3.4. Early Stage Cancer and Metabolomics

Early detection of cancer is very important for a complete recovery. Therefore, many researchers have searched for possible biomarkers of early cancer detection. In biomarker discovery research using metabolomics, evaluations of early cancer detection have been carried out. In the study by Kobayashi *et al.* a diagnostic model for pancreatic cancer was established using GC-MS-based serum metabolomics and multiple logistic regression analysis accompanied by the stepwise method [48]. This established model had a high sensitivity of 77.8% in resectable pancreatic cancer, namely relatively early stage pancreatic cancer, while sensitivities of CA19-9 and CEA were 55.6% and 44.4%, respectively. In serum lipid analysis for colorectal cancer, the metabolite profile data based on palmitic amide, oleamide, hexadecanedioate, octadecanoate acid, eicosatrienoate, LPC(18:2), LPC(20:4), LPC(22:6), myristate and LPC(16:0) exerted a sensitivity of 0.981 in early stage colorectal cancer patients compared to healthy volunteers [40]. In the analysis of plasma amino acids, alterations in levels of amino acids were observed in early stage lung, gastric, colorectal, breast, and prostate cancer [50]. Thus, the metabolites in biological fluids seem to be changed at the early stage of cancers, and metabolomics may be a powerful strategy for biomarker discovery, although detailed validation is still lacking at this point in time.

3.5. The Relationship between Metabolite Alterations and Cancer

Recently, studies aimed at biomarker candidate discovery based on amino acid-specific metabolite profiling have also been performed [50]. Moreover, it was demonstrated that high-mobility group box 1 protein (HMGB1) is released during the development and progression of colorectal cancer and subsequently induces muscle tissues to supply glutamine to cancer cells [51]. These findings suggest that increased HMGB1 levels lead to alterations in the blood amino acid profile and increased glutamine levels in colorectal tumors. In the paper by Miyagi *et al.* [50], the plasma level of tryptophan was significantly decreased in five types of cancer, i.e. lung, gastric, colorectal, breast and prostate cancer compared with healthy controls. Tryptophan is converted to kynurenine by indoleamine-2,3-dioxygenase, and it has been demonstrated that indoleamine-2,3-dioxygenase is over-expressed in cancer cells [52]. The possibility that indoleamine-2,3-dioxygenase may cause immune escape of various different tumors [52–55] has also been suggested, and that over-expression of indoleamine-2,3-dioxygenase in tumors may increase tryptophan metabolism, leading to a decreased of tryptophan in cancer patients. In addition, as shown in Table 1, the level of lactate seems to be upregulated in various gastroenterological cancers. Lactate is synthesized from pyruvate in the anaerobic condition, and it is known that this lactate synthesis is upregulated in cancer cells. This phenomenon is called "the Warburg effect" [56], so this reaction possibly promotes lactate synthesis, leading to an increased level of lactate. Thus, it seems that there are not only specific metabolite alterations in certain cancers but also common metabolite alterations in various cancers. These metabolite alterations are more likely to be reflected by the results of the metabolite biomarker candidates. Therefore, to draw firm conclusions about metabolite biomarker candidates for gastroenterological cancer it is important to understand the relationship between metabolite alterations and cancer, and moreover to elucidate the reasons for the observed alterations in the metabolite profile.

4. Future of Metabolomics-Based Disease Diagnosis

4.1. Procedures for Long-Term and Large-Scale Metabolomics Research

In the future, metabolomics is expected to be used in the clinical setting to screen for a variety of diseases including gastroenterological cancers. If metabolomics technology in screening programs enables early diagnosis, it can result in marked improvements in patients' quality of life. Recently, the Human Serum Metabolome (HUSERMET) Consortium recommended the procedures for long-term and large-scale metabolomic studies involving thousands of human serum/plasma samples [57]. Subsequently, a method for the global metabolite profiling of animal and human tissues has also been proposed [58]. The HUSERMET Consortium recommended the methods for sample collection, sample preparation, and data acquisition for LC-MS- and GC-MS-based studies and also pointed out that the most important stage in large-scale metabolomic studies is appropriate sample collection, because the systematic failure at the beginning of the investigation could invalidate the whole study. In addition, they proposed the protocols for large-scale GC-MS-based metabolomic studies, which describe the number of samples that should be prepared each week and the number of samples that should be measured in a day and recommend the usage of the retention index instead of retention time. The use of standard operating procedures based on validated protocols is important for studies attempting to find novel metabolite biomarker candidates. Quality control and assurance (QC/QA) is also important in the long-term and large-scale metabolomic studies [57,59–62]. Samples for QC are analyzed every batch throughout all measurement batches, and signal intensity, peak shape, retention time, separation resolution, mass accuracy, and the amount of detectable peaks are checked by using data obtained from QC samples. The pooled biological fluid samples and standard compound mixture samples may be used as QC samples. Before starting the batch measurement, it may be required to confirm the status of the injector, the mass spectrometer, and so on. Instrument tuning including mass calibration and sensitivity check is also required routinely as well as after the instrument maintenance. Recently, the analyzing workflow for the large-scale non-targeted serum metabolite profiling by LC-MS was visualized in a PubMed-indexed video journal [63].

4.2. Sampling for Biomarker Discovery Research by Metabolomics

In the clinical setting, the analyses of biomarkers present in serum/plasma, saliva, urine, and tissue samples have been prevalent. However, collecting tissues is invasive, and therefore serum/plasma, saliva, and urine are well used. Especially serum/plasma biomarkers are preferable due to the ease of collection, and there are a variety of biomarker studies using serum/plasma. In the metabolomics research field, serum/plasma are also well used, and metabolomics-based studies evaluating the differences between plasma and serum metabolite levels have been carried out recently. In the report by Yu *et al.* [64], the serum levels of 104 of 122 metabolites were found to be about 10% higher than their plasma levels, and nine metabolites displayed serum levels that were more than 20% higher than their plasma levels. Yu *et al.* demonstrated that both plasma and serum metabolite data exhibited good reproducibility, but the plasma data displayed better reproducibility than the serum data. In another report by Yin *et al.* [65], it was stated that the exposure of blood to room temperature led to increased levels of hypoxanthine and sphingosine 1-phosphate, and hence, Yin *et al.* suggested that the following

procedures should be employed during sample collection: the use of ethylenediaminetetraacetic acid (EDTA)-plasma samples is recommended for situations involving the metabolomic analysis of clinical samples; hemolyzed samples should be excluded; blood should be placed in ice water immediately after collection and should not be stored for longer than 2 h; the use of non-refrozen plasma is recommended (repeated freezing should not be performed), and MS data should be carefully examined for unexpected signals (the selection of blood collection tubes is also important because chemical noise derived from blood collection tubes can interfere with data analysis). In addition, the intra- and/or inter-day variance of metabolite levels has to be taken into consideration. Intra- and/or inter-day variance data has been reported for some metabolites [39,66,67]; for example, a previous study found that the tryptophan levels observed in the afternoon and at night were significantly lower than those detected in the morning. During pre-treatment and the subsequent measurement process, some metabolites might be unstable, and thus, it is necessary to confirm their corresponding metabolites and to eliminate any unstable metabolites from the subsequent analyses.

As for urine, the need to correct the obtained metabolite concentrations is an issue, although the correction using creatine and creatinine levels has been described in a great number of studies. However, collecting urine is non-invasive, and urine requires less sample pretreatment, because the protein level in urine is lower leading to a lack of complexity. Thus, urine has a number of advantages as an analytical material over other biological fluids [68–70], and in the future it may be recognized that urine is the most suitable biological fluids for the metabolomic approach to obtain meaningful diagnostic information.

Recently, metabolomic studies using saliva have also been carried out in the medical research field [20,21]. In humans, there are the three paired major salivary glands—the parotid gland, the submandibular gland, and the sublingual gland—and saliva is secreted from these major salivary glands. Saliva contains various DNAs, mRNAs, proteins including enzymes and antibodies, metabolites, and other molecules. Some of these molecules pass into the saliva from the blood stream via transcellular or paracellular routes. Therefore, saliva may correspond to blood regarding the reflection the physiological state of the body, and may be useful as a material containing disease biomarkers. Saliva collection is easy and noninvasive, and moreover, no specialized equipment is needed to obtain saliva [71]. Now, salivary diagnostics is recognized as one of the main approaches in biomedical basic and clinical areas [72], and it has been demonstrated that molecules in saliva may be associated with disease conditions [73–75]. To date, the number of metabolomics studies using saliva is small, but the potential of saliva metabolomics as a biomarker discovery approach has been proven by the accumulated results from saliva metabolomics.

4.3. Validation for Biomarker Discovery Research by Metabolomics

After discovering novel metabolite biomarker candidates whilst paying careful attention to the above issues, validation testing should be performed. In the disease biomarker discovery research, the use of samples obtained from other facilities is also important. The proposal by Yin *et al.* as shown above [65], lists considerable issues when the validation research is carried out in other facilities. In addition, during validation, it might be better to use different instruments from those utilized to detect novel metabolite biomarker candidates. Furthermore, techniques other than MS should be employed.

When MS is used, it is necessary to prepare stable isotopes corresponding to the metabolite biomarker candidates if possible. The quantitative performance of mass spectrometers is affected by various factors such as ion suppression [76]. Therefore, stable isotopes are required to obtain detailed quantitative information about alterations in the levels of the target molecules [77]. Stable isotopes will also be essential for quantitative evaluations if the metabolomics-based research using MS is to result in practical clinical applications. During the validation process and in clinical practice, the use of multiple reaction monitoring (MRM) coupled with stable isotopes and triple quadrupole (QqQ) MS is a powerful method for measuring the levels of targeted metabolites, because MRM based on QqQ-MS leads to molecule detection with high sensitivity, selectivity, reproducibility as well as a broad dynamic analysis range. MRM coupled with stable isotope dilution using QqQ-MS is a longtime and principal method to quantify small molecules and also a powerful method for quantitative measurement of targeted proteins [78–80] Recently, an analysis of mouse blood metabolites using GC/QqQ-MS was validated, although the study did not use stable isotopes [81]. An metabolomic article describes the quantification of metabolites in serum/plasma carried out by LC-MS coupled with stable isotopes as internal standards, which are contained in the AbsoluteIDQTM p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) [82]. Through a strict validation process, the candidates can be narrowed down to several metabolites, and some biomarker candidates that exhibit high repeatability can be utilized for clinical application after assay optimization.

4.4. Assay Optimization of Mass Spectrometry-Based Metabolomics

Regarding the assay optimization of MS-based analysis systems, some problems remain to be resolved, for example, it would be useful if the following processes could be automated: (1) metabolite extraction; (2) the pre-treatment process; (3) data analysis including peak alignment, annotation, and identification; and (4) the output of the obtained results. Regarding the automation of metabolite extraction and the pre-treatment process, dried blood spot sampling, in which blood is blotted and dried on filter paper, and supercritical fluid extraction (SFE), which is an extraction technique involving the use of supercritical carbon dioxide, have been studied. When SFE is performed, it is not necessary to perform sample pre-treatment, and SFE is also suitable for extracting hydrophobic compounds. Recently, SFE was combined with MS, and the analysis of blood metabolites using this system accompanied by dried plasma spotting is currently being investigated [83]. Then, in biomarker research, the analysis of volatile organic compounds (VOCs), which include molecules such as alcohols, aldehydes, ketones, and other heterocyclic compounds, has been performed with combination of headspace-solid phase microextraction (HS-SPME) and GC-MS [84–86]. In this combination approach, the solvent extraction step for volatile analysis is not needed. Moreover, the analyzing system for VOCs in blood was constracted usng in-tube extraction (ITEX), which is superior to HS-SPME [87]. The systems using HS-SPME and ITEX do not need manual metabolite extraction from biological fluids, and so this may be useful for assay optimization. In addition, some studies have used an automated system for sample preparation before MS measurement [88,89]. Although the study regarding the automation of each process is ongoing, at present the metabolites are manually extracted via liquid-liquid and solid-phase extraction, and other processes are also largely performed manually.

4.5. Mass Spectrometry Data Preprocessing, Peak Alignment and Peak Identification

For automatic MS data preprocessing, peak alignment, and molecule identification, software for metabolome analysis are freely available or can be purchased. Examples of the software include MetAlign, XCMS, MZmine, AIoutput, and MRMPROBS [90–94]. For metabolite identification in metabolome analysis, the metabolite database including mass spectrum and retention time/retention time index may be used. Construction of an in-house database is needed, but METLIN and MassBank are also available for metabolite peak identification based on the fragment ion data [95]. To obtain the metabolite information such as the biological/biochemical characteristics and the related pathway, the Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG), Recon X and so on are convenient [96–98]. Especially, HMDB includes spectral data for human metabolites.

Thus, the technology for metabolome analysis is being innovated, the information is accumulated, resulting in development of the metabolomics-based biomarker discovery research. However, easier extraction, pretreatment, and data analysis methods are required to make metabolome analysis more practical. Therefore, it is hoped that an automated analysis system that performs all of the required processes from metabolite extraction to data output will be developed.

5. Conclusions

The Japanese population has the highest life expectancy in the world. Due to its aging society, the working population in Japan has been rapidly decreasing, and now the most populous age group is the 60-69-year-olds. As a result, the medical costs of elderly people in Japan have increased every year, and these increases are regarded as a financial problem for the national government. Therefore, the development of a low-cost and easy diagnostic approach for detecting diseases at an early stage is needed to reduce medical expenses. Similar problems have arisen in various developed countries. Recently, various types of clinical samples have been subjected to metabolome analysis using GC-MS, LC-MS, CE-MS, matrix-assisted laser desorption ionization (MALDI)-MS, NMR spectrometry, or FT-IR spectrometry in order to discover novel biomarkers and elucidate the onset mechanisms of diseases. It is important to obtain disease-specific metabolome profiles in order to increase our understandings of diseases. Novel findings based on these disease-specific metabolome profiles are useful not only for discovering new biomarkers and elucidating the onset mechanisms of diseases, but also for developing novel therapeutic strategies, although accomplishing these aims will probably require the integration of omics data obtained from genomics-, transcriptomics-, and proteomics-based approaches as well as data acquired using metabolomics. The metabolomics-based research will hopefully increase our understanding of various diseases and lead to the elucidation of novel metabolite biomarkers. In addition, the development of metabolomics-based screening processes that only require a single drop of blood and allow diseases to be diagnosed at an early stage is greatly desired.

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Author Contributions

Shin Nishiumi planned and wrote the manuscript. Makoto Suzuki, Takashi Kobayashi, Atsuki Matsubara, Takeshi Azuma and Masaru Yoshida commented on and modified the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Supercritical fluid extraction as a preparation method for mass spectrometry of dried blood spots

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ABSTRACT

The potential of supercritical fluid extraction (SFE) as a preparation method for mass spectrometry of dried blood spots (DBS) was examined. SFE is generally used for the extraction of hydrophobic compounds, but hydrophilic metabolites such as amino acids, amines, and nucleic-acid-related metabolites could be extracted by adding a low level of methanol as a modifier. Under the optimized conditions, over 200 metabolites were detected from a dried serum spot, of which over 160 metabolites could be analyzed stably (RSD <20%). These results show that SFE is an effective extraction method of metabolites with a wide range of polarity in DBS.

1. Introduction

There are many advantages of dried blood spot (DBS) sampling compared with the conventional blood sampling method, including lower sample consumption and easier handling [1–4]. The former advantage enables easier application of blood sampling to newborns and reduction of the use of experimental animals. The latter contributes to reduce costs for transport and the preservation of samples.

Owing to these advantages, DBS has been applied for newborn mass screening [5,6] and for HIV tests [7]. Furthermore, combined with mass spectrometry (MS), the application of DBS is being extended to therapeutic drug monitoring (TDM) [8–10], pharmacokinetic study [11,12], and metabolome analysis [13,14]. Although there are several problems about false positive detection such as cross-talk or in-source decay, MS is a highly sensitive and selective detection method, and is free from the problem of cross-reactivity [15,16], which is a major defect of an indirect detection method that uses antibody response. Furthermore, MS can target multiple metabolites at one analysis [17]. Generally, MS's drawback is low quantitative capability due to ion suppression, but this problem can be solved by using stable isotopes of target compounds [18]. The combination of DBS with MS is a promising strategy for biochemical analysis.

Supercritical fluid extraction (SFE) is an extraction method that has features such as rapidity, high selectivity, and low solvent consumption [19,20]. It is generally used for the extraction of hydrophobic compounds such as fat-soluble vitamins [19,21], carotenoids [22], fatty acids [23], and aliphatic hydrocarbons [24], owing to the high hydrophobicity of supercritical carbon dioxide

Abbreviations: DBS, dried blood spot; MS, mass spectrometry; TDM, therapeutic drug monitoring; SFE, supercritical fluid extraction; $SCCO_2$, supercritical carbon dioxide; LC/MS/MS, liquid chromatography/tandem mass spectrometry; BHT, dibutylhydroxytoluene; DSS, dried serum spot; DWBS, dried whole blood spot; PC, phosphatidylcholine; PE, phosphatidylethanolamine; OSE, organic solvent extraction; SFC, supercritical fluid chromatography.

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($SCCO_2$). In our previous study [25], we applied SFE as an extraction method for phospholipids in DBS. However, other metabolites including hydrophilic metabolites were not targeted in that study. Recently, on the other hand, it has been reported that hydrophilic compounds such as amino acids could be extracted from crops by SFE with a relatively large amount of modifier [26,27]. This suggests that SFE can target the wide variety of metabolites contained in DBS.

In this study, SFE's potential as a preparation method for MS using DBS was evaluated. To specify SFE's applicable range of polarity, the extracts obtained from SFE of DBS were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS), which target lipids (phospholipids, fatty acids, acylcarnitines, bile acids) as well as hydrophilic compounds (amino acids, amines, nucleic-acid-related metabolites). The extraction conditions were optimized and the extraction efficiency of SFE was compared to that of the commonly used organic solvent extraction to characterize SFE as a DBS extraction method.

2. Experimental

2.1. Chemicals

CO_2 used for SFE was purchased from Iwatani Corporation (Tokyo, Japan). As an extraction medium, methanol (HPLC grade) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Acetone and dibutylhydroxytoluene (BHT) used in the extraction procedure were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). For LC/MS/MS, water and acetonitrile (LC/MS grade) were purchased from Wako Pure Chemical Industries Ltd. and methanol (LC/MS grade) was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Formic acid (LC/MS grade, Wako Pure Chemical Industries Ltd.) and ammonium acetate (1 M solution, HPLC grade, Wako Pure Chemical Industries Ltd.) were used as additives for the mobile phase. 2-Bromohypoxanthine purchased from Sigma–Aldrich (MO, USA) and dilauroylphosphatidylcholine (PC 12:0-12:0) purchased from Avanti Polar Lipids (AL, USA) were used as internal standards.

2.2. Samples

BondElut DMS card (Agilent Technologies, CA, USA) was punched using a dedicated hole puncher (3 mm, I.D.). As an antioxidant, 30 μl of 0.5% BHT (Wako Pure Chemical Industries Ltd.) in acetone (HPLC grade, Wako Pure Chemical Industries) was added to the hollowed out pieces [28]. After drying, 3 μl of human serum or whole blood from a healthy subject, including 30 μM 2-bromohypoxanthine and 2.4 μM PC 12:0-12:0 as internal standards, was dropped on the punched pieces and dried for over 2 h at room temperature. These samples are referred to as dried serum spots (DSS) and dried whole blood spots (DWBS), respectively, in this article.

2.3. Extraction conditions

In this study, a prototype SFE system made by Shimadzu Corporation (Kyoto, Japan) was used as an SFE instrument. Extraction was performed in dynamic mode. For the collection of extracts, the bubbling method was used. Time for extraction was 5 min because 5 min was sufficient for minimum flow rate condition in this study (methanol 0.3 ml/min). Other extraction conditions are shown in the main text and the figure legends. Methods for organic solvent extraction were as follows: 300 μl of methanol was added to the dried blood spot and left to stand for an hour. After centrifugation (15,000 × g, 4 °C, 5 min), the supernatant was collected into a new tube. The collected extracts were dried by centrifugal concentration, and reconstituted with 50 μl of water (Kanto Chemical) for hydrophilic metabolite analysis or methanol for lipid analysis.

2.4. Analytical conditions

Analyses were carried out using a Nexera LC system (Shimadzu Corp.) equipped with two LC-30AD pumps, a DGU-20As degasser, a SIL-30AC auto sampler, a CTO-20AC column oven, and a CBM-20A control module, coupled with an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corp.). The hydrophilic metabolites were separated using a pentafluorophenyl column (Discovery HS F5, 150 mm × 2.1 mm, 3 μm, SUPELCO, PA, USA) with a guard column (20 mm × 2.1 mm, 3 μm), while lipids were separated using an octadecylsilylated silica column (Inertsustain C18, 100 mm × 2.1 mm, 3 μm, GL Sciences, Tokyo, Japan) with a guard column (10 mm × 3 mm, 5 μm). The mobile phase for hydrophilic metabolites was constituted with A: 0.1% formic acid in water and B: acetonitrile. The flow rate was 0.3 ml/min and the column oven temperature was 40 °C. The gradient program for mobile phase B was as follows: 0 min, 0%; 7 min, 0%; 20 min, 40%; 20.1 min, 100%; 25 min, 100%; 25.1 min, 0%; and 35 min, 0%. The mobile phase for lipids consisted of A: 20 mM ammonium acetate in water and B: methanol. The flow rate was 0.4 ml/min and the column oven temperature was 40 °C. The gradient program for mobile phase B was as follows: 0 min, 80%; 13 min, 98%; 30 min, 98%; 30.1 min, 80%; and 35 min, 80%. The target metabolites of these systems are shown in Supplementary Table 1. Typical MRM chromatograms of these metabolites are shown in Supplementary Fig. 1. Hydrophilic metabolites were evaluated by peak area whereas lipids were evaluated by peak height because some lipid isomers could not be separated completely.

3. Result and discussion

3.1. Optimization of SFE conditions

First, basic conditions for extraction were investigated using methanol as a modifier. Direct collection caused splatter of the extracts. Therefore, extracts contained in the mixture of CO_2 and methanol were bubbled and trapped in methanol, at the bottom of a test tube. In order to determine what kind of metabolites can be extracted using this system, extraction of a dried serum spot (DSS) was performed under the intermediate conditions (pressure, 20 MPa; temperature, 30 °C; methanol, 0.6 ml/min; CO_2, 2.4 ml/min) and the extracts were analyzed under two different LC/MS/MS conditions that target either lipid or hydrophilic metabolites (data not shown). As a result, in addition to the formerly reported phosphatidylcholines (PC) and phosphatidylethanolamines (PEs) [25], hydrophobic metabolites such as acylcarnitines and bile acids were detected by analysis with the extracts. Furthermore, many hydrophilic metabolites such as amino acids, nucleic-acid-related metabolites, and betaines (carnitine, choline, etc.) were also extracted by SFE. These results clearly show that DSS-SFE samples contain the metabolites with a variety of polarities.

Next, parameters of SFE such as temperature, pressure, and modifier were optimized by comparing extraction efficiency of the metabolites representative of each metabolite class under the testing conditions (amino acids, isoleucine; nucleic-acid-related metabolites, hypoxanthine; betaines, choline; other hydrophilic metabolites, creatinine; bile acids, glycodeoxycholic acid; free fatty acids, linoleic acid; PC, PC 18:0-18:2/18:1-18:1; PE, PE 18:0-18:2/18:1-18:1 using DSS as samples. First, extraction temperature (30, 50, 80 °C) was investigated under the following conditions: CO_2, 2.4 ml/min; methanol, 0.6 ml/min; pressure, 20 MPa: time for extraction, 5 min. Although 80 °C was the only condition that met the supercritical conditions among these conditions [29], there was no dramatic improvement in extraction efficiency (Fig. 1A). These

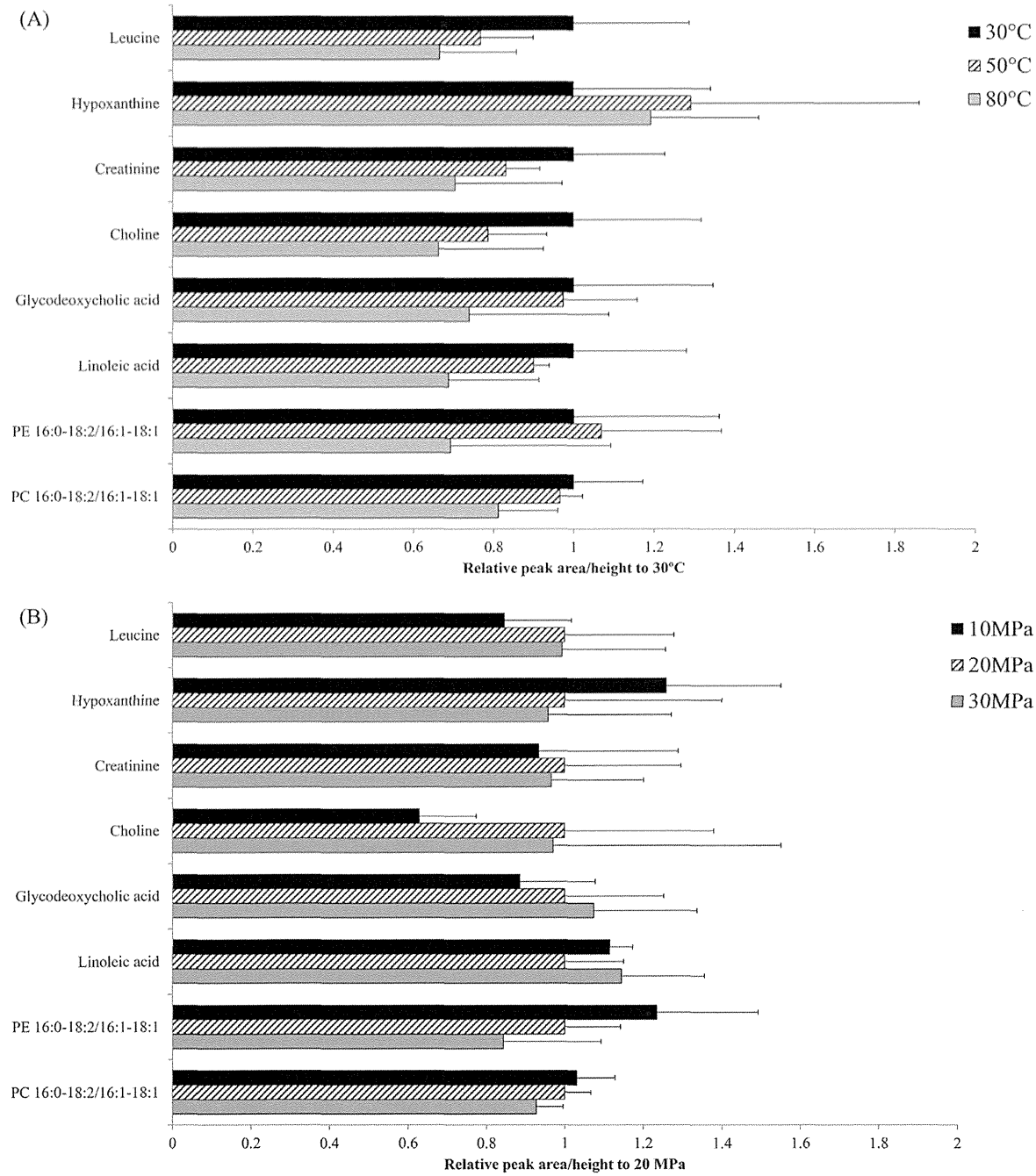

Fig. 1. Effects of temperature (A) and pressure (B) for SFE of various metabolites in dried serum spots (DSS). DSS ($n = 5$) were extracted under conditions of various temperatures (30 °C, 50 °C, 80 °C) and pressures (10 MPa, 20 MPa, 30 MPa), and then analyzed by LC/MS/MS. The value is shown as the relative peak height (for hydrophilic metabolites)/area (for lipids) to that at 30 °C (A) or that at 20 MPa (B). Other conditions: pressure for (A), 20 MPa; temperature for (B), 30 °C; CO_2 flow rate, 2.4 ml/min; methanol flow rate, 0.6 ml/min; time for extraction, 5 min.

results indicate that whether the extraction medium was supercritical or subcritical state did not affect much on extraction efficiency. Actually, the extraction efficiency tended to decrease as the temperature increased. In general, the density of $SCCO_2$ decreases when temperature is increased. Therefore, it stands to reason that the solvent power decreased as temperature increased [30]. In addition, the breakdown of the analytes was another possible reason for this result. Secondly, extraction pressure (10, 20, 30 MPa) was investigated under the following conditions: CO_2, 2.4 ml/min; methanol, 0.6 ml/min; temperature, 30 °C; time for extraction, 5 min. Generally, the increase of pressure makes the density and the solvent power of $SCCO_2$ increase [30]. However, as a result, pressure did not markedly affect the extraction efficiency (Fig. 1B). There are two possible reasons for these results. The first is that the time for extraction was more than sufficient for this sample volume; therefore, the solvent power did not affect the results much. The second reason is that, in this subcritical condition, pressure has a minimal effect on extraction efficiency. Finally, the effect of solvent constitution was investigated (Fig. 2). Temperature and pressure were fixed at 30 °C and 20 MPa, respectively, and various

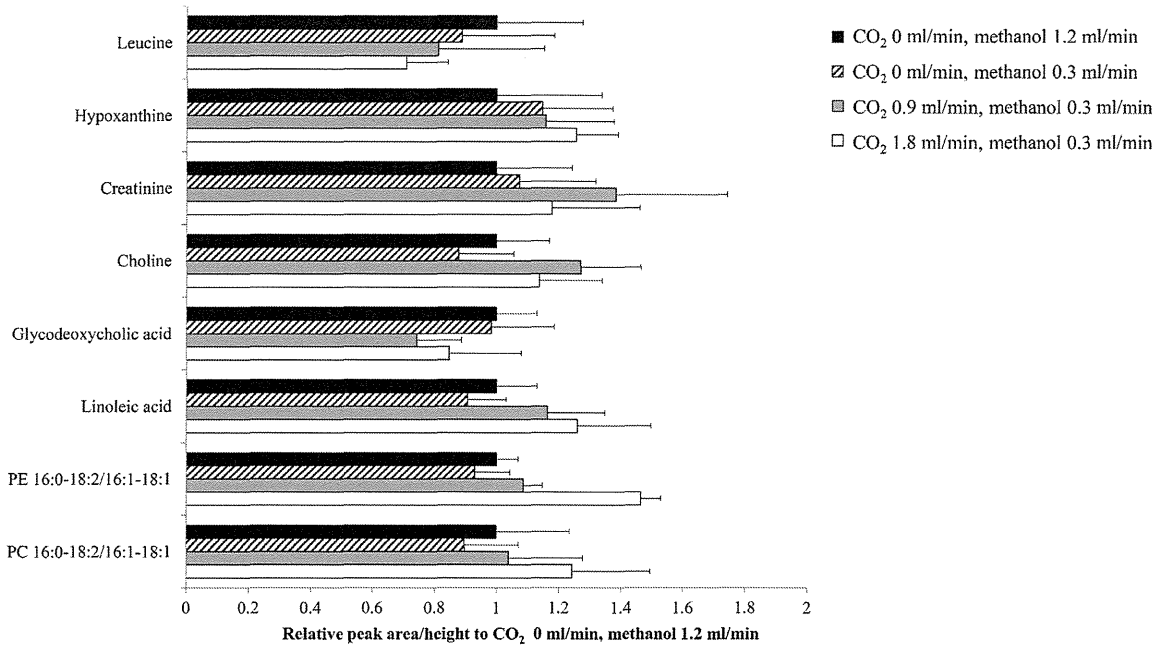

Fig. 2. Effect of solvent constitution for SFE of various metabolites in dried serum spots (DSS). DSS ($n=5$) were extracted under various $SCCO_2$/methanol compositions (0.3 ml/min methanol, 1.2 ml/min methanol, 0.9 ml/min $SCCO_2$ and 0.3 ml/min methanol, 1.8 ml/min $SCCO_2$ and 0.3 ml/min methanol) and analyzed by LC/MS/MS. The value is shown as the relative peak height (for hydrophilic metabolites)/area (for lipids) to that of CO_2 at 0 ml/min, methanol at 1.2 ml/min. Other conditions: temperature, 30 °C; pressure, 20 MPa; time for extraction, 5 min.

$SCCO_2$/methanol compositions (0.3 ml/min methanol, 1.2 ml/min methanol, 0.9 ml/min $SCCO_2$ and 0.3 ml/min methanol, 1.8 ml/min $SCCO_2$ and 0.3 ml/min methanol) were investigated. The results of 0.3 ml/min methanol only were nearly the same as those of 1.2 ml/min methanol only. This indicates that 5 min was enough time for DSS extraction. The effect of CO_2 addition can be estimated using 0.3 ml/min methanol with 0, 0.9, and 1.8 ml/min $SCCO_2$. As a result, the more CO_2 added, the higher the extraction efficiency of lipids tends to be; on the other hand, that of hydrophilic metabolites has a tendency to be lower. These results indicate that, at least to a certain extent, there is an effect of $SCCO_2$ on hydrophobicity. However, the difference is not drastic and it seemed that methanol is a dominant factor in the DBS extraction process.

3.2. Comparison of SFE with conventional organic solvent extraction

Next, SFE was compared with organic solvent extraction (OSE), which is the commonly used preparation method for DBS. From the above-mentioned results, extraction conditions were determined as follows: pressure, 20 MPa; temperature, 30 °C; methanol, 0.3 ml/min; and CO_2, 1.8 ml/min; time for extraction, 5 min. For analysis targeting metabolites with wide range of polarity, it is difficult to determine the only extraction condition which is best for all target metabolites. In this study, therefore, methanol that was the same solvent for modifier of SFE was chosen as organic solvent to minify the difference in extraction condition for easier understand of the results. For SFE conditions, lower CO_2 flow rate was better for hydrophilic metabolites, while higher CO_2 flow rate was better for lipids. In this study, with a view to construct an online coupling to supercritical fluid chromatography (SFC), a single extraction condition was chosen. For online SFE system, in order to trap metabolites in a trap column, low modifier concentration was desired. Therefore, the highest CO_2 flow rate (1.8 ml/min) was chosen. As a result, 37 hydrophilic metabolites including amino acids, betaines,

Table 1
Number of metabolites detected from dried blood spot extracted by supercritical fluid extraction (SFE) or organic solvent extraction (OSE).

	Cation		Lipids	
	Detected	RSD <20%	Detected	RSD <20%
SFE	37	17	195	144
OSE	40	24	185	127

Table 2
List of metabolites whose peak intensity showed a significant difference between supercritical fluid extraction (SFE) and organic solvent extraction (OSE) of dried serum spots.

Significantly high in SFE samples	Significantly high in OSE samples
LPC 14:0 (sn-1)	Glycine
PC 16:0e-16:0	Guanosine
PC 16:0-18:2/16:1-18:1	Hypoxanthine
PC 18:1-18:1/18:0-18:2	Alanine
PC 17:0-20:4	Arginine
PC 18:0-20:4	Citrulline
PC 18:1-22:6	Glutamine
PE 16:1-18:1/16:0-18:2	Glutamate
PE 16:0-18:1	Histidine
PE 16:0-20:4	Homoserine
PE 18:1-18:2	Lysine
PE 18:1-18:1/18:0-18:2	Serine
FA 14:1 Myristoleic acid*	Threonine
FA 14:0 Myristic acid	Tryptophan
FA 15:0 Pentadecylic acid*	Tyrosine
FA 16:1 Palmitoleic acid	Dimethylglycine
FA 17:1 10-Heptadecanoic acid	Taurine
FA 21:0 Heneicosanoic acid*	Kynurenine
FA 22:1 (n-9) Erucic acid*	Phosphocholine*
FA 23:0 Tricosanoic acid	
FA 24:1 (n-9) Nervonic acid	
FA 24:0 Lignoceric acid	
FA 25:0 Pentacosanoic acid	
FA 27:0 Heptacosanoic acid	

* Detected by one extraction method only.

Table 3
List of metabolites detected only from dried serum spot (DSS) or dried whole blood spot (DWBS) by supercritical fluid extraction.

Detected only from DSS	Detected only from DWBS
LPC 17:1 (sn-2)	LPE 22:6 (sn-1)
LPC 19:0 (sn-1)	LPE 22-6 (sn-2)
	PE 16:0-16:1/14:0-18:1
	PE 16:0-16:0
	PE 16:0p-18:1
	PE 16:1-18:2/16:0-18:3
	PE 16:0p-20:5
	PE 18:0p-18:1
	PE 17:0-18:1
	PE 18:0e-18:1
	PE 18:2-18:3
	PE 16:0-20:5
	PE 16:0-22:4
	PE 18:1-20:3/18:2-20:2
	AC 18:0
	AC 18:1
	AC 18:2

nucleic acids, and 195 lipids including (lyso)phosphatidylcholines, (lyso)phosphatidylethanolamines, bile acids, and acylcarnitines were detected from DSS-SFE samples, whereas 40 hydrophilic metabolites and 185 lipids were detected from DSS-OSE (Table 1, Supplementary Table 1). These results clearly show that SFE with the modifier methanol had no disadvantages in hydrophilic metabolite extraction. The number of stably analyzed (RSD <20%) metabolites from SFE samples was 17 hydrophilic metabolites and 144 lipids. Many of the metabolites that showed significantly higher intensity by SFE were phospholipids and fatty acids, some of which were detected only from SFE samples (Table 2, Supplementary Table 1). These results indicate that the extraction efficiency of hydrophobic metabolites was enhanced by $SCCO_2$. Dried whole blood spots (DWBS) were extracted in the same way. It is difficult to store whole blood samples. Dried spot analysis can be one of the solutions to this problem. As a result, many PEs were detected only from DWBS (Table 3, Supplementary Table 1). PEs are one of the main phospholipids in the blood cells that are removed in the serum sample [31]. These results suggest that DWBS are useful samples for the detection of the metabolites that cannot be targeted in the case of serum analysis.

4. Conclusion

In this study, the potential of SFE as a DBS preparation method was examined. Generally, SFE has been considered as effective for hydrophobic compound extraction. However, the addition of methanol as a modifier significantly improved SFE's applicable range of polarity, and there were no problems in hydrophilic metabolite extraction from DSS or DWBS. As a result, over 200 metabolites were detected by LC/MS/MS, of which over 160 metabolites were analyzed stably (RSD <20%). It was shown that the addition of CO_2 improved the extraction efficiency of hydrophobic metabolites.

Furthermore, it is easy to connect SFE with analytical instruments and to construct an online extraction system. Actually, we have developed a high-throughput screening system for phospholipids using supercritical fluid chromatography (SFC)/MS coupled with online SFE [25]. For online extraction, the trapping of target compounds before the separation step is essential, but the most difficult point to be investigated. In SFC, organic solvents increase the power for elution. Therefore, the trapping of target compounds cannot be achieved if a high level of organic solvent is used for the extraction step. This study revealed that SFE requires a low level of methanol to extract hydrophilic metabolites and showed the possibility that many hydrophilic metabolites can be the targets of online SFE-SFC/MS/MS. Several studies were conducted to achieve online extraction of multiple metabolites, and they did not use chromatographic separation before MS analysis [32,33]. These systems cannot separate isomers and cannot avoid ion suppression, which are the main defects of MS. Online SFE-SFC/MS can solve these problems. SFE of DBS, coupled with SFC/MS, is expected to be an ideal analytical technique for TDM, pharmacokinetic study, and multiple biomarker quantification.

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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.jchromb.2014.08.013.

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