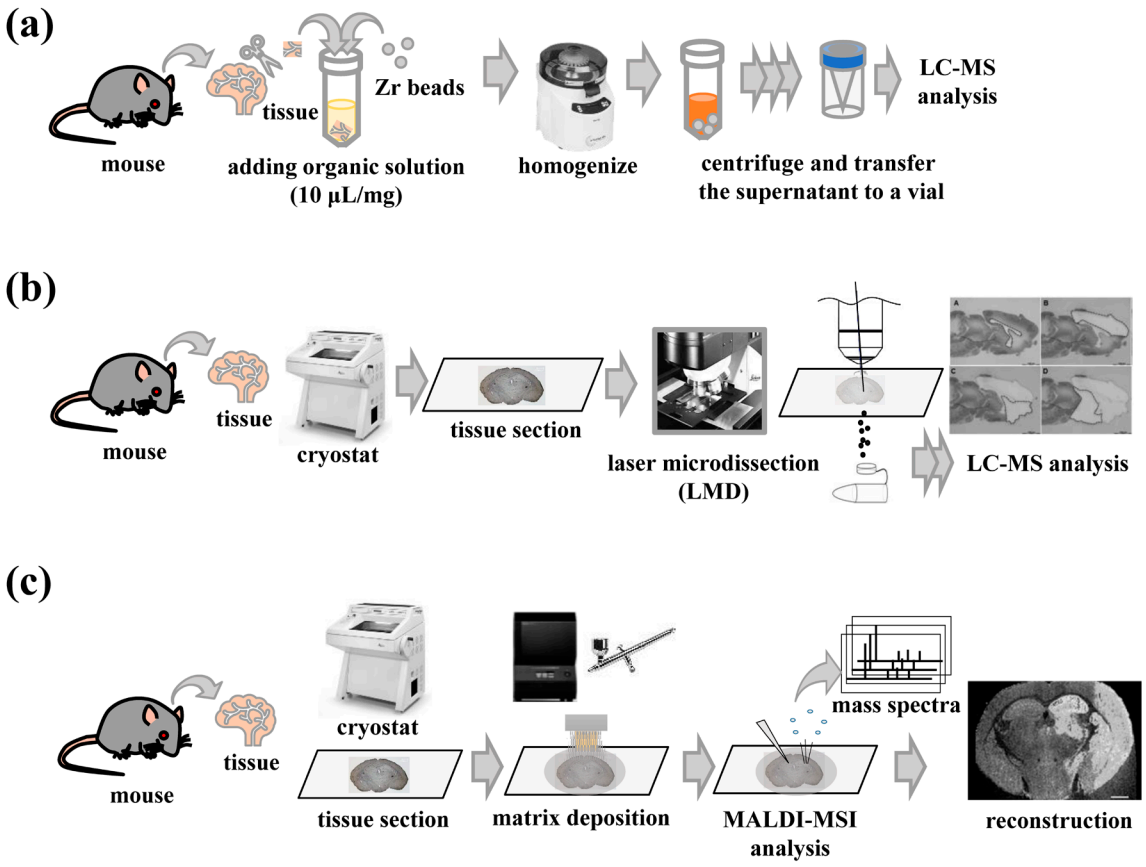


Fig. 3. Strategies of metabolic profiling in tissue.
(a) Bead-homogenizing procedure of whole or partial tissue by organic solution
(b) Laser microdissection (LMD) procedure of a tissue section
(c) MALDI-MSI procedure of a tissue section

information.

3.3. *LMD for metabolic profiling*

As shown in Fig. 3(b), LMD can capture the characteristics of a wide range of tissues, such as the brain, kidney, liver, and spleen, which are assumed to be widely distributed to some extent, whereas it cannot observe the cell level of distribution[59, 60]. Wang et al. demonstrated the localization of sphingosine 1-phosphate (S1P), which is one of the lipid mediators in the red and white pulp on the spleen, quantified by LC-MS/MS, showing the difference in S1P concentration in the LMD sections[61].

While the tissue distribution of metabolites in LMD sections highly corresponds with the results of partially homogenized tissue analysis by LC-MS/MS, LMD works at room temperature due to the combination with a microscopic system. Therefore, the stability of temperature for target metabolites should be evaluated in advance by LMD assay because some metabolites, such as S1P, have a phosphate group in the chemical structure, and the observed time depends on the reduction in tissue at room temperature[59]. In some cases, a heat stabilizer technique is utilized to suppress the decomposition of tissue metabolites; however, the other effects of heating specimens should be considered[62].

3.4. *MALDI-MSI for metabolic profiling*

Recently, MSI technology has been improved and utilized in many studies to reveal the localization of metabolites in tissue sections[63]. MALDI is the most widely used technique for MSI, whereas other ionization technologies, such as desorption electrospray ionization, secondary ion mass spectrometry imaging or liquid extraction surface analysis, have been developed[64, 65]. Although this review does not describe much about the development of MSI instruments, MSI is utilized in the research field of biomarker discovery, and many biological molecules have been identified with the development of MSI technology. The typical strategy of biomarker discovery by MSI is shown in Fig. 4. After preparing the tissue section and obtaining the visualized image by the MSI protocol, as shown in Fig. 3(c), the localized spots were selected by imaging software. Then, biomarker candidates were extracted by multivariate analysis, and a similar process to the protocol of biomarker discovery by G-Met was used. Therefore, the combination of G-Met by LC-MS and distribution analysis by MSI using tissue samples has promoted research that takes advantage of the benefits of both meth-

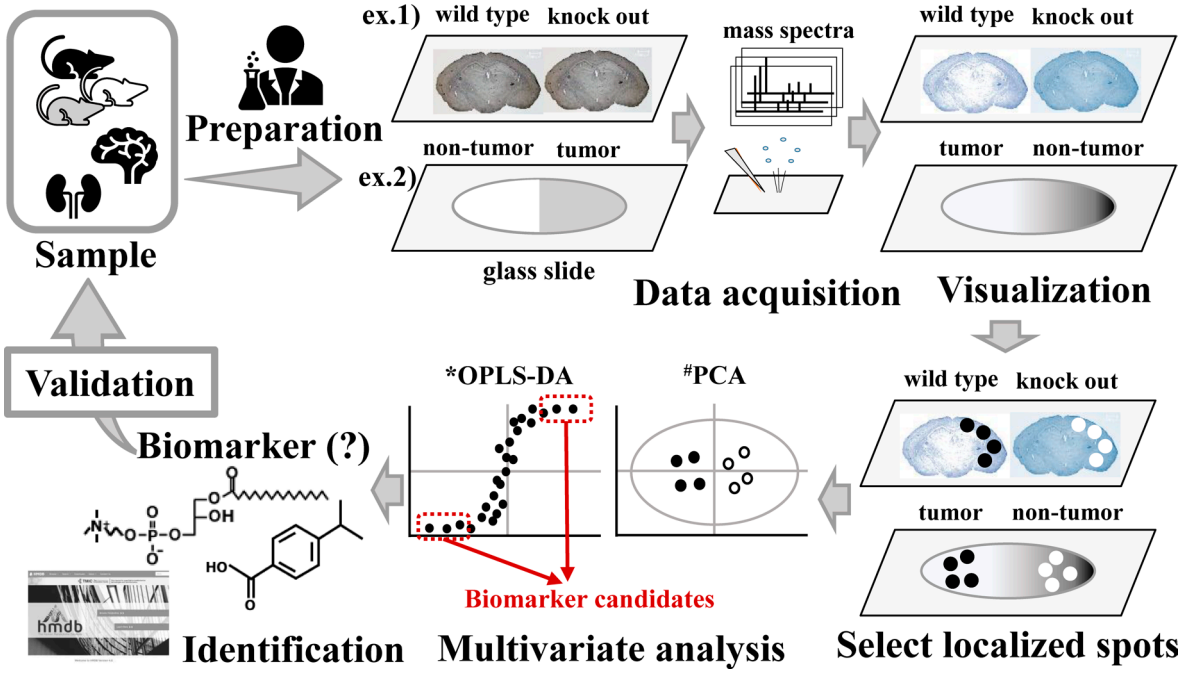


Fig. 4. Typical strategy of biomarker discovery by MSI.

Experiment 1 (ex.1) or ex.2 was the representative comparison between wild type and knockout or nontumor and tumor analysis by MSI.

*OPLS-DA: orthogonal partial least square discriminant analysis

#PCA: principal component analysis

Table 1. Combination analysis of biomarker discovery by LCMS and MSI

Disease	Model/human	Specimine	Molecule/s	LCMS	MSI	Ref.
clear cell renal cell carcinoma	human	kidney	lipid species	LC-FTMS	MALDI-LTQ-FTMS	14)
hepatocellular carcinoma (HCC)	human	liver	triglycerides	LC-QTOF/MS	DESI-MSI	52)
diabetic kidney disease (DKD)	DKD model mice	kidney	phenylsulfate	LC-QTOF/MS	MALDI-TOF/MS	55)
ischemia−reperfusion injury	middle cerebral artery occlusion	brain	amino acid, nucleotides, tricarboxylate cycle metabolites	LCMS-IT-TOF	MALDI-TOF/MS, QIT, TOF/TOF	66)
glaucoma	optic nerve crush mice	retina	L-acetylcarnitine, phosphatidylcholine	LC-FTMS, LC-QTOF/MS	MALDI-TOF/MS	67)
diabetic	db/db mice	Whole body, brain, liver, kidney, heart, spleen, lung, muscle, and pancreas	alanine, aspartate, and glutamate metabolism	LC-FTMS	airflow-assisted desorption electrospray ionization (AFADESI)-MSI	79)
acute liver failure	acetaminophen-induced acute liver failure mice	liver	oxidized phosphatidylcholines	LC-FTMS, LC-MS/MS	MALDI-MS/MS, MALDI-MSI	80)
knee osteoarthritis	human	tibial plateau tissue	N-glycans	LC-QTOF/MS	MALDI-TOF/TOF	81)
Niemann-Pick disease type C1 (NPC1)	NPC1 model mice	brain	phosphoinositides	LC-ESI-QTOF/MS, LC-MS/MS	MALDI-TOF/TOF	83)
adenocarcinoma	human	lung	phosphatidylcholines, sphingomyelins	LC-QTOF/MS	MALDI-TOF/MS	84)
cauda equina compression (CEC)	CEC model rat	spinal cord, dorsal root ganglia	lysophospholipids	LC-MS/MS	MALDI-TOF/MS	85)
sarciopenia	adenine-induced chronic kidney disease (CKD) model mice	skeletal muscle	indoxyl sulfate	LC-MS/MS	MALDI-TOF/MS, MALDI-QIT-TOF/MS	86)
CKD	adenine-induced chronic kidney disease (CKD) model mice	kidney	indoxyl sulfate, p-cresyl sulfate	LC-MS/MS	MALDI-TOF/MS, MALDI-QIT-TOF/MS	87)
preeclampsia (PE)	sFlt-1-overexpressing PE mice	placenta	carnitine and acylcarnitine	LC-FTMS, LC-QTOF/MS	MALDI-TOF/MS	88)
traumatic brain injury (TBI)	TBI rat	brain	peroxidized phosphatidylethanolamine	LC-FTMS	gas cluster ion beam secondary ion mass spectrometry	89)

ods[13, 14]. A summary of the combination studies based on a previous publication is listed in Table 1.

Irie et al. analyzed rat transient middle cerebral artery occlusion (MCAO) brain tissue after ischemia−reperfusion to characterize the detailed metabolomic response to pathological alterations[66]. They compared the spatially resolved metabolic state between ischemic and contralateral hemispheres of the MCAO brain, and coronally sliced tissues were subjected to MSI. They also measured the metabolites extracted from three different cerebral regions, including the whole cortex (CTX), hippocampus and corpus striatum (CPu), by LC-MS, and significant metabolic changes in the CTX and CPu were observed after reperfusion, demonstrating that the correlation between MSI and LC-MS data was relatively high in the CTX and CPu.

Sato et al. analyzed retinal samples from a mouse model of glaucoma in which retinal ganglion cell (RGC) death was induced by nerve crush (NC) treatment by G-Met analysis and identified molecules that were significantly increased or decreased on NC day 2, day 4, or day 7 compared with their controls[67]. The identified metabolites were detected in RGCs, and histological sections were obtained for MSI. Two metabolites, L-acetylcarnitine and phospholipids, were increased not only preceding the peak of RGC

death in the whole retina but also in the RGC layer.

Nagai et al. identified hepatocellular carcinoma (HCC) biomarkers in human liver samples by G-Met and demonstrated their localization in cryosections using the desorption electrospray ionization (DESI)-MSI technique[52]. From their multivariate analysis, *m/z* 904.83 and *m/z* 874.79 were significantly high and low, respectively, in tumor samples and were identified as TGs and clearly localized in the tumor or nontumor areas of the cryosection. Thus, the strategy of combining LC-MS and MSI contributes to the understanding of molecular function as a disease biomarker.

However, even if a biomarker was identified with LC-MS-based metabolic profiling, some molecules could not be detected by MSI due to the compatibility with the optimal matrix for MALDI-MSI to promote ionization. In addition, the absolute number of ionized molecules is reduced by physical means because the laser irradiation of MALDI is focused on a small number of cells at 10−50 μm spatial resolution[68].

In these cases, chemical derivatization is utilized to overcome the detection limit for specific molecules and promising techniques in visualizing minor and poorly ionizable molecules by MALDI-MSI[69]. A target analyte is derivatized by introducing a molecular tag to enhance its ionization efficiency. The tag also increases the molecular weight to avoid the effect of spectral noise. Recently, a method for on-tissue derivatization using chemical reagents, which are directly deposited onto tissue, was developed. For instance, Girard's reagent T and 2-picolylamine enhance the detection of corticosteroids and free fatty acids, respectively[70–72]. Specifically, detection in the m/z region, which is not easily affected by increased ionic strength or ion suppression, allows separation from background ions and high sensitivity detection.

Iwama et al. developed a novel on-tissue derivatization method using Phos-tag, a zinc complex that specifically binds to a phosphate monoester group, and made it possible to image lysophosphatidic acid and S1P in the mouse brain by MSI[73]. Uruno et al. utilized *N*-ethylmaleimide to avoid nonspecific reactions to the thiol residue of glutathione (GSH) by MALDI-MSI and demonstrated that GSH levels in various parts of the brain are increased in the Alzheimer's disease model mouse brain, perhaps due to increased nuclear factor-erythroid 2-related factor 2 activity[74].

In addition, there have been many improvements in recent years, such as the development of a two-step laser irradiation technique that allows ions to be detected with stable intensity even when the spatial resolution is reduced[75]. Sugiyama et al. generated an atlas of serotonin, dopamine, and norepinephrine levels in the whole brain of the mouse, obtained by the analysis of continuous cryosection, and the technique was utilized to observe the dynamics of molecules by MALDI-MSI[76].

Other new technologies, such as desorption ionization using a through-hole alumina membrane (DIUTHAME), based on premanufactured nanostructured membranes to be deposited on top of a tissue section[77], show promising features for easy and reproducible sample preparation and have demonstrated a higher lateral resolution of 5 μm at high mass resolution using atmospheric-pressure MSI of biomolecules from native tissues from different organisms in a previous publication[78].

Thus, novel methods that enable highly precise evaluation of candidate biomarker molecules identified by LC-MS have been developed and are expected to be used in the future.

3.5. Technical limitations of LC-MS and MSI

Although MS-based techniques are utilized for biomarker discovery, there are still some technical limitations. For instance, in the data preprocessing step, time-dependent variation could sometimes be observed on the score plot of PCA by LC-MS analysis. Normalization by the result of intermediate QC analysis is able to work to suppress the effect of the variation; however, it is impossible to apply the result on a large scale, such as over tens of hundred sample analyses in the clinic and cohort. Moreover, even if biomarker candidates have been extracted in several studies, it is difficult to annotate accurate molecular structures by hand using database searching due to isomeric and isobaric molecules, especially lipid species. Recently, software using fragment mass spectra has been developed[90]. It is a promising approach and is utilized for the accurate identification of molecular structures and estimating future improvements.

In contrast, technical limitations of sample preparation and system limitations of data acquisition by MSI could be observed. For instance, due to the size limitation of laser irradiation of the MSI system, it is still difficult to observe the molecular distribution at the single-cell and organelle levels. In addition, the stability of the laser control system of MSI is essential for reproducible imaging and maintaining the higher sensitivity detection of molecules. Although

the molecular localization could be observed at 0.6μm of spatial resolution, which reached the single-cell level of detection by a t-MALDI 2 MSI system[75], higher abundant molecules could be detected on the tissue section, and more improvement is still needed to see lower abundant molecules. In addition, when the spatial resolution reaches the single-cell level, higher quality tissue sections are required for MSI analysis. A conductive adhesive film has been developed for cutting high-quality tissue sections and has been applied for MALDI-MSI[58]. Therefore, future technological improvements are needed for MSI analysis for biomarker discovery.

4. Conclusion

Metabolic profiling is a promising approach for future precision medicine to identify disease-specific and sensitive biomarkers. MS-based methodologies have already been developed, and the technique seems almost mature, especially in terms of the comprehensibility and sensitivity of LC-MS. However, the optimal sample preparation procedure must be selected according to the type of targeted molecule. In addition, the annotated biomarker should be evaluated by another methodology to clarify the biological function. In this case, the combination of LC-MS and MSI is utilized for metabolic profiling for biomarker discovery using tissue samples, and we expect that further significant technological improvements will be made in MSI methodology in future studies.

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Conflict of Interest

The author declares no conflict of interest.

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