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The use of on-line SFE-SFC/MS/MS to analyze disease biomarkers in dried serum spots compared with serum analysis using LC/MS/MS

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ABSTRACT

RATIONALE

The analytical stability and throughput of biomarker assays based on dried serum spots (DSS) are strongly dependent on the extraction process and determination method. In the present study, an on-line system based on supercritical fluid extraction-supercritical fluid chromatography coupled with tandem mass spectrometry (SFE-SFC/MS/MS) was established for analyzing the levels of disease biomarkers in DSS.

METHODS

The chromatographic conditions were investigated using the ODS-EP, diol, and SIL-100A columns. Then, we optimized the SFE-SFC/MS/MS method using the diol column, focusing on candidate biomarkers of oral, colorectal, and pancreatic cancer that were identified using liquid chromatography (LC)/MS/MS.

RESULTS

By using this system, 4 hydrophilic metabolites and 17 hydrophobic metabolites were simultaneously detected within 15 min. In an experiment involving clinical samples, PC 16:0-18:2/16:1-18:1 exhibited 93.8% sensitivity and 64.3% specificity, whereas PC 17:1-18:1/17:0-18:2 showed 81.3% sensitivity and 92.9% specificity for detecting

oral cancer. In addition, assessments of the creatine levels demonstrated 92.3% sensitivity and 78.6% specificity for detecting colorectal cancer.

CONCLUSIONS

The results of this study indicate that our method has great potential for clinical diagnosis and would be suitable for large-scale screening.

Keywords

Supercritical fluid extraction; Supercritical fluid chromatography; Mass spectrometry; Dried serum spot; Disease biomarker

INTRODUCTION

Cancer is a leading cause of death, accounting for an estimated 8.2 million deaths worldwide in 2012.[1] The early, rapid, and accurate diagnosis of cancer using disease biomarker assays is necessary to improve patient prognosis and survival. The detection of changes in the levels of metabolites can be used to determine the physiological state of individuals, which makes it possible to diagnose diseases, and hence, analyzing the metabolites in biospecimens has become an important part of clinical practice.[2,3] Serum contains a wide variety of metabolites, and some of these metabolites might be useful biomarkers of cancer.[4,5] It is important to validate candidate biomarkers in large population studies based on stable and targeted quantitative analytical method. However, some of the metabolites found in serum exhibit poor stability during sample processing, transportation, and/or storage.[6,7] In order to obtain accurate results, clear standard operating procedures for sample

collection, storage, and handling have to be employed. To achieve this, it is necessary to develop easy-to-use techniques.

Dried blood spot (DBS) sampling, a recently developed technique, is advantageous due to its reduced invasiveness, minimal sample volume requirements, and the ease of sample storage (no special laboratory processing is required), which makes it suitable for clinical applications, such as newborn screening and viral disease management.[8–10] In addition, dried serum spot (DSS) sampling is currently used in folate assays.[11] However, the pre-analytical treatments for dried spotting techniques commonly include extraction with organic solvents and further purification steps, such as filtration and/or solid phase extraction, which require optimization based on the physicochemical properties of the target metabolites.

Supercritical fluid extraction (SFE) is an efficient extraction technique involving the use of supercritical carbon dioxide, and it can be used as an alternative to conventional solvent-based extraction techniques.[12,13] In our previous study, hydrophobic and hydrophilic metabolites were extracted from DBS using SFE with the addition of methanol as a modifier, and then analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS).[14] In addition, during SFE the sample preparation and subsequent analysis can be performed using fully automated instruments, which helps to minimize analyte loss during the extraction procedure and increase sample throughput. Moreover, SFE can be coupled with an on-line chromatographic instrument, such as a supercritical fluid chromatography (SFC) system, which are very compatible with SFE as they use supercritical carbon dioxide as a mobile phase.[15] SFC is frequently used to analyze hydrophobic compounds

including lipids,[16] organic pesticides,[17] and fat-soluble vitamins.[18] Recently, Sen et al. presented an analysis of hydrophilic urinary metabolites (for the purpose of metabolic phenotyping) based on the SFC technique.[19] Therefore, on-line SFE-SFC could be used to analyze both the hydrophobic and hydrophilic metabolites in DSS. Furthermore, its combination with MS/MS would enable target metabolite detection with high sensitivity, selectivity, and reproducibility within a broad dynamic range.[20]

The aim of this study is to determine the feasibility of performing disease biomarker analysis of DSS using on-line SFE-SFC/MS/MS. In this study, we carried out serum analysis to identify disease biomarker candidates using LC/MS/MS with three types of methods, which targeted hydrophobic, cationic, and anionic metabolites, respectively. Then, we developed a method for analyzing biomarker candidates in DSS using SFE-SFC/MS/MS. Finally, the utility of the SFE-SFC/MS/MS system for disease diagnosis was assessed using clinical samples.

EXPERIMENTAL

Chemicals and materials

Ultrapure water, acetonitrile (LC/MS grade), chloroform, ammonium acetate (1 M solution, high-performance liquid chromatography grade), formic acid (LC/MS grade), and acetic acid (LC/MS grade) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and methanol (LC/MS grade) was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Tributylamine obtained from Sigma-Aldrich (MO, USA) was used as the ion-pair reagent. Dilauroylphosphatidylcholine (PC 12:0-12:0), which was purchased from Avanti Polar Lipids (AL, USA), was used as an internal standard for

hydrophobic metabolites, whereas 2-bromohypoxanthine and 10-camphorsulfonate, which were purchased from Sigma-Aldrich (MO, USA), were used as internal standards for hydrophilic metabolites. Bond Elut dried matrix spotting (DMS) cards were purchased from Agilent Technologies, Inc. (CO, USA). The carbon dioxide used for the SFE-SFC/MS/MS was purchased from Iwatani Corporation (Tokyo, Japan).

Subjects

The study protocol was approved by the ethics committee of Kobe University Graduate School of Medicine (Kobe, Japan), and the study was conducted between February 2009 and January 2015. The human samples were used in accordance with the guidelines of Kobe University Hospital, and written informed consent was obtained from all subjects. The serum samples from the cancer patients were collected following diagnosis at Kobe University Hospital and related hospitals. The cancer patients were clinically or pathologically diagnosed by physicians and/or pathologists. The serum samples from the healthy volunteers were obtained from Kobe University Hospital and other institutions. The subjects' clinical characteristics are shown in Supplementary Table S1. No clinical abnormalities were detected in the healthy volunteers during medical check-ups, which included a physical assessment, a blood test, a urine test, diagnostic imaging, an endoscopic examination, and/or a medical interview. In addition, the serum and plasma samples were collected from 3 healthy volunteers to assess the influence of sample preparation procedure on metabolite analysis. The serum and plasma samples were prepared from blood samples collected in the morning using the standard venous blood sampling protocol. After blood sampling, the blood tube containing clot activator and separation gel for serum

preparation was gently mixed and kept at room temperature for more than 30 min until the complete coagulation occurred. The blood tube containing EDTA-2Na for plasma preparation was gently mixed and kept at room temperature for 0, 15, or 30 min. Then, the blood tubes were centrifuged at 3000 rpm for 10 min at 4°C, and the serum and plasma were transferred to clean tubes and stored at -80°C before being used.

Procedure for preparing the serum samples

For the analysis of hydrophobic metabolites, 10 µL of serum were mixed with 80 µL of methanol and 10 µL of 500 ppb PC 12:0-12:0 dissolved in methanol as an internal standard, and then the solution was centrifuged at 16,000 g for 5 min at 4°C. The resultant supernatant was subjected to LC/MS/MS analysis.

To extract hydrophilic metabolites, 50 µL of serum were mixed with 900 µL of a solvent mixture (methanol : water : chloroform = 2.5:1:1) containing 1 µM 2-bromohypoxanthine and 10-camphorsulfonate as internal standards. The mixture was subsequently shaken at 1,400 rpm for 30 min at 37°C, before being centrifuged at 16,000 g for 3 min at 4°C. The resultant supernatant (630 µL) was transferred to a clean tube. Then, 280 µL of water were added, and the mixture was mixed well. After the mixture had been centrifuged at 16,000 g for 5 min at 4°C, 500 µL of the resultant supernatant were centrifugally filtrated through a 3-kDa filter device (Millipore, MA, USA) at 14,000 g for 1 hr to remove any proteins. The filtrate was lyophilized using a freeze dryer, dissolved in water, and analyzed by LC/MS/MS. The plasma samples were treated with the same procedure as described above.

Procedure for preparing the DSS samples

As internal standards, 1 μL of 500 ppb PC 12:0-12:0 dissolved in methanol and 1 μL of 100 μM 2-bromohypoxanthine dissolved in water were added to punched pieces (3 mm, I.D.) of DMS card. Subsequently, 1 μL of human serum was dropped onto the pieces of DMS card, 10 μL of acetonitrile were added, and then the card was dried for over 2 hr at room temperature. The pipettings of 1 μL volume were performed using Eppendorf 2000 series reference pipet (capacity: 0.5 to 10μL) with precision of <1.8 % and accuracy of ±2.5 %. Finally, the obtained DSS was enclosed in the extraction vessel and analyzed by SFE-SFC/MS/MS.

LC/MS/MS analysis

The LC/MS/MS analyses were performed using a Nexera LC system (Shimadzu Corp.) equipped with two LC-30AD pumps, a DGU-20A_5 degasser, an SIL-30AC autosampler, a CTO-20AC column oven, and a CBM-20A control module, coupled with an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corp.). The MS was equipped with an electrospray ionization (ESI) source under the following conditions: nebulizing gas flow, 3 L/min; desolvation line temperature, 250°C; heat block temperature, 400°C; drying gas flow, 15 L/min; interface voltage for positive mode, +4.5 kV; and interface voltage for negative mode, -3.5kV. Collision-induced dissociation gas pressure was set to 230 kPa.

Hydrophobic metabolites were separated using an octadecylsilylated silica (ODS) 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 hydrophobic

metabolites 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%. Blank runs (injecting isopropanol) between actual sample runs were employed to remove carry-over contamination.

Cation analysis was carried out using a pentafluorophenylpropyl column (Discovery HS F5, 150 × 2.1 mm, 3 μm; SUPELCO, PA, USA) and a guard column (20 × 2.1 mm, 3 μm). The mobile phase for the cation analysis was composed of 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%.

Anion analysis was performed using an ODS column (InertSustain C18, 150 × 2.1 mm, 3 μm; GL Sciences, Tokyo, Japan). The mobile phase for the anion analysis consisted of A: water containing 15 mM acetic acid and 10 mM tributylamine, and B: methanol. The flow rate was 0.3 mL/min, and the column oven temperature was 35°C. The gradient program for mobile phase B was as follows: 0 min, 0%; 0.5 min, 0%; 20 min, 75%; 20.1 min, 98%; 24 min, 98%; 24.1 min, 0%; and 30 min, 0%.

SFE-SFC/MS/MS analysis

The SFE-SFC/MS/MS analysis was performed using a Nexera UC system (Shimadzu Corp.) equipped with an SFE-30A auto extractor with a rack changer, an LC-30AD$_{SF}$

carbon dioxide pump, an SFC-30A back pressure regulator, an LC-30AD modifier pump, an LC-20AD$_{XR}$ make-up pump, a DGU-20A$_{5R}$ degasser, a CTO-20AC column oven, and a CBM-20A control module, coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corp.). The MS was equipped with a heated ESI source under the following conditions: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface temperature, 300°C; desolvation line temperature, 250°C; heat block temperature, 400°C; drying gas flow, 10 L/min; interface voltage for positive mode, +4.0 kV; and interface voltage for negative mode, -3.0 kV. Collision-induced dissociation gas pressure was set to 270 kPa. In addition, an SIL-30AC autosampler was connected during the analysis of standard compound solutions with SFC/MS/MS. The mobile phase consisted of A: carbon dioxide and B: methanol with 0.1% formic acid. The flow rate was 0.8 mL/min, and the SFE and column oven temperature was 40°C. The back pressure was regulated at 10 MPa in order to maintain the supercritical state of the mobile phase.

The assessments of chromatographic retention and separation were performed with a polar group embedded ODS column (Inertsil ODS-EP, 150 mm × 2.1 mm, 3 μm; GL Sciences, Tokyo, Japan), a pure silica gel column (Inertsil SIL-100A, 150 mm × 2.1 mm, 3 μm; GL Sciences, Tokyo, Japan), and a diol column (Inertsil diol, 150 mm × 2.1 mm, 3 μm; GL Sciences, Tokyo, Japan). During the SFC/MS/MS-based column assessment, the gradient program for mobile phase B was as follows: 0.01 min, 10%; 5 min, 10%; 5.01 min, 10%; 15 min, 40%. For the SFE-SFC/MS/MS-based DSS analysis using the diol column, which was performed in static extraction mode, the gradient program for mobile phase B was as follows: 0.01 min, 10% (static SFE); 4 min, 10% (dynamic SFE); 5 min, 10% (end SFE); 5.01 min, 10%; 15 min, 60%. The

washing program (100% mobile phase B, 3 mL/min, 3 min) after actual sample runs were employed to remove carry-over contamination.

Data analysis

The hydrophobic metabolites detected by LC/MS/MS were putatively identified based on multiple reaction monitoring transitions and their retention times using an in-house library, which targeted lipids (phospholipids, acylcarnitines, fatty acids, bile acids). Phospholipids included in the library were verified based on the precursor m/z and a head group-specific positive fragment (m/z 184.1) for phosphocholines or head group-specific neutral loss (NL 141) for phosphoethanolamines. The structural details of the constituent fatty acids were assigned based on the numbers of C-atoms and double bonds in their negative fragments. Acylcarnitines included in the library were verified based on the precursor m/z and specific positive fragment (m/z 85.05). Fatty acids and bile acids included in the library were identified using authentic chemical standards. A list of the peaks detected in the sera using LC/MS/MS is provided in Supplementary Table S2. In SFE-SFC/MS/MS, the hydrophobic metabolites were putatively identified using same procedure as mentioned above.

The hydrophilic metabolites detected by LC/MS/MS and SFE-SFC/MS/MS were identified based on the precursor ions, single specific product ions, and retention times acquired from authentic chemical standards that had been analyzed using the same analytical methods.

Peak picking and integration were automatically performed using the LabSolutions software (ver. 5.65; Shimadzu Corp.), and the results were then checked manually. The peak area of each metabolite was normalized to that of the internal standard. Then, the obtained corrected peak area values were statistically evaluated using EZR, which is a graphical user interface for the software R.[21] The candidate biomarkers were identified using the Steel–Dwass test (p <0.05) during comparisons between each type of cancer and the healthy volunteers. Spearman's rank correlation coefficients were used to test the strength of the relationships between the corrected peak areas of the biomarker candidates detected in the DSS using SFE-SFC/MS/MS and those obtained during the analysis of serum using LC/MS/MS. The Bland-Altman plots were used to assess the systematic differences between two measurements. Receiver operating characteristic (ROC) analysis was used for each biomarker candidate to evaluate their diagnostic performance.

RESULTS AND DISCUSSION

Identification of disease biomarker candidates in serum

Cancer is a complex disease that develops through a multi-stage carcinogenic process related to metabolic pathways.[22] Hence, variation is seen between the metabolite profiles of different types of cancer. Recently, the use of metabolome analysis of biofluids, e.g., blood, urine, or saliva, for biomarker identification has increased, and such analyses are performed using MS-based techniques because they exhibit high selectivity and sensitivity during the identification and quantification of metabolites.[23] In the present study, we carried out serum analysis using LC/MS/MS to identify disease biomarker candidates for three types of cancer, oral, colorectal, and

pancreatic cancer (Supplementary Table S1). To detect a wide variety of metabolites, we employed three types of method, which targeted hydrophobic metabolites (lipids), cationic metabolites (cations), and anionic metabolites (anions), respectively. As a result, 208, 41, and 33 metabolites were detected during the analysis of hydrophobic, cationic, and anionic metabolites, respectively. Of them, the metabolites that exhibited stable extraction efficiency values during DBS analysis using SFE were selected according to the method described in our previous study[14] in order to screen for disease biomarker candidates that would be applicable to SFE-SFC/MS/MS (Supplementary Table S2). Of the remaining metabolites, we identified disease biomarker candidates that demonstrated *p*-values of <0.05 in the Steel-Dwass test during comparisons between each type of cancer and the healthy volunteers. As shown in Table 1, the levels of 5 metabolites in oral cancer patients, 16 metabolites in colorectal cancer patients, and 11 metabolites in pancreatic cancer patients were significantly altered compared with those of the healthy volunteers, respectively. We targeted these metabolites to optimize our SFE-SFC/MS/MS procedures.

Optimization of SFE-SFC/MS/MS for DSS analysis

In order to establish stable conditions for analyzing the levels of biomarker candidates using SFE-SFC/MS/MS, it is critical to assess the ability of analytical columns to retain biomarker candidates under the extraction conditions and then elute them and produce reasonable peak shapes under the separation conditions. Supercritical fluid carbon dioxide has low polarity (similar to hexane), which makes it suitable for normal phase chromatography. On the other hand, lipids can be efficiently separated based on their fatty acid species using SFC methods and an ODS column (reversed

phase chromatography).[24] In our column assessment, we investigated the utility of the ODS-EP column, which can separate polar lipid species based on their polar head groups and fatty acyl moieties, using the SFC method.[25] In addition, the diol and SIL-100A columns were also investigated. Specifically, we evaluated the chromatographic retention and peak shapes of internal standards, including 2-bromohypoxanthine for hydrophilic metabolites and PC 12:0-12:0 for hydrophobic metabolites using SFC/MS/MS. During this analysis, 10% methanol containing 0.1% formic acid (isocratic) was used as the initial modifier for 5 min, and then the concentration of the modifier was increased to 40% over 10 min (Figure 1). When the ODS-EP column was used, 2-bromohypoxanthine and PC 12:0-12:0 were retained under the initial conditions, although it was difficult to produce reasonable peak shapes for quantification under the separation conditions. On the other hand, the diol and SIL-100A columns were able to retain both compounds under the initial conditions and to elute them with improved peak shapes under the separation conditions. As it produced narrower peaks, the diol column was selected for further use in the present study.

In order to further optimize our SFE-SFC/MS/MS-based procedures for analyzing biomarker candidates, we prepared quality control (QC) serum; i.e., pooled serum obtained from healthy volunteers. Then, the optimal modifier gradient conditions for use in combination with the static extraction mode were assessed using DSS prepared from the QC serum. In this experiment, we employed 10% methanol containing 0.1% formic acid during the SFE, since the extraction efficiency remained stable when the modifier concentration was 10%, but not when it was 5% (data not shown). As a result, 4 hydrophilic metabolites and 17 hydrophobic metabolites were simultaneously

detected using the following protocol: 10% modifier for 5 min, followed by an increase in the modifier concentration to 60% over 10 min (Figure 2).

Feasibility of using SFE-SFC/MS/MS for disease diagnosis

The developed method was applied to clinical samples to investigate the feasibility of using it for disease diagnosis. In our study, the available volumes of some clinical samples were limited and so these could only be used in the LC/MS/MS analysis. Therefore, after excluding the samples with insufficient volume, we analyzed the levels of the biomarker candidates in DSS prepared from the sera of 18 oral cancer patients, 13 colorectal cancer patients, 18 pancreatic cancer patients, or 10 healthy volunteers using SFE-SFC/MS/MS (Supplementary Table S1). To evaluate the analytical performance of our method, the SFE-SFC/MS/MS dataset was statistically compared with the LC/MS/MS dataset for the same subjects. Specifically, we assessed the Spearman's rank correlation coefficients for the relationships between the corrected peak areas of the biomarker candidates detected in the DSS using SFE-SFC/MS/MS and those obtained during the analysis of serum using LC/MS/MS. In addition, inter-batch analytical stability values were calculated using QC samples that were analyzed using SFE-SFC/MS/MS (n=7) and LC/MS/MS (n=5). The concentrations of hydrophilic metabolites in clinical samples were estimated by one-point calibration using the calibrator containing 100 μM authentic chemical standards. The limits of detection were calculated based on S/N=3. As a result, strong correlations were detected between the levels of 3 hydrophilic metabolites and 5 hydrophobic metabolites according to SFE-SFC/MS/MS and the levels of the same metabolites according to LC/MS/MS (ρ >0.7) (Table 2). The metabolites that

demonstrated low correlation coefficients might have been subjected to inadequate chromatographic separation from their structural isomers and/or have been influenced by matrix effects; i.e., ion suppression or enhancement.[26] Moreover, the hydrophilic metabolites in the QC samples displayed high relative standard deviation values, which might have been caused by inter-batch variations in extraction efficiency. Subsequently, the Bland-Altman plots were used to evaluate the measurements of choline, creatine, and hypoxanthine in DSS using SFE-SFC/MS/MS against those in serum analysis using LC/MS/MS (Figure 3). The measured concentrations of choline, creatine, and hypoxanthine in DSS analysis using SFE-SFC/MS/MS showed mean biases of 29.0%, 39.7%, and -33.3%, respectively, compared with those obtained in serum analysis using LC/MS/MS, which might have been caused by matrix effects. It is possible to compensate for matrix effects and variations in extraction efficiency by utilizing stable isotope-labeled internal standards as biomarker candidates, which would increase the accuracy and robustness of our quantification process.[27,28]

Subsequently, ROC analysis was used to calculate area under the curve (AUC), sensitivity, and specificity values for each biomarker candidate to evaluate their diagnostic performance (Supplementary Table S3). In this analysis, the ROC curves that exhibited AUC of >0.8 during SFE-SFC/MS/MS were compared with those obtained using LC/MS/MS (Figure 4). In DSS analysis using SFE-SFC/MS/MS, PC 16:0-18:2/16:1-18:1 exhibited 93.8% sensitivity and 64.3% specificity for detecting oral cancer, whereas PC 17:1-18:1/17:0-18:2 showed 81.3% sensitivity and 92.9% specificity for detecting the disease. Phosphatidylcholine is a major phospholipid component of eukaryotic cells which participates in lipid metabolism, signal transduction mechanisms, and in membrane structure.[29] Thus, the higher levels of

phosphatidylcholine in serum might be involved in increased cell proliferation in the tumor tissue. In addition, assessments of the creatine levels in DSS based on SFE-SFC/MS/MS demonstrated 92.3% sensitivity and 78.6% specificity for detecting colorectal cancer. The creatine/creatine kinase system plays a key role in cellular energy buffering and transport. Several types of malignant cells showed low creatine content.[30] Therefore, the level of creatine might be useful as a marker for detecting cancer. Our method exhibited comparable diagnostic performance to serum analysis using LC/MS/MS, suggesting that DSS analysis using SFE-SFC/MS/MS has potential for disease diagnosis.

In our study, human serum was employed for the identification of disease biomarker candidates and then the use of dried spotting technique. Serum contains variable amounts of blood cell derived metabolites that are released during the coagulation cascade. Pre-analytical phases including sampling, transport, intermediate storage, sample preparation, and biobanking condition are particularly important factors of data quality.[31] To assess the influence of sample preparation procedure on metabolite analysis, we analyzed the levels of disease biomarker candidates in serum and plasma collected from the same healthy volunteers by using LC/MS/MS (Supplementary Table S4). As a result, choline, hypoxanthine, and xanthine had the significantly higher levels in serum than in plasma, possibly due to the release from the clot. Yu et al. showed the good reproducibility of metabolite measurements in both plasma and serum, and high correlations between plasma and serum metabolite concentrations from the same individuals, although some metabolites including lysophosphatidylcholine were higher in serum.[32] During the storage phase for serum preparation, phosphatidylcholine might be hydrolyzed to form

lysophosphatidylcholine by phospholipases, which are released during the clotting process. Recent biochemical evidence shows that lysophosphatidylcholine is converted to lysophosphatidic acid by autotaxin, which is implicated in cell motility and tumor progression.[33] To evaluate the disease specific changes of lipid species, it is critical to eliminate the pre-analytical hydrolysis. The results of our experiment showed no significant differences in the levels of lipid species in serum compared with those in plasma (Supplementary Table S4). Also, the levels of lipid species in plasma were not affected by the storage times at room temperature before blood tube centrifugation. Moreover, Breier et al. reported that the majority of metabolites in serum, which was stored on cool packs for 24 hr before centrifugation, were stable and unaffected by tube type and one or two freeze-thaw cycles, although some amino acids and fatty acids became unstable within 3 or 6 hr before centrifugation.[34] In our experiment, the level of choline in plasma was significantly increased by the influence of storage times at room temperature before blood tube centrifugation. In addition, Ishikawa et al. reported the differences in lipid metabolite profiles between plasma and serum samples, and showed the markedly decreased levels of lipids, unsaturated fatty acids, and oxidative fatty acids by 10 cycles of freeze-thawing[35], suggesting that lipid metabolites might be degraded or metabolized during the process of pre-analytical phases. Thus, further clinical validation of biomarker candidates should be performed under standardized operating procedures of pre-analytical phases.

In small-scale screening, some metabolite biomarkers might produce false-positives or false-negatives due to confounding factors, such as the effects of diet.[36,37] Furthermore, the diagnostic performance of biomarker candidates might be affected by comorbidities associated with lipidomic abnormalities. Recently, lipidomic studies

of diabetes[38] and hyperlipidemia[39] represent lipid species abnormalities, which are in part similar as assumed to be specific for the cancer patients. Therefore, a large number of well-verified clinical samples from multiple institutions and with the detailed sample information including medical history have to be analyzed to further validate the biomarker candidates identified in this study. Our SFE-SFC/MS/MS-based method has the ability to quantify hydrophobic and hydrophilic metabolites in DSS in a single run. Thus, it could be used for large-scale clinical validation and would represent a practical tool for clinical diagnosis.

CONCLUSIONS

In the present study, we established a new SFE-SFC/MS/MS-based method for analyzing disease biomarker candidates in DSS. The optimized method, which was performed with a diol column, was able to analyze the levels of a number of biomarker candidates, including 4 hydrophilic metabolites and 17 hydrophobic metabolites, simultaneously within 15 min. Moreover, an analysis of the diagnostic performance of this method during SFE-SFC/MS/MS-based DSS analysis of the levels of PC 16:0-18:2/16:1-18:1 and PC 17:1-18:1/17:0-18:2 (for oral cancer), or creatine (for colorectal cancer) showed that it exhibited comparable diagnostic performance to that seen during serum analysis using LC/MS/MS, which suggests that our method could be applied to disease diagnosis. Further optimization using stable isotope-labeled internal standards will make it possible to compensate for matrix effects and increase the analytical accuracy of our procedure. Our method has great potential for increasing the use of dried spotting techniques and might be a useful tool for large-scale screening.

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Figure captions

Figure 1
MRM chromatograms of 2-bromohypoxanthine and PC 12:0-12:0 obtained using SFC/MS/MS and an (A) ODS-EP column, (B) diol column, or (C) SIL-100A column
The modifier conditions were as follows: 10% (isocratic) for 5 min, followed by an increase in the modifier concentration to 40% over 10 min.

Figure 2
MRM chromatograms of the disease biomarker candidates in DSS obtained using SFE-SFC/MS/MS
The modifier conditions were as follows: 10% modifier for 5 min (static extraction mode), followed by an increase in the modifier concentration to 60% over 10 min.

Figure 3
Bland-Altman plots of the measured concentrations of (A) choline, (B) creatine, and (C) hypoxanthine in DSS analysis using SFE-SFC/MS/MS and in serum analysis using LC-MS/MS

The center dotted line represents the bias between the two measurements. The upper and lower dotted lines represent the 95% limits of agreement between the two measurements.

Figure 4
ROC curves of (A) PC 16:0-18:2/16:1-18:1 and (B) PC 17:1-18:1/17:0-18:2 for diagnosing oral cancer, or (C) creatine for diagnosing colorectal cancer, based on their levels in cancer patients vs. healthy volunteers.

Electronic supplementary material

Table S1
Characteristics of the study subjects

Table S2
List of the metabolites detected in sera using LC/MS/MS

Table S3
Diagnostic performance of disease biomarker candidates

Table S4
Comparison of the levels of disease biomarker candidates in serum and plasma by using LC/MS/MS

Tables

Table 1

Disease biomarker candidates identified in serum using LC/MS/MS analysis

	Oral cancer		Colorectal cancer		Pancreatic cancer	
Metabolite	*p*-value[b]	Fold change[c]	*p*-value[b]	Fold change[c]	*p*-value[b]	Fold change[c]
Choline	0.024*	0.640	0.068	0.675	0.386	0.815
Creatine	0.914	0.896	0.039*	0.562	0.447	0.731
Hypoxanthine	0.901	1.103	0.260	1.263	0.014*	1.546
Xanthine	0.998	1.151	0.824	1.085	0.039*	1.317
LPC 20:4 (sn-1)	0.901	1.089	0.708	1.144	0.036*	1.431
LPC 22:6 (sn-1)	0.824	1.069	0.936	1.011	0.027*	1.452
LPC 22:6 (sn-2)	0.534	1.151	0.749	1.110	0.008*	1.579
PC 14:0-16:1	0.293	1.045	0.001*	1.179	0.020*	1.193
PC 15:0-18:2	0.029*	1.438	0.009*	1.456	0.329	1.356
PC 18:1e-16:0/18:0e-16:1[a]	0.469	1.119	0.024*	1.344	0.215	1.170
PC 17:0-16:0/18:0-15:0	0.386	1.138	0.029*	1.352	0.175	1.196
PC 18:0e-16:0[a]	0.622	1.119	0.027*	1.437	0.121	1.326
PC 16:0-18:2/16:1-18:1	0.002*	1.180	0.005*	1.212	0.022*	1.155
PC 16:1e-20:3[a]	0.946	1.059	0.215	1.213	0.048*	1.301
PC 17:1-18:1/17:0-18:2	0.010*	1.335	0.010*	1.340	0.366	1.191
PC 16:0-20:4/16:1-20:3	0.666	1.079	0.187	1.116	0.039*	1.205
PC 18:1-18:1/18:0-18:2	0.057	1.152	0.039*	1.219	0.103	1.142
PC 18:0-18:0	0.490	1.383	0.095	1.401	0.001*	2.278
PC 20:0-18:1	0.447	1.147	0.095	1.214	0.002*	1.511
PE 16:0-20:4	0.997	1.034	0.033*	1.587	0.806	1.111

PE 16:0-20:1/18:0-18:1	0.244	1.256	0.048*	1.671	0.708	1.195
PE 18:0p-20:4[a]	0.027*	1.347	0.008*	1.523	0.215	1.459
PE 18:2-20:4/18:1-20:5/16:0-22:6 /16:1-22:5/20:2-18:4	0.708	1.138	0.039*	1.387	0.578	1.175
FA 20:5 (n-3) Eicosapentaenoic acid	0.406	0.843	0.027*	0.590	0.993	1.622
FA 22:6 (n-3) Docosahexaenoic acid	0.534	0.907	0.048*	0.686	0.708	1.634
FA 22:5 (n-6) Docosapentaenoic acid	0.276	0.853	0.036*	0.651	0.806	1.572

[a] Ether-linked species of plasmanyl (e) and plasmenyl (p) analogs of glycerophospholipids

[b] *p*-values were calculated using the Steel–Dwass test, and the asterisks indicate *p*-values of <0.05.

[c] The fold changes in the metabolite levels of the oral cancer patients (n=18), colorectal cancer patients (n=18), or pancreatic cancer patients (n=18) compared with those seen in the healthy volunteers (n=16) are shown.

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FA, fatty acid

Table 2

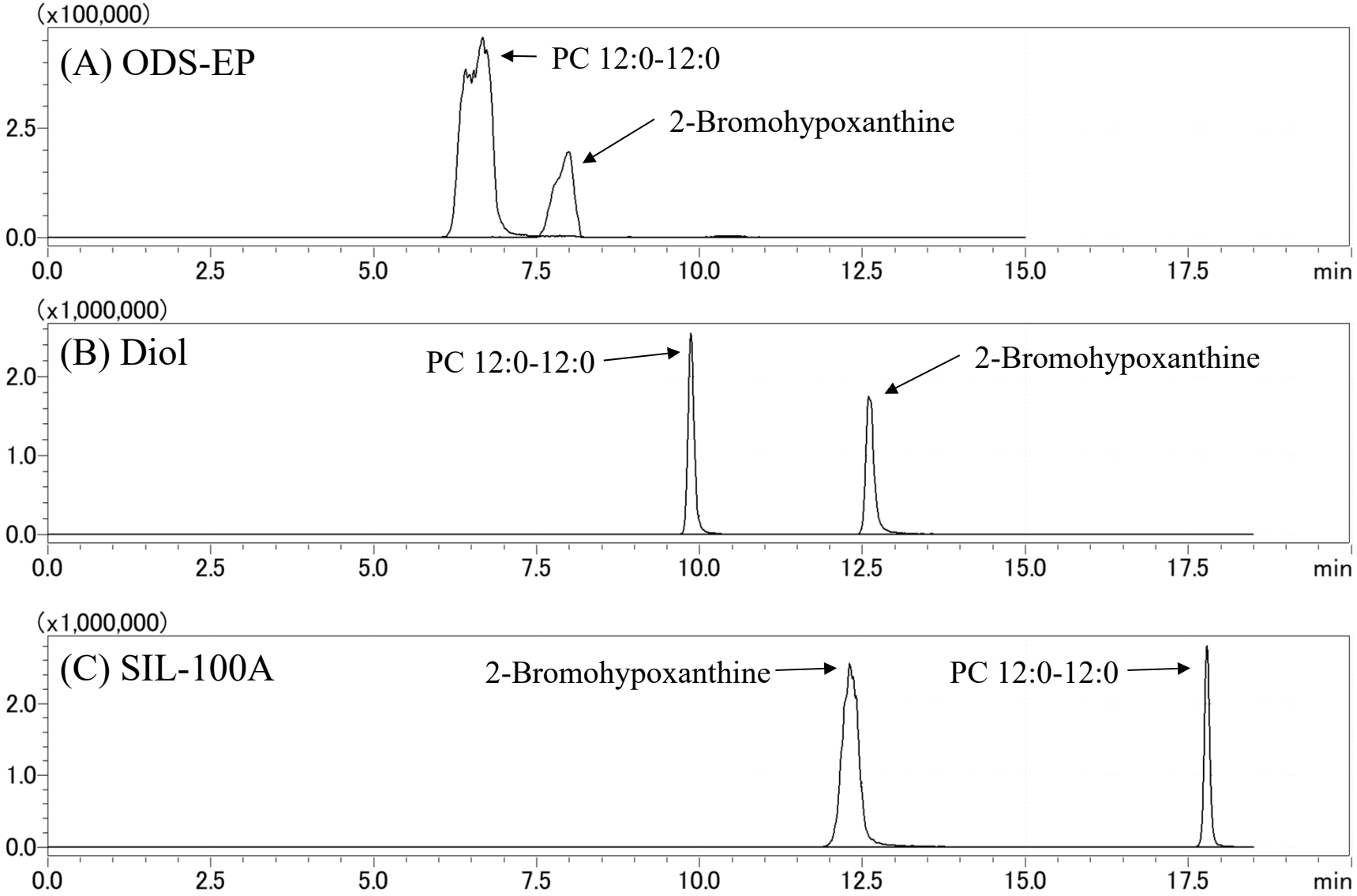
Disease biomarker candidates whose SFE-SFC/MS/MS and LC/MS/MS data exhibited strong correlations

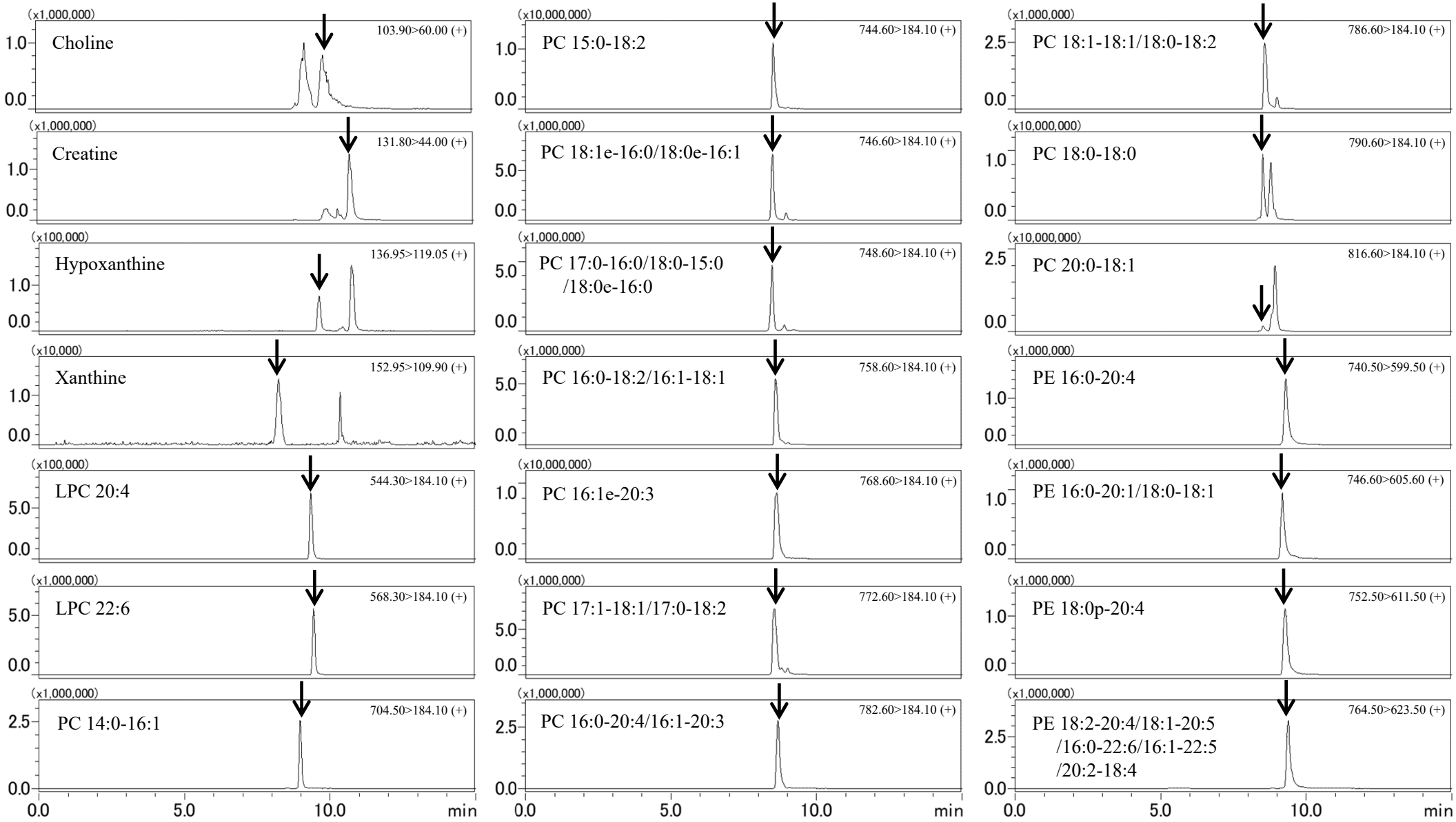
Metabolite	ρ[a]	SFE-SFC/MS/MS of DSS				LC/MS/MS of serum			
		%RSD of QC samples (n=7)	Concentration of clinical samples (μM)		Limit of detection (μM)	%RSD of QC samples (n=5)	Concentration of clinical samples (μM)		Limit of detection (μM)
			Mean	Range			Mean	Range	
Choline	0.863	22.6	65.0	16.9 – 215	0.507	20.8	50.4	13.8 – 155	0.0287
Creatine	0.903	18.3	111	37.1 – 213	0.254	21.4	79.5	14.9 – 209	0.0523
Hypoxanthine	0.749	21.0	20.0	1.24 – 45.6	0.230	8.04	30.0	0.15 – 62.8	0.0528
PC 17:1-18:1/17:0-18:2	0.728	5.29	N.A.[b]	N.A.[b]	N.A.[b]	9.89	N.A.[b]	N.A.[b]	N.A.[b]
PC 18:1-18:1/18:0-18:2	0.748	8.64	N.A.[b]	N.A.[b]	N.A.[b]	9.18	N.A.[b]	N.A.[b]	N.A.[b]
PE 16:0-20:4	0.726	8.07	N.A.[b]	N.A.[b]	N.A.[b]	9.04	N.A.[b]	N.A.[b]	N.A.[b]
PE 16:0-20:1/18:0-18:1	0.792	14.0	N.A.[b]	N.A.[b]	N.A.[b]	8.98	N.A.[b]	N.A.[b]	N.A.[b]
PE 18:2-20:4/18:1-20:5/16:0-22:6 /16:1-22:5/20:2-18:4	0.729	9.89	N.A.[b]	N.A.[b]	N.A.[b]	9.69	N.A.[b]	N.A.[b]	N.A.[b]

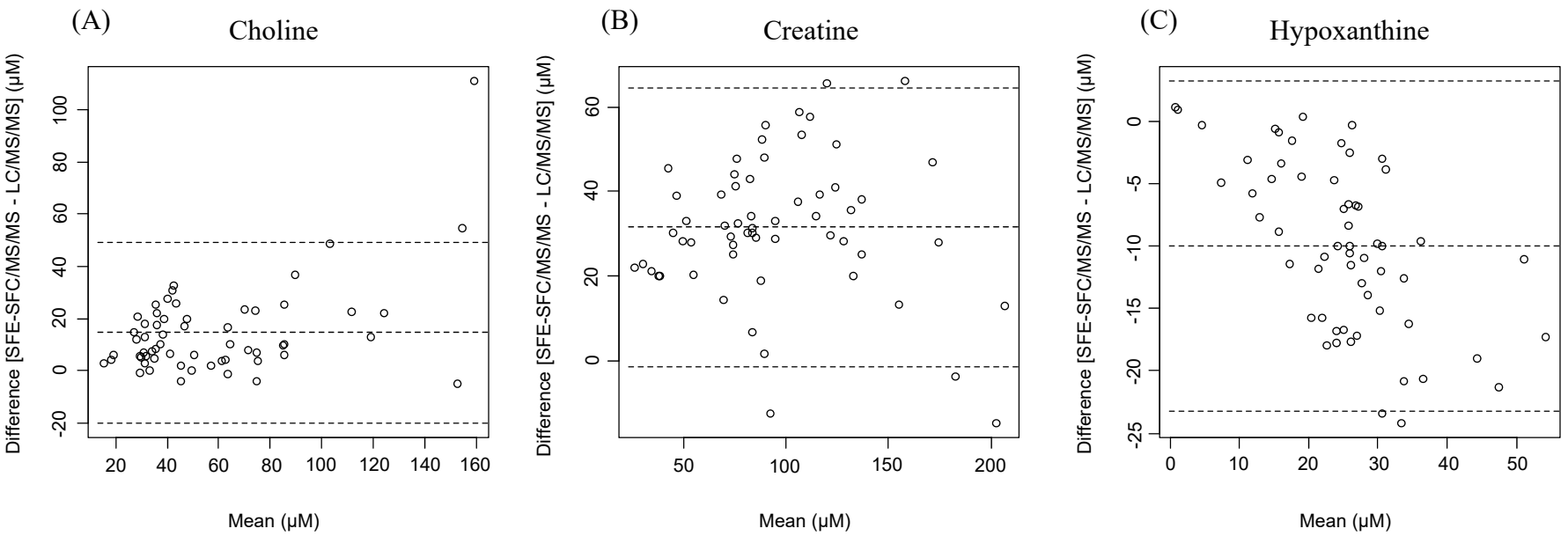
[a] Spearman's rank correlation coefficients were used to test the strength of the relationships between the two sets of data.

[b] Calibrations were not performed due to the lack of authentic chemical standards.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine


(x100,000)
(A) ODS-EP
PC 12:0-12:0
2-Bromohypoxanthine
2.5
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
min
(x1,000,000)
(B) Diol
PC 12:0-12:0
2-Bromohypoxanthine
2.0
1.0
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
min
(x1,000,000)
(C) SIL-100A
2-Bromohypoxanthine
PC 12:0-12:0
2.0
1.0
0.0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
min


(x1,000,000)
Choline
103.90>60.00 (+)
(x1,000,000)
Creatine
131.80>44.00 (+)
(x100,000)
Hypoxanthine
136.95>119.05 (+)
(x10,000)
Xanthine
152.95>109.90 (+)
(x100,000)
LPC 20:4
544.30>184.10 (+)
(x1,000,000)
LPC 22:6
568.30>184.10 (+)
(x1,000,000)
PC 14:0-16:1
704.50>184.10 (+)
(x10,000,000)
PC 15:0-18:2
744.60>184.10 (+)
(x1,000,000)
PC 18:1e-16:0/18:0e-16:1
746.60>184.10 (+)
(x1,000,000)
PC 17:0-16:0/18:0-15:0
/18:0e-16:0
748.60>184.10 (+)
(x1,000,000)
PC 16:0-18:2/16:1-18:1
758.60>184.10 (+)
(x10,000,000)
PC 16:1e-20:3
768.60>184.10 (+)
(x1,000,000)
PC 17:1-18:1/17:0-18:2
772.60>184.10 (+)
(x1,000,000)
PC 16:0-20:4/16:1-20:3
782.60>184.10 (+)
(x1,000,000)
PC 18:1-18:1/18:0-18:2
786.60>184.10 (+)
(x10,000,000)
PC 18:0-18:0
790.60>184.10 (+)
(x10,000,000)
PC 20:0-18:1
816.60>184.10 (+)
(x1,000,000)
PE 16:0-20:4
740.50>599.50 (+)
(x1,000,000)
PE 16:0-20:1/18:0-18:1
746.60>605.60 (+)
(x1,000,000)
PE 18:0p-20:4
752.50>611.50 (+)
(x1,000,000)
PE 18:2-20:4/18:1-20:5
/16:0-22:6/16:1-22:5
/20:2-18:4
764.50>623.50 (+)
0.0
5.0
10.0
min


(A)
Choline
Difference [SFE-SFC/MS/MS - LC/MS/MS] (μM)
Mean (μM)
(B)
Creatine
Difference [SFE-SFC/MS/MS - LC/MS/MS] (μM)
Mean (μM)
(C)
Hypoxanthine
Difference [SFE-SFC/MS/MS - LC/MS/MS] (μM)
Mean (μM)

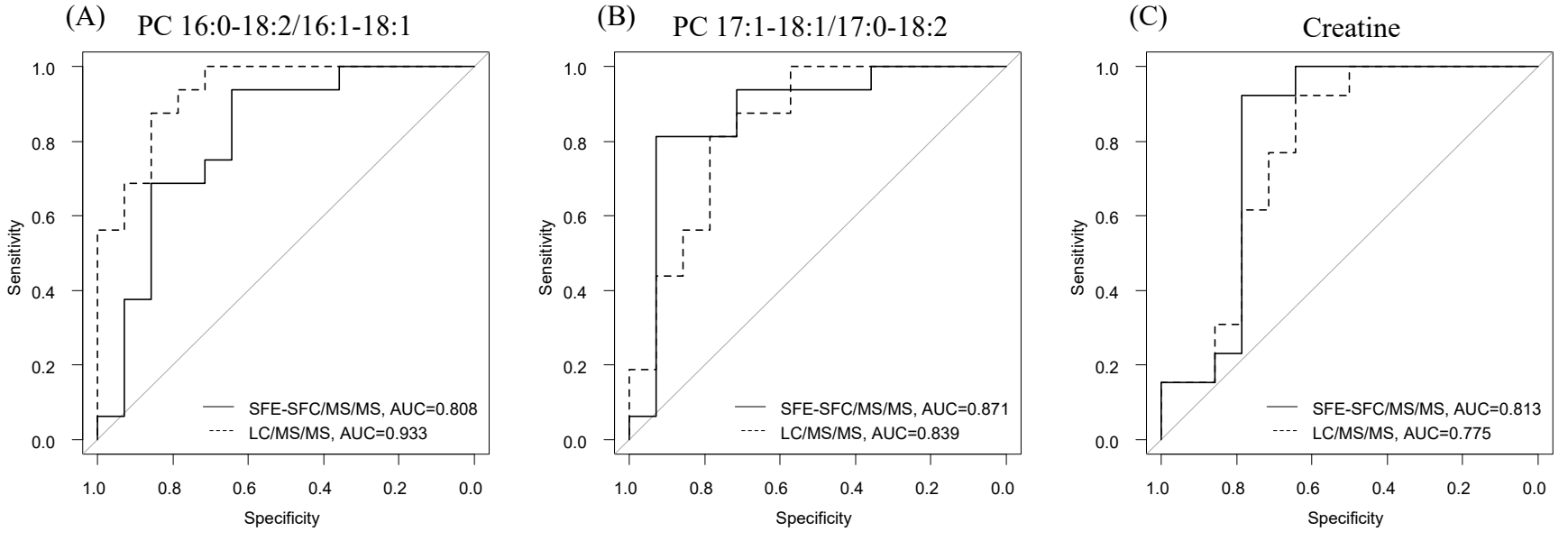

(A)
PC 16:0-18:2/16:1-18:1
Sensitivity
Specificity
SFE-SFC/MS/MS, AUC=0.808
LC/MS/MS, AUC=0.933
(B)
PC 17:1-18:1/17:0-18:2
Sensitivity
Specificity
SFE-SFC/MS/MS, AUC=0.871
LC/MS/MS, AUC=0.839
(C)
Creatine
Sensitivity
Specificity
SFE-SFC/MS/MS, AUC=0.813
LC/MS/MS, AUC=0.775

Table S1
Characteristics of the study subjects

	Oral cancer (n=18)	Colorectal cancer (n=18)	Pancreatic cancer (n=18)	Healthy volunteers (n=16)
Serum analysis using LC/MS/MS				
Number	18	18	18	16
Male/female	10 / 8	11 / 7	8 / 10	9 / 7
p -value[1] (vs. healthy volunteers)	1.000	1.000	0.732	-
Age				
Mean	62.7	68.5	69.7	60.3
SEM	4.24	2.16	1.75	2.37
Range	23 - 88	51 - 88	53 - 83	45 - 73
p -value[2] (vs. healthy volunteers)	0.787	0.140	0.0250	-
Stage	I : 9 II : 9	0 : 15 I : 3	0 : 2 IA : 3 IB : 3 IIA : 5 IIB : 5	-
DSS analysis using SFE-SFC/MS/MS				
Number	18	13	18	10
Male/female	10 / 8	7 / 6	8 / 10	3 / 7
p -value[1] (vs. healthy volunteers)	0.254	0.402	0.689	-
Age				
Mean	62.7	66.8	69.7	58.3
SEM	4.24	2.59	1.75	3.65
Range	23 - 88	51 - 88	53 - 83	45 - 73
p -value[2] (vs. healthy volunteers)	0.785	0.353	0.102	-
Stage	I : 9 II : 9	0 : 12 I : 1	0 : 2 IA : 3 IB : 3 IIA : 5 IIB : 5	-

[1] The differences were evaluated using Fisher's exact test.

[2] The differences were evaluated using the Steel-Dwass test.

Table S2
List of the metabolites detected in sera using LC/MS/MS

Metabolite	Method	*m/z*	Retention time (min)	Stable detection in DBS by SFE[2]
LPC 14:0 (*sn* -1)	Lipid	468.30>184.10	2.88	*
LPC 14:0 (*sn* -2)	Lipid	468.30>184.10	3.20	*
LPC 16:0p[1]	Lipid	480.30>184.10	5.70	
LPC 15:0 (*sn* -1)	Lipid	482.30>184.10	3.56	*
LPC 15:0 (*sn* -2)	Lipid	482.30>184.10	3.95	*
LPC 16:0e[1]	Lipid	482.30>184.10	5.91	
LPC 16:1 (*sn* -1)	Lipid	494.30>184.10	3.29	**
LPC 16:1 (*sn* -2)	Lipid	494.30>184.10	3.63	*
LPC 16:0 (*sn* -1)	Lipid	496.30>184.10	4.38	**
LPC 16:0 (*sn* -2)	Lipid	496.30>184.10	4.82	**
LPC 17:1 (*sn* -1)	Lipid	508.30>184.10	4.03	
LPC 17:1 (*sn* -2)	Lipid	508.30>184.10	4.44	
LPC 17:0 (*sn* -1)	Lipid	510.40>184.10	5.29	*
LPC 17:0 (*sn* -2)	Lipid	510.40>184.10	5.76	*
LPC 18:3 (*sn* -1)	Lipid	518.30>184.10	3.14	**
LPC 18:3 (*sn* -2)	Lipid	518.30>184.10	3.45	
LPC 18:2 (*sn* -1)	Lipid	520.30>184.10	3.85	*
LPC 18:2 (*sn* -2)	Lipid	520.30>184.10	4.22	**
LPC 18:1 (*sn* -1)	Lipid	522.40>184.10	4.87	**
LPC 18:1 (*sn* -2)	Lipid	522.40>184.10	5.30	**
LPC 18:0 (*sn* -1)	Lipid	524.40>184.10	6.26	**
LPC 18:0 (*sn* -2)	Lipid	524.40>184.10	6.75	**
LPC 19:0 (*sn* -1)	Lipid	538.40>184.10	7.25	
LPC 19:0 (*sn* -2)	Lipid	538.40>184.10	7.73	
LPC 20:5 (*sn* -1)	Lipid	542.30>184.10	3.15	**
LPC 20:5 (*sn* -2)	Lipid	542.30>184.10	3.44	**
LPC 20:4 (*sn* -1)	Lipid	544.30>184.10	3.87	**
LPC 20:4 (*sn* -2)	Lipid	544.30>184.10	4.21	**
LPC 20:3 (*sn* -1)	Lipid	546.40>184.10	4.51	**
LPC 20:3 (*sn* -2)	Lipid	546.40>184.10	4.91	*
LPC 20:2 (*sn* -1)	Lipid	548.40>184.10	5.48	*
LPC 20:2 (*sn* -2)	Lipid	548.40>184.10	5.94	**

LPC 20:1 (*sn* -1)	Lipid	550.40>184.10	6.71	*
LPC 20:1 (*sn* -2)	Lipid	550.40>184.10	7.17	*
LPC 20:0 (*sn* -1)	Lipid	552.40>184.10	8.20	
LPC 20:0 (*sn* -2)	Lipid	552.40>184.10	8.70	
LPC 22:6 (*sn* -1)	Lipid	568.30>184.10	3.90	**
LPC 22:6 (*sn* -2)	Lipid	568.30>184.10	4.23	**
LPC 22:4 (*sn* -1)	Lipid	572.40>184.10	5.24	
LPC 22:4 (*sn* -2)	Lipid	572.40>184.10	5.68	
LPC 22:0 (*sn* -1)	Lipid	580.40>184.10	10.03	
LPC 22:0 (*sn* -2)	Lipid	580.40>184.10	10.55	
PC 14:0-16:1	Lipid	704.50>184.10	13.54	**
PC 16:0-14:0	Lipid	706.50>184.10	13.72	**
PC 15:0-16:1	Lipid	718.50>184.10	13.39	
PC 16:0p-16:0[1]	Lipid	718.50>184.10	15.36	**
PC 16:0-15:0	Lipid	720.50>184.10	14.07	*
PC 16:0e-16:0	Lipid	720.50>184.10	15.63	**
PC 14:0-18:2/16:1-16:1	Lipid	730.50>184.10	13.19	*
PC 14:0-18:1/16:0-16:1	Lipid	732.50>184.10	14.02	*
PC 16:0-16:0	Lipid	734.60>184.10	14.83	**
PC 15:0-18:2	Lipid	744.60>184.10	13.79	**
PC 16:0e-18:2[1]	Lipid	744.60>184.10	15.00	**
PC 16:1e-18:1[1]	Lipid	744.60>184.10	15.68	**
PC 15:0-18:1/16:0-17:1	Lipid	746.60>184.10	14.53	**
PC 18:1e-16:0/18:0e-16:1[1]	Lipid	746.60>184.10	15.82	**
PC 17:0-16:0/18:0-15:0	Lipid	748.60>184.10	15.82	**
PC 18:0e-16:0[1]	Lipid	748.60>184.10	17.04	**
PC 14:0-20:5	Lipid	752.50>184.10	12.43	**
PC 16:1-18:3/14:0-20:4	Lipid	754.50>184.10	13.13	*
PC 14:0-20:3	Lipid	756.60>184.10	13.49	*
PC 16:1-18:2/16:0-18:3	Lipid	756.60>184.10	13.76	*
PC 16:0-18:2/16:1-18:1	Lipid	758.60>184.10	14.36	**
PC 16:0-18:1	Lipid	760.60>184.10	15.07	**
PC 16:0-18:0	Lipid	762.60>184.10	16.08	**
PC 15:0-20:5	Lipid	766.50>184.10	13.09	*

PC 16:0e-20:5[1]	Lipid	766.50>184.10	14.10 *
PC 16:0p-20:4[1]	Lipid	766.50>184.10	14.74 *
PC 15:0-20:4	Lipid	768.60>184.10	13.72 *
PC 16:1e-20:3[1]	Lipid	768.60>184.10	14.93 **
PC 17:1-18:2	Lipid	770.60>184.10	14.06 *
PC 18:1e-18:2[1]	Lipid	770.60>184.10	15.24 **
PC 18:2e-18:1[1]	Lipid	770.60>184.10	16.06 **
PC 17:1-18:1/17:0-18:2	Lipid	772.60>184.10	14.87 **
PC 18:0p-18:1/18:1e-18:1[1]	Lipid	772.60>184.10	16.21 **
PC 16:0e-20:2[1]	Lipid	772.60>184.10	17.04 **
PC 17:0-18:1/17:1-18:0/16:0-19:1	Lipid	774.60>184.10	15.70 **
PC 16:1-20:5	Lipid	778.50>184.10	12.77 *
PC 14:0-22:6	Lipid	778.50>184.10	13.04 *
PC 14:0-22:5/16:1-20:4/16:0-20:5	Lipid	780.60>184.10	13.69 *
PC 18:2-18:2/18:1-18:3	Lipid	782.60>184.10	13.86 *
PC 16:0-20:4/16:1-20:3	Lipid	782.60>184.10	14.23 **
PC 18:1-18:2/16:0-20:3/18:0-18:3	Lipid	784.60>184.10	14.63 **
PC 18:1-18:1/18:0-18:2	Lipid	786.60>184.10	15.50 **
PC 18:0-18:1	Lipid	788.60>184.10	16.34 **
PC 16:0p-22:6[1]	Lipid	790.60>184.10	14.64 *
PC 18:0-18:0	Lipid	790.60>184.10	18.33 **
PC 15:0-22:6	Lipid	792.60>184.10	13.64 *
PC 18:1e-20:5[1]	Lipid	792.60>184.10	14.58 **
PC 16:0e-22:6[1]	Lipid	792.60>184.10	14.89 **
PC 17:0-20:5/17:1-20:4	Lipid	794.60>184.10	14.05 *
PC 16:0e-22:5/18:0e-20:5[1]	Lipid	794.60>184.10	15.18 **
PC 18:0p-20:4[1]	Lipid	794.60>184.10	15.95 **
PC 17:0-20:4	Lipid	796.60>184.10	14.80 **
PC 18:1e-20:3[1]	Lipid	796.60>184.10	15.87 **
PC 18:0e-20:4[1]	Lipid	796.60>184.10	16.20 **
PC 17:0-20:3/19:1-18:2	Lipid	798.60>184.10	15.25 **
PC 19:1-18:1/19:0-18:2	Lipid	800.60>184.10	16.13 **
PC 19:0-18:1/18:0-19:1	Lipid	802.60>184.10	17.09 **

PC 18:2-20:5/16:1-22:6	Lipid	804.60>184.10	13.17 *
PC 18:2-20:4/16:0-22:6	Lipid	806.60>184.10	14.14 *
PC 18:1-20:4	Lipid	808.60>184.10	14.53 **
PC 18:0-20:5	Lipid	808.60>184.10	14.77 **
PC 18:1-20:3	Lipid	810.60>184.10	14.89 **
PC 18:0-20:4	Lipid	810.60>184.10	15.36 **
PC 18:1-20:2/18:0-20:3	Lipid	812.60>184.10	15.90 **
PC 16:0-22:2	Lipid	814.60>184.10	15.90 **
PC 18:0-20:2	Lipid	814.60>184.10	16.76 **
PC 20:0-18:1	Lipid	816.60>184.10	19.56 **
PC 18:1e-22:6[1]	Lipid	818.60>184.10	15.03 *
PC 17:0-22:6	Lipid	820.60>184.10	14.69 **
PC 18:0p-22:5[1]	Lipid	820.60>184.10	16.07 **
PC 19:0-20:3	Lipid	826.60>184.10	17.31 **
PC 20:4-20:4	Lipid	830.60>184.10	13.68 **
PC 20:3-20:4	Lipid	832.60>184.10	14.09 *
PC 18:1-22:6	Lipid	832.60>184.10	14.43 **
PC 20:2-20:4/18:1-22:5	Lipid	834.60>184.10	14.85 *
PC 18:0-22:6	Lipid	834.60>184.10	15.27 **
PC 18:0-22:5	Lipid	836.60>184.10	15.69 **
PC 20:1-20:3/18:0-22:4	Lipid	838.60>184.10	16.39 **
PC 18:1-22:0	Lipid	844.70>184.10	15.97
PC 19:0-22:6	Lipid	848.60>184.10	15.90 **
LPE 16:0 (*sn*-1)	Lipid	454.30>313.30	4.44 **
LPE 16:0 (*sn*-2)	Lipid	454.30>313.30	4.88 **
LPE 18:2 (*sn*-1)	Lipid	478.30>337.30	3.91 **
LPE 18:2 (*sn*-2)	Lipid	478.30>337.30	4.29 **
LPE 18:1 (*sn*-1)	Lipid	480.30>339.30	4.91 **
LPE 18:1 (*sn*-2)	Lipid	480.30>339.30	5.36 **
LPE 18:0 (*sn*-1)	Lipid	482.30>341.30	6.33 **
LPE 18:0 (*sn*-2)	Lipid	482.30>341.30	6.81 **
LPE 20:5 (*sn*-1)	Lipid	500.30>359.30	3.19
LPE 20:5 (*sn*-2)	Lipid	500.30>359.30	3.47
LPE 20:4 (*sn*-1)	Lipid	502.30>361.30	3.94 **
LPE 20:4 (*sn*-2)	Lipid	502.30>361.30	4.28 **

LPE 22:6 (*sn* -1)	Lipid	526.30>385.30	3.95 *
LPE 22:6 (*sn* -2)	Lipid	526.30>385.30	4.26 *
PE 16:0-16:1/PE 14:0-18:1	Lipid	690.50>549.50	14.02 *
PE 16:0p-18:1[1]	Lipid	702.50>561.50	15.69 *
PE 16:0e-18:1	Lipid	704.60>563.60	15.84 *
PE 16:0-18:3	Lipid	714.50>573.50	13.80 **
PE 16:0-18:2/16:1-18:1	Lipid	716.50>575.50	14.41 **
PE 16:0-18:1	Lipid	718.50>577.50	15.06 **
PE 16:0-18:0	Lipid	720.60>579.60	16.31 *
PE 16:0p-20:5[1]	Lipid	722.50>581.50	14.13 **
PE 16:0p-20:4[1]	Lipid	724.50>583.50	14.78 **
PE 16:0p-20:3/16:0e-20:4[1]	Lipid	726.50>585.50	14.95 **
PE 18:1p-18:1/18:0p-18:2/18:0e-18:3[1]	Lipid	728.50>587.50	16.13 *
PE 17:0-18:2	Lipid	730.50>589.50	14.89 *
PE 18:0e-18:2[1]	Lipid	730.50>589.50	16.11
PE 18:0p-18:1[1]	Lipid	730.50>589.50	17.21 **
PE 17:0-18:1	Lipid	732.60>591.60	15.69 **
PE 18:0e-18:1[1]	Lipid	732.60>591.60	17.33 *
PE 18:2-18:3	Lipid	738.50>597.50	13.31 **
PE 16:0-20:5	Lipid	738.50>597.50	13.69 **
PE 18:2-18:2	Lipid	740.50>599.50	13.90 **
PE 16:0-20:4	Lipid	740.50>599.50	14.26 **
PE 18:1-18:2	Lipid	742.50>601.50	14.58 **
PE 18:1-18:1/18:0-18:2	Lipid	744.60>603.60	15.50 **
PE 16:0-20:1/18:0-18:1	Lipid	746.60>605.60	16.35 **
PE 16:0p-22:6[1]	Lipid	748.60>607.60	14.68 **
PE 18:0p-20:5/18:1p-20:4/16:0e-22:6[1]	Lipid	750.50>609.50	14.90 **
PE 18:1p-20:3/16:0p-22:4[1]	Lipid	752.50>611.50	15.20 **
PE 18:0p-20:4[1]	Lipid	752.50>611.50	15.98 **
PE 17:0-20:4	Lipid	754.50>613.50	14.93
PE 18:1e-20:3[1]	Lipid	754.50>613.50	16.23 **
PE 16:1-22:6/18:2-20:5	Lipid	762.50>621.50	13.32 *
PE 18:2-20:4/18:1-20:5/16:0-22:6/16:1-22:5/20:2-18:4	Lipid	764.50>623.50	14.14 **

PE 18:1-20:4	Lipid	766.50>625.50	14.51	**
PE 18:0-20:5	Lipid	766.50>625.50	14.80	*
PE 18:0-20:4	Lipid	768.60>627.60	15.39	**
PE 20:1-18:2	Lipid	770.60>629.60	15.39	*
PE 18:0-20:3	Lipid	770.60>629.60	16.00	*
PE 18:0p-22:6/18:1p-22:5[1]	Lipid	776.50>635.50	15.85	**
PE 18:0p-22:5/18:1p-22:4[1]	Lipid	778.50>637.50	16.12	*
PE 18:1-22:6	Lipid	790.50>649.50	14.42	
PE 18:0-22:6	Lipid	792.60>651.60	15.29	
PE 18:0-22:5	Lipid	794.60>653.60	15.74	
FA 14:1 (n-5) Myristoleic acid	Lipid	225.20>225.20	2.56	
FA 16:1 (n-7) Palmitoleic acid	Lipid	253.10>253.10	3.85	*
FA 16:0 Palmitic acid	Lipid	255.05>255.05	5.12	*
FA 17:1 (n-7) *cis* -10-Heptadecanoic acid	Lipid	267.20>267.20	4.72	*
FA 18:4 (n-3) Stearidonic acid	Lipid	275.20>275.20	2.99	
FA 18:3 (n-3) α-Linolenic acid/(n-6) gamma-Linolenic acid	Lipid	276.90>276.90	3.66	*
FA 18:2 (n-6) Linoleic acid	Lipid	278.95>278.95	4.50	**
FA 18:1 (n-9)c Oleic acid/(n-7)c Vaccenic acid	Lipid	280.90>280.90	5.65	**
FA 20:5 (n-3) Eicosapentaenoic acid	Lipid	300.90>300.90	3.66	**
FA 20:4 (n-6) Arachidonic acid	Lipid	303.10>303.10	4.49	**
FA 20:3 (n-6) Dihomo-gamma-linolenic acid/(n-9) Mead acid	Lipid	305.05>305.05	5.28	**
FA 20:2 (n-6) *cis* -11-14-Eicosadienoic acid	Lipid	307.30>307.30	6.38	*
FA 20:1 (n-9) *cis* -11-Eicosenoic acid	Lipid	309.30>309.30	7.62	*
FA 22:6 (n-3) Docosahexaenoic acid	Lipid	326.95>326.95	4.40	**
FA 22:5 (n-6) Docosapentaenoic acid	Lipid	329.20>329.20	5.10	**
FA 22:4 (n-6) Docosatetraenoic acid	Lipid	331.30>331.30	6.09	**
FA 24:1 (n-9) Nervonic acid	Lipid	365.30>365.30	11.36	**
Glycodeoxycholic acid	Lipid	448.10>448.10	1.41	**
AC 2:0	Lipid	204.10>85.05	0.77	
AC 4:0	Lipid	233.20>85.05	0.80	
AC 6:0	Lipid	261.20>85.05	0.91	
AC 8:0	Lipid	289.20>85.05	1.00	
AC 10:0	Lipid	317.30>85.05	1.31	
AC 12:0	Lipid	345.30>85.05	1.86	
AC 14:0	Lipid	373.30>85.05	2.92	

AC 14:1	Lipid	371.30>85.05	2.30	
AC 16:0	Lipid	401.40>85.05	4.45	**
AC 16:1	Lipid	399.30>85.05	3.28	
AC 18:0	Lipid	429.40>85.05	6.33	**
AC 18:1	Lipid	427.40>85.05	4.95	*
AC 18:2	Lipid	425.40>85.05	3.88	*
Glycine	Cation	76.00>30.00	1.64	*
Alanine/Sarcosine	Cation	90.00>43.95	1.75	*
Choline	Cation	103.90>60.00	3.26	**
Serine	Cation	106.00>59.90	1.58	*
Creatinine	Cation	114.05>43.90	3.46	**
Proline	Cation	116.05>70.10	2.15	**
N-Acetylglycine	Cation	117.95>76.10	2.56	
Valine	Cation	118.00>72.00	3.39	*
Threonine	Cation	120.05>56.10	1.70	*
Homoserine	Cation	120.05>74.15	1.70	*
Tyramine	Cation	121.00>77.05	13.10	
Cysteine	Cation	122.25>58.95	1.72	
Taurine	Cation	126.00>44.10	1.52	
4-Hydroxy-proline	Cation	131.70>85.90	1.66	**
Creatine	Cation	131.80>44.00	2.61	**
N-Acetyl-alanine	Cation	131.95>89.90	4.22	**
Isoleucine	Cation	132.00>86.00	7.69	*
Leucine/Norleucine	Cation	132.00>86.00	8.64	*
Ornithine	Cation	133.00>70.10	2.02	*
Asparagine	Cation	133.00>74.00	1.58	*
Aspartate	Cation	134.00>73.90	1.60	
Hypoxanthine	Cation	136.95>119.05	4.08	**
Tryptamine	Cation	144.00>116.95	16.38	
Glutamine	Cation	147.00>83.90	1.67	*
Lysine	Cation	147.10>84.15	2.04	*
Glutamate	Cation	147.95>83.95	1.68	*
Methionine	Cation	149.95>55.95	3.85	*
Xanthine	Cation	152.95>109.90	4.08	**
Histidine	Cation	156.00>110.10	2.06	*

Carnitine	Cation	161.80>60.00	3.88	**
Phenylalanine	Cation	166.00>120.20	13.11	*
Arginine	Cation	175.20>70.10	2.23	*
Serotonin	Cation	177.00>159.95	15.23	
Tyrosine	Cation	182.00>90.95	8.13	*
Phosphocholine	Cation	184.00>86.00	1.59	
Tryptophan	Cation	204.80>188.15	16.40	*
Kynurenine	Cation	209.00>191.95	13.36	*
Cystathionine	Cation	223.00>87.95	1.60	
Cystine	Cation	240.95>151.85	1.55	
Cytidine	Cation	244.00>111.95	5.04	
Uridine	Cation	245.00>113.05	4.18	**
Pyruvate	Anion	87.2>43.05	8.73	
Lactate	Anion	89.2>43	6.98	
3-Hydroxypropionate	Anion	89.2>59.15	5.56	
Phosphoric acid	Anion	97.15>79	6.21	
2-Hydroxyisobutyrate	Anion	103.15>57.1	9.14	
3-Hydroxybutyrate	Anion	103.15>59.1	7.38	
3-Hydroxyisobutyrate	Anion	103.15>73.1	7.13	
Glycerate	Anion	105.2>75.05	5.94	
Fumarate	Anion	115.2>71.05	12.38	
Levulinate	Anion	115.25>71.1	8.49	
Succinate	Anion	117.2>73.05	9.94	
3-Hydroxy-3-methylbutanoate	Anion	117.25>59.1	9.88	
2-Hydroxy-3-methylbutyrate	Anion	117.25>71.1	11.49	
Benzoate	Anion	121.25>77.05	14.12	
4-Methyl-2-oxovalerate	Anion	129.25>85.1	14.56	
Ethylmalonate	Anion	131.15>87.1	11.48	
2-Hydroxyisocaproate	Anion	131.25>85.1	13.70	
Malate	Anion	133.2>115.05	10.81	
Threonate	Anion	135.15>75.05	5.87	
p -Hydroxybenzoate	Anion	137.1>93.1	9.11	
Acetylsalicylate	Anion	137.1>93.1	14.82	
2-Ketoglutarate	Anion	145.1>101	11.62	
2-Hydroxyglutarate	Anion	147.15>129.05	11.01	

2-Hydroxyphenylacetate	Anion	151.15>107.05	14.24
β-Phenyllactate	Anion	165.15>147.1	14.50
Urate	Anion	168.95>140.9	4.95
Glycerol phosphate	Anion	171.1>79.05	7.64
cis -Aconitate	Anion	173.1>85.1	12.78
2-Isopropylmalate	Anion	175.2>115.1	12.37
Hippurate	Anion	178.15>77.1	13.52
4-Hydroxyphenyllactate	Anion	181.15>163.1	11.17
Glucuronate	Anion	193.15>113.05	5.29
Gluconate	Anion	195.15>75.1	5.79

[1] Ether-linked species of plasmanyl (e) and plasmenyl (p) analogs of glycerophospholipids

[2] *detected, **stably analyzed (RSD<20%) (A. Matsubara et al., J. Chromatogr. B. 969 (2014) 199–204.)

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; FA, fa

Table S3
Diagnostic performance of disease biomarker candidates

(A) ROC analysis aimed at discriminating between samples from patients with oral cancer (n=18) and healthy volunteers (n=10)

Metabolite	SFE-SFC/MS/MS of DSS			LC/MS/MS of serum		
	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
Choline	0.750	0.714	0.621	0.625	1.000	0.790
PC 15:0-18:2	0.875	0.643	0.786	0.750	0.786	0.813
PC 16:0-18:2/16:1-18:1	0.938	0.643	0.808	0.875	0.857	0.933
PC 17:1-18:1/17:0-18:2	0.813	0.929	0.871	0.813	0.786	0.839
PE 18:0p-20:4	0.563	0.786	0.652	0.750	0.929	0.763

(B) ROC analysis aimed at discriminating between samples from patients with colorectal cancer (n=13) and healthy volunteers (n=10)

Metabolite	SFE-SFC/MS/MS of DSS			LC/MS/MS of serum		
	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
Creatine	0.923	0.786	0.813	0.923	0.643	0.775
PC 14:0-16:1	0.846	0.571	0.687	0.923	0.857	0.879
PC 15:0-18:2	0.923	0.500	0.681	0.923	0.714	0.857
PC 18:1e-16:0/18:0e-16:1	0.846	0.714	0.742	0.769	0.857	0.841
PC 17:0-16:0/18:0-15:0	0.923	0.571	0.731	0.769	0.857	0.841
PC 18:0e-16:0	0.923	0.571	0.731	0.769	0.857	0.852
PC 16:0-18:2/16:1-18:1	0.462	0.857	0.610	0.923	0.643	0.846
PC 17:1-18:1/17:0-18:2	0.846	0.714	0.753	0.923	0.786	0.830
PC 18:1-18:1/18:0-18:2	0.692	0.643	0.588	0.846	0.714	0.780
PE 16:0-20:4	0.308	1.000	0.610	0.615	1.000	0.819
PE 16:0-20:1/18:0-18:1	0.308	1.000	0.549	0.692	0.857	0.786
PE 18:0p-20:4	0.462	0.786	0.555	0.692	0.929	0.786
PE 18:2-20:4/18:1-20:5/16:0-22:6	0.615	0.643	0.516	0.846	0.714	0.802

(C) ROC analysis aimed at discriminating between samples from patients with pancreatic cancer (n=18) and healthy volunteers (n=10)

Metabolite	SFE-SFC/MS/MS of DSS			LC/MS/MS of serum		
	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
Hypoxanthine	0.438	0.929	0.719	0.875	0.857	0.853
Xanthine	0.688	0.643	0.554	0.813	0.714	0.772
LPC 20:4 (sn-1)	0.688	0.500	0.558	0.750	0.857	0.808
LPC 22:6 (sn-1)	0.938	0.286	0.518	0.750	0.929	0.848
LPC 22:6 (sn-2)				0.875	0.786	0.862
PC 14:0-16:1	0.563	0.571	0.451	0.813	0.857	0.821
PC 16:0-18:2/16:1-18:1	0.313	0.857	0.482	0.875	0.714	0.790
PC 16:1e-20:3	0.563	0.714	0.594	0.813	0.714	0.763
PC 16:0-20:4/16:1-20:3	0.563	0.643	0.545	0.688	0.786	0.768
PC 18:0-18:0	0.750	0.357	0.478	0.813	0.929	0.915
PC 20:0-18:1	0.625	0.714	0.621	0.875	0.857	0.879

Table S4
Comparison of the levels of disease biomarker candidates in serum and plasma by using LC/MS/MS

	Corrected peak area (Mean ± SD)[b]				*p*-value[e]					
Metabolite	Serum[c]	Plasma (0 min)[d]	Plasma (15 min)[d]	Plasma (30 min)[d]	Serum vs Plasma (0 min)	Serum vs Plasma (15 min)	Serum vs Plasma (30 min)	Plasma (0 min) vs Plasma (15 min)	Plasma (0 min) vs Plasma (30 min)	Plasma (15 min) vs Plasma (30 min)
Choline	0.444 ± 0.0867	0.282 ± 0.0705	0.343 ± 0.0701	0.393 ± 0.0891	0.0164*	0.0891	0.740	0.0891	0.0356*	0.740
Creatine	1.55 ± 0.872	1.33 ± 0.873	1.41 ± 0.701	1.78 ± 0.967	0.312	0.876	0.996	0.836	0.519	0.687
Hypoxanthine	0.129 ± 0.141	0.0116 ± 0.0165	0.0121 ± 0.0144	0.0196 ± 0.0178	0.027*	0.0203*	0.0596	0.989	0.456	0.521
Xanthine	0.0421 ± 0.0293	0.00774 ± 0.00736	0.0125 ± 0.0112	0.0178 ± 0.0107	0.00932*	0.0277*	0.229	0.575	0.0574	0.632
LPC 20:4 (sn-1)	0.941 ± 0.218	0.993 ± 0.309	0.997 ± 0.203	0.950 ± 0.250	0.979	0.996	0.999	0.999	0.979	0.911
LPC 22:6 (sn-1)	0.351 ± 0.0950	0.386 ± 0.131	0.373 ± 0.0869	0.354 ± 0.106	0.962	0.940	0.999	0.979	0.962	0.836
LPC 22:6 (sn-2)	0.424 ± 0.136	0.426 ± 0.150	0.445 ± 0.115	0.412 ± 0.136	0.999	0.940	0.990	1.00	0.979	0.632
PC 14:0-16:1	34.0 ± 4.29	31.7 ± 2.58	32.6 ± 3.05	31.6 ± 3.33	0.687	0.911	0.687	0.911	0.999	0.911
PC 15:0-18:2	2.18 ± 0.552	2.13 ± 0.453	2.22 ± 0.679	2.13 ± 0.612	0.911	0.996	1.00	0.979	0.999	0.979
PC 18:1e-16:0/18:0e-16:1[a]	8.05 ± 0.952	7.82 ± 1.48	8.31 ± 1.66	8.15 ± 2.05	0.575	1.00	0.979	0.940	0.911	0.999
PC 17:0-16:0/18:0-15:0	0.734 ± 0.0775	0.703 ± 0.119	0.749 ± 0.131	0.727 ± 0.158	0.464	1.00	0.996	0.876	0.911	0.979
PC 18:0e-16:0[a]	0.622 ± 0.163	0.594 ± 0.118	0.632 ± 0.197	0.609 ± 0.226	0.999	1.00	0.911	0.999	0.911	0.962
PC 16:0-18:2/16:1-18:1	158 ± 13.7	156 ± 15.8	163 ± 23.2	156 ± 23.7	0.990	0.979	0.940	0.836	0.996	0.790
PC 16:1e-20:3[a]	12.7 ± 1.51	12.3 ± 1.89	13.0 ± 1.72	12.6 ± 1.56	0.979	0.979	0.979	0.836	0.979	0.940
PC 17:1-18:1/17:0-18:2	6.90 ± 1.57	6.72 ± 1.39	7.25 ± 1.93	7.11 ± 2.07	0.990	0.999	0.996	0.999	0.999	0.990
PC 16:0-20:4/16:1-20:3	64.4 ± 10.7	63.0 ± 11.5	65.4 ± 9.65	64.4 ± 8.09	0.990	0.996	0.940	1.00	1.00	0.990
PC 18:1-18:1/18:0-18:2	162 ± 9.14	157 ± 14.3	167 ± 14.2	161 ± 14.9	0.836	0.632	0.996	0.575	0.979	0.911
PC 18:0-18:0	0.232 ± 0.0704	0.205 ± 0.0723	0.217 ± 0.0844	0.249 ± 0.127	0.836	0.962	1.00	1.00	0.836	0.836
PC 20:0-18:1	11.9 ± 2.42	10.5 ± 1.29	11.7 ± 2.98	11.5 ± 1.88	0.740	0.940	0.962	0.876	0.519	0.999
PE 16:0-20:4	0.0766 ± 0.0135	0.0693 ± 0.0133	0.0772 ± 0.00850	0.0773 ± 0.0146	0.632	0.996	0.999	0.229	0.632	0.996
PE 16:0-20:1/18:0-18:1	0.223 ± 0.0274	0.211 ± 0.0280	0.232 ± 0.0223	0.230 ± 0.0311	0.790	0.790	0.940	0.360	0.519	0.996
PE 18:0p-20:4[a]	0.176 ± 0.0402	0.178 ± 0.0426	0.196 ± 0.0431	0.174 ± 0.0444	0.999	0.687	0.999	0.836	0.979	0.519
PE 18:2-20:4/18:1-20:5/16:0-22:6 /16:1-22:5/20:2-18:4	0.318 ± 0.0621	0.305 ± 0.0480	0.321 ± 0.0757	0.301 ± 0.0783	0.979	1.00	0.836	0.996	0.962	0.940
FA 20:5 (n-3) Eicosapentaenoic acid	0.0336 ± 0.0118	0.0285 ± 0.00971	0.0270 ± 0.00827	0.0278 ± 0.00681	0.740	0.575	0.790	0.990	0.999	0.996
FA 22:6 (n-3) Docosahexaenoic acid	0.142 ± 0.0336	0.130 ± 0.0402	0.121 ± 0.0292	0.119 ± 0.0253	0.876	0.519	0.519	0.962	0.979	0.999
FA 22:5 (n-6) Docosapentaenoic acid	0.102 ± 0.0186	0.0931 ± 0.0207	0.0896 ± 0.0137	0.0947 ± 0.0107	0.836	0.519	0.911	0.911	0.999	0.876

[a] Ether-linked species of plasmanyl (e) and plasmenyl (p) analogs of glycerophospholipids

[b] The values of mean and SD were calculated using corrected peak area obtained from 3 analytical replicates of each serum or plasma collected from 3 healthy volunteers.

[c] The blood tube containing clot activator and separation gel for serum preparation was gently mixed and kept at room temperature until complete coagulation occurred after blood sampling, and then centrifuged at 3,000 rpm for 10 min at 4°C.

[d] The blood tube containing EDTA-2Na for plasma preparation was gently mixed and kept at room temperature for 0, 15, or 30 min after blood sampling, and then centrifuged at 3,000 rpm for 10 min at 4°C.

[e] *p*-values were calculated using the Steel–Dwass test, and the asterisks indicate *p*-values of <0.05.

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FA, fatty acid