治療もおこなえるメリットがある一方，患者の負担が大きく前処置の煩雑さ，穿孔などのリスク，検査に対する羞恥心などから，二次検診としての受診率の低さが問題となっている．これらの問題点から，より低侵襲，高感度，高特異度の大腸癌検診法が求められている．近年，バイオマーカーの検索としてメタボロミクスが注目されている．生体分子を網羅的に解析することはオミックス解析とよばれており，その種類はDNAを解析するゲノミクス，メッセンジャーRNA（mRNA）を解析するトランスクリプトミクス，蛋白質を解析するプロテオミクス，代謝物を解析するメタボロミクスがあげられる．メタボロミクスはおもな特徴として，ヒトゲノム（約25,000種類）やプロテオミクス（約1,000,000種類）とくらべて約4,000種類と対象が少ないため，解析が比較的容易である．また，メタボロミクスは有機酸，アミノ酸，脂肪酸，糖類などさまざまな低分子化合物を解析の対象としているが，これらの物質は動物依存性がないうえに，いままでの研究でさまざまな手法で取り扱われてきたものであることから，生理学，病理学的な知識，データベースが蓄積されている．また，代謝物はいわゆる生体のセントラルドグマの下流にあたることから，上流の解析にあたるゲノミクスやプロテオミクスとくらべて生体の現在の病態をリアルタイムに反映し変動することで，疾患の早期発見のバイオマーカーの探索に適していると考えられる．これまでは，低分子化合物がさまざまな化学的特徴をもち不安定であることから，恒常性，再現性のある網羅的な解析が困難であった．しかし，近年，質量分析（mass spectorometory：MS）を使用した測定が広くおこなわれるようになり，安定した網羅的な代謝物解析が可能となった．オミックス解析で用いられるMSは，その対象となる分子の水溶性（極性），または，揮発性に応じてガスクロマトグラフィー（gas chromatography：GC），液体クロマトグラフィー（liquid chromatography：LC），キャピラリー電気泳動（capillary electrophoresis：CE）などの分離分析法とエレクトロスプレーイオン法（electrospray ionization：ESI）やマトリックス支援レーザー脱離イオン化（matrix assisted laser desorption ionization：MALDI）などのイオン化法を組み合わせておこなわれる．そのなかでもGCは揮発性の低分子の解析にすぐれており，汎用性，再現性が高いことからも多くの医学領域の研究で使用されている．次項では，GC/MSを用いたメタボローム解析について，簡単に解説する．

1 GS/MSを用いたメタボローム解析とは

GC/MSとはガスクロマトグラフィー（GC）と質量分析（MS）とを連結した装置を用いておこなう分析手法である．GCは，揮発性があり，熱に対して安定した分子の分析に適しており，その対象は炭化水素，脂肪酸，アミノ酸，有機酸，糖類など多岐にわたる．通常，生体試料をそのままの状態でGC/MSに供することは不適切で，試料に対し，GCに適した化学的特徴を付加する誘導体化という前処置を加えてから，GCに導入される．GCへ導入される試料はまず加熱され気化する．気化した試料は数十m長で内径数百μmのキャピラリーカラムに導入される．ここでオーブンによりカラムを低温から徐々に高温に変化させることにより，分子ごとの沸点やカラムとの親和性の違いを利用し，化合物は分離していく．カラムを通過した化合物は順にMS部に導入され測定されていく．ここで順にMSへ導入される分子に対し，一般的に用いられる電子イオン化法では，一定の電子エネルギーを化合物に与えることで物質を断片化・イオン化させ，これを検出器によって電気的に計測する．この断片化・イオン化のパターンは，イオン化の方法によってほぼ一定しており，検出器に到達する時間とイオン化パターンをデータベース化することで，対象とする分子を一斉に測定することが可能となる．また，

表 1. 学習セット，検証セットの症例の特徴

	学習セット		検証セット	
	CRC	HV	CRC	HV
症例数	60	60	59	63
性別				
男性	39	39	30	32
女性	21	21	29	31
年齢				
平均値	67.7	64.5	64.8	62.8
BMI	21.9	22.1	22.5	22.2
TNM stage				
0	12	-	15	--
Ⅰ	12	—	11	—
Ⅱ	12	--	3	—
Ⅲ	12	—	11	—
Ⅳ	12	—	19	—

(Nishiumi S *et al*, 2012[7]より改変引用)

MSより得られるデータはマススペクトラムとして検出されるが，これは夾雑物のデータも含まれるため，これらのデータを目的に応じて再構築し，MSデータを解析する．われわれのグループはガスクロマトグラフィー（GC/MS）を使用し，大腸癌を含めた消化器疾患に対するさまざまなバイオマーカーを検討し，報告してきた[3)~6)]．本稿では，われわれのグループがGC/MSを使用しおこなった血清のメタボロミクスによる大腸癌に対する新たな診断的アプローチ[7)]について紹介する．

2 血清のメタボロミクス解析を使用した新しい大腸癌の診断モデル

本研究では，神戸大学医学部附属病院，および，その他2施設から集められた大腸癌患者（colorectal cancer patients：CRC）119例と健常者（healthy volunteers：HV）123例を対象とし，HVは登録時に問診，血液検査を施行し，異常のない症例を対象とした．対象者はすべて，絶食の状態で午前中に検体を採取した．なお，本研究は神戸大学大学院医学研究科倫理委員会の承認を得ており，対象となる症例に対してはすべて研究内容の説明をおこない，書面による同意を取得した．CRCはまずUICC分類にもとづき大腸癌症例をStage 0～Ⅳの症例を各12人ずつに振り分け，CRCと対比し年齢，性別を調整した学習セット（CRC60人，HV60人）を作成，残りの症例を検証セット（CRC59人，HV63人）に振り分けた．学習セット，検証セットの特徴を表1に示す．前処置として各血清50 μlをクロロホルム・水・メタノールの混合液を加え，上層のみ凍結乾燥した．凍結乾燥後をおこなった後，メトキシアミン/ピリジン溶液によってオキシム化し，さらに，*N*-メチル-*N*-トリメチルシリルトリフルオロアセトアミドによってトリメチル誘導体化したうえで，GC/MSによる解析をおこなった．GC/MSにはGCMS-QP2010Ultra（島津製作所社製）を用いた．キャピラリーカラムにはCP-SIL8 CB low bleed/MS（30 m長×内径0.25 mm，膜厚0.25 μm，アジレント・テクノロジー社製）を用いた．GC/MSによる解析の結果，血清から131の物質が検出され，検討により定量性の安定した27の代謝物に

表 2. 多重ロジスティック回帰分析

代謝物	回帰係数	標準誤差	p 値	95%信頼区間
切片	−0.832	1.539	<0.0001	−5.621
2-ヒドロキシ酪酸	286.59	71.90	<0.0001	440.1
アスパラギン酸	33.87	14.29	0.0178	63.85
キヌレニン	1634.96	569.3	0.0041	2.830E+03
シスタミン	78.78	26.82	0.0033	137.3

(Nishiumi S *et al.* 2012[7] より改変引用)

表 3. 腫瘍マーカーと診断モデルの感度，特異度の対比

	CEA	CA19-9	診断モデル
学習セット			
感度	35%	16.7%	85%
特異度	96.7%	100%	85%
検証セット			
感度	33.9%	13.6%	83.1%
特異度	96.8%	100%	81%

(Nishiumi S *et al.* 2012[7] より改変引用)

限定し，検証をおこなった．それぞれの分子を単独で評価した場合，単独で感度，特異度ともに80%を超える分子は存在しなかった．これにより単独の代謝物での大腸癌の診断に優位なバイオマーカーの検出は困難と考えられ，より有効な診断モデルの作成のため，ステップワイズ法により2-ヒドロキシ酪酸，アスパラギン酸，キヌレニン，シスタミンの4つの物質を選択し，多重ロジティック回帰分析（表2）により以下の診断モデルを作成した．

$p = 1/[1 + e2\,|28.32 + 286.59\,(2\text{-ヒドロキシ酪酸}) + 33.87(\text{アスパラギン酸}) + 1634.96(\text{キヌレニン}) + 78.78(\text{シスタミン})|\,]$.

学習セット解析におけるこの診断モデルの受信者動作特性曲線（receiver operating characteristic curve：ROC 曲線）の area under the curve（AUC）は 0.9970 で，至適カットオフ値は 0.4945 であり，この場合の診断モデルの感度，特異度はそれぞれ 85%であった．同じ学習セットを使用して CEA，CA19-9 の感度，特異度も検討した結果，CEA は感度 35.0%，特異度 96.7%，CA19-9 は感度 16.7%，特異度 100%であった．さらに，検討セット（CRC59 人，HV60）でも同様の検証をおこなった結果，診断モデルの感度は 83.1%，81%であった．また，この診断モデルはとくに Stage Ⅰ～Ⅱの早期大腸癌に 82.8%と高い感度を示した．学習セット，検討セットでの診断モデル，CEA，CA19-9 での検討結果を表3に示す．今回，作成した大腸癌の診断モデルで使用した4つの代謝物はいずれも大腸癌症例の血清や組織のメタボローム解析での上昇が報告されている[8)~12)]．また，血清を使用した大腸癌のバイオマーカーの研究は世界中でおこなわれており，これまで遺伝子や，アミノ酸や脂肪酸など複数の代謝物が大腸癌症例で上昇を認め，血清のバイオマーカーとして報告されている[13)~15)]．われわれ

の作成した診断モデルの早期大腸癌に対する感度はいずれもこれらのバイオマーカーと同等以上であった．近年，医療や診断方法の発展により，さまざまな癌に対する治療成績が向上している．そのなかで大腸癌が依然死亡率の高い癌である大きな問題点として，初期の段階では症状の出現に乏しいため早期発見が困難であり，発見時に進行，転移をきたしていることが多いことなどがあげられる．スクリーニング法として使用されている便潜血検査は，エビデンスが確立されており大腸癌の死亡率の改善に貢献しているが，早期の癌に対する感度，特異度が低いことや偽陽性の高さなどの問題点もある．今回われわれの作成した大腸癌の診断モデルはとくに早期大腸癌（Stage Ⅰ～Ⅱ）に対する感度が非常に高く，少量の血清で解析をおこなえることから簡便性，低侵襲性もあわせもっており，今後の大腸癌の新しいスクリーニング法として臨床への応用に期待がもてるものであると考えられる．

おわりに

これまでメタボロミクスによる大腸癌の診断モデルの検討，有用性について述べたが，質量解析計を使用したメタボローム解析はその汎用性から分野にとらわれずさまざまな研究，解析が可能となっている．現在，臨床の現場でもメタボロミクス解析への注目が集まっており，質量解析計による研究データの蓄積が急速におこなわれている．今後，これらの蓄積されたデータの解析により，メタボローム解析はバイオマーカーのみならず病態の解明，疾患予防など含めさまざまな分野での応用が期待される．

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Metabolomics Evaluation of Serum Markers for Cachexia and Their Intra-Day Variation in Patients with Advanced Pancreatic Cancer

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Abstract

Purpose: Cancer cachexia is a multifactorial syndrome characterized by progressive loss of weight and muscle atrophy. Using metabolomics, we investigated serum markers and their intra-day variation in advanced pancreatic cancer patients with cachexia.

Methods: Patients were enrolled in two groups: those with or without cachexia. Blood samples collected at 6:30 AM, 11:30 AM, 4:30 PM, and 9:30 PM were analyzed using metabolomics, and serum levels of IL-6, TNF-α, and leptin were measured and compared between the two groups. Intra-day variation was then evaluated.

Results: Twenty-one patients were enrolled in total. In the cachexia group (n = 9), median body weight loss rate over 6 months was greater, performance status was poorer, and anorexia was more severe than in the non-cachexia group (n = 12). Each metabolites level showed substantial intra-day variation, and some of them displayed significant differences between the two groups. Levels of paraxanthine remained markedly lower in the cohort with cachexia at all measurement points. Besides, median IL-6 and TNF-α levels appeared higher and leptin concentration appeared lower in the cachexia group, albeit without statistical significance.

Conclusion: Some metabolites and some serological marker levels were affected by cancer cachexia. Although paraxanthine levels were consistently lower in patients with cachexia, we identified that many metabolites indicated large intra- and inter-day variation and that it might be necessary to pay attention to intra-day variation in metabolomics research.

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Introduction

Cachexia is a multifactorial syndrome characterized by progressive loss of weight and muscle atrophy that cannot be fully reversed by conventional nutritional support, thereby leading to progressive functional impairment [1]. Its pathophysiology is considered to involve a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism. The agreed diagnostic criterion for cachexia is weight loss of greater than 5%, or weight loss greater than 2% in individuals already showing depletion, according to current body weight and height (body mass index <20 kg/m^2) or skeletal muscle mass [2].

Cachexia is reported in approximately 80% of advanced pancreatic cancer patients who typically develop a decreased dietary intake and a range of symptoms such as anorexia, early satiety, anxiety, and depression [3]. Cachexia has been shown to worsen prognosis and has also been associated with impairment of physical function, increased psychological distress, a reduction in tolerance of and response to therapy, a decrease in quality of life, and reduced duration of survival [4,5]. However, while substantial research is currently focused on determining the mechanism behind cachexia development, no precise understanding has yet been obtained.

Fasting hormones, such as leptin and ghrelin; pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interferon gamma, and interleukin 6 (IL-6); insulin-like growth factor-1 (IGF-1); and the tumor-secreted proteolysis-inducing factor have all been implicated to some extent in cachexia development [6–9]. An

improved understanding of the integrative physiology of cancer cachexia may thus yield further novel therapeutic approaches.

Metabolomics (metabolome analysis) may prove useful in identifying the dynamic metabolic response of a living system to pathophysiological stimuli [10,11]. In metabolite profiling, the selected metabolites in a particular environment are identified and then subjected to quantitative or semi-quantitative assessment. This approach is useful for facilitating understanding of known metabolic pathways and biological alterations in mammalian homeostasis and living systems in pathophysiology [12].

Metabolism is known to be subject to circadian rhythms. The circadian timekeeping system in mammalian homeostasis is a hierarchical multi-oscillator network, with the suprachiasmatic nucleus acting as the central pacemaker [13,14], synchronizing to daily light-dark cycles and coordinating circadian metabolism and physiology. A comprehensive understanding of the pathophysiology of cancer cachexia may require consideration of the influence of intra-day variation [15].

Here, we investigated the difference in serum metabolite levels in pancreatic cancer patients with and without cachexia and analyzed the pattern and intra-day variation in metabolite levels using metabolomics.

Methods

Patient selection criteria

The study (trial registration ID: UMIN000002384) was conducted in hospitalized patients at Kobe University Hospital with pancreatic cancer that was locally advanced or metastatic and not amenable to curative surgical resection. Additional eligibility criteria were age ≥20 years, histologically confirmed adenocarcinoma or adenosquamous carcinoma of the pancreas, and adequate organ function (serum total bilirubin <1.5× upper limit of normal [ULN], aspartate aminotransferase [AST] <2.5×ULN, alanine aminotransferase [ALT] <2.5×ULN, and serum creatinine <1.5×ULN). No chemotherapy in the previous 7 days, no surgery or definitive irradiation in the previous 4 weeks, and palliative irradiation in the previous 2 weeks were permitted. Other exclusion criteria included active multiple primary cancer, serious pre-existing medical condition such as uncontrolled infection, diabetes mellitus, and concomitant use of steroids.

To discern small variations in metabolites, this study included cohorts with and without cachexia, defined as follows: the cohort with cachexia included those with an Eastern Cooperative Oncology Group performance status (ECOG PS) 1 to 4, grade 1 to 4 anorexia, and weight loss greater than 10% over the past 6 months; while that without cachexia included patients with a ECOG PS 0 to 2, grade 0 to 1 anorexia, serum albumin levels exceeding 3.5 mg/dL, and weight loss less than 5% over the past 6 months. Patients who didn't meet these cohort criteria were also excluded in this study.

All patients provided written informed consent, and study approval was obtained from the Institutional Review Board of Kobe University Hospital.

Objectives and outcomes

The objective of this observational study was to investigate the difference in serum metabolite levels between pancreatic cancer patients with and without cachexia and to explore the pattern and intra-day variations in metabolite levels using metabolomics. Eligible subjects were assigned to either cohort with cachexia or that without cachexia. Primary endpoint was identification of cachexia-related metabolites using metabolomics. Secondary endpoints were intra-day variation in the metabolites involved in cachexia and changes in the level of serological markers involved in cachexia, such as inflammatory cytokines (e.g. IL-6, TNFα) and leptin, in the presence or absence of cachexia. Patient characteristics and medication information were recorded throughout the study. Adverse events were evaluated using the CTCAE v4.0.

The present study featured 10 subjects per group, as although this study was exploratory in nature and therefore involved no statistical rationale for sample size calculation, preceding studies on metabolomics have indicated significant results with a sample size of approximately 10 subjects per group. However, if a more useful analysis method found is found that may be implemented in the study, the analysis methods may be altered and the necessary sample size recalculated.

Serum collection and preparation

Blood samples from hospitalized subjects were collected at 6:30 AM (after waking in early morning), 11:30 AM (late morning), 4:30 PM (early evening), and 9:30 PM (before retiring at night) at equal intervals in a single day to analyze intra-day variation. After collection of whole blood, samples were allowed to clot at room temperature, and serum was separated by centrifugation at 3,000×g for 10 min at 4°C and stored at −80°C until use.

To extract low-molecular-weight metabolites, 50 μl of serum was mixed with 250 μl of a solvent mixture (MeOH: H_2O:$CHCl_3$ = 2.5:1:1) containing 10 μl of 0.5 mg/ml 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) dissolved in distilled water, and then the solution was shaken at 1,200 rpm for 30 min at 37°C before being centrifuged at 16,000×g for 5 min at 4°C. A total of 225 μl of the obtained supernatant was transferred to a clean tube, and 200 μl of distilled water was added. After mixing, the solution was centrifuged at 16,000×g for 5 min at 4°C, and 250 μl of the resultant supernatant was transferred to a clean tube before being lyophilized using a freeze dryer. For oximation, 40 μl of 20 mg/ml methoxyamine hydrochloride (Sigma-Aldrich) dissolved in pyridine was mixed with a lyophilized sample, and the mixture was shaken at 1,200 rpm for 90 min at 30°C. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; 20 μl) (GL Science, Tokyo, Japan) was then added for derivatization, and the mixture was incubated at 1,200 rpm for 30 min at 37°C. The mixture was then centrifuged at 16,000×g for 5 min at 20°C, and the resultant supernatant was subjected to gas chromatography-mass spectrometry (GC/MS) measurement.

Serum leptin levels were measured via radioimmunoassay as described previously in SRL Inc. (Tokyo, Japan) [16,17]. The limit of sensitivity was 0.5 ng/ml, and the intra- and interassay coefficients of variation both ranged from 2.5% to 5.0% over the sample concentration range. Serum IL-6 was detected using a Chemiluminescent Enzyme Immunoassay from SRL Inc. (Tokyo, Japan) [18]. The limit of sensitivity was 4.0 pg/ml, and the intra- and interassay coefficients of variation ranged from 2.2% to 3.8% and 3.6% to 8.6%, respectively, over the sample concentration range. Serum TNFα was detected using an Enzyme-Linked ImmunoSorbent Assay from SRL Inc. (Tokyo, Japan). Assay results ranged from 0.6 to 2.8 pg/ml.

GC/MS analysis and data processing

GC/MS analysis was performed using a GCMS-QP2010 Ultra (Shimadzu Co., Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; inner diameter, 30 mm×0.25 mm; film thickness, 0.25 μm; Agilent Co., Palo Alto, CA, USA), in accordance with a previously described method [19]. The front inlet temperature was 230°C, and the flow rate of helium gas through the column was 39.0 cm/sec. The column temperature was held at 80°C for 2 min and then raised by 15°C/min to

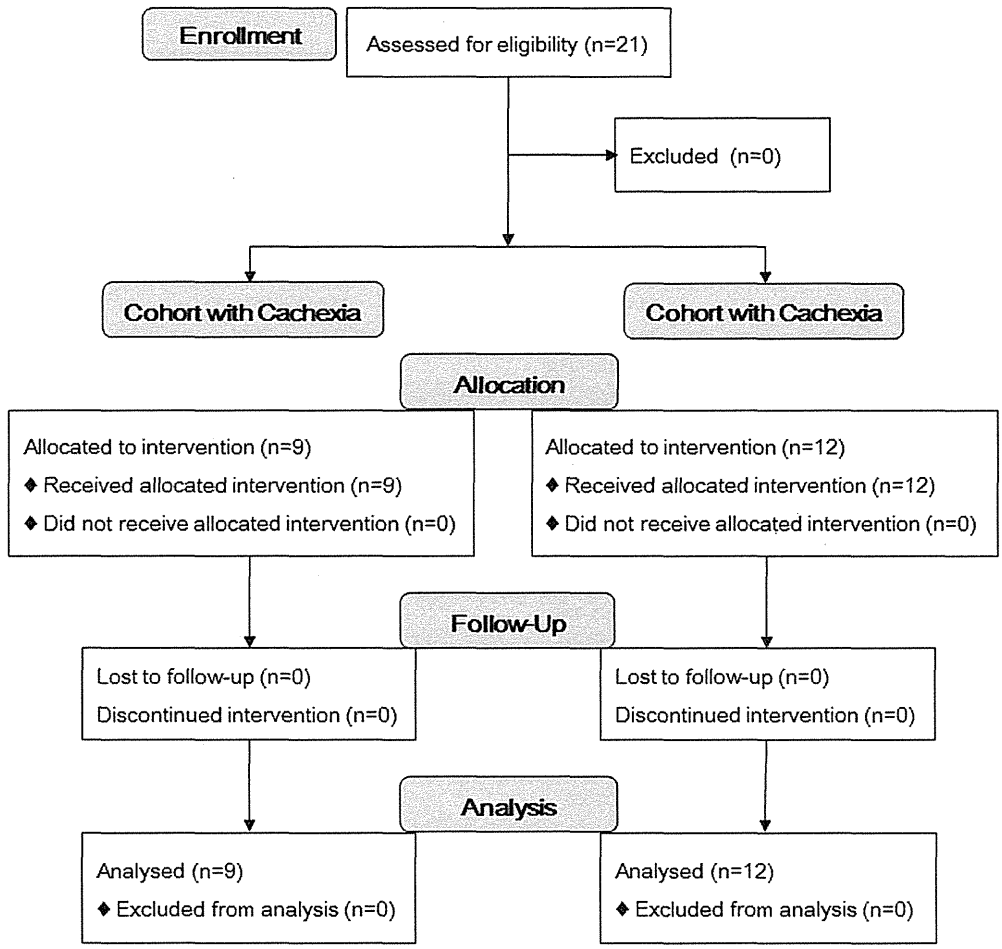

Figure 1. CONSORT Flow Diagram.
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330°C and held there for 6 min. The transfer line and ion-source temperatures were 250°C and 200°C, respectively. Twenty scans per second were recorded over the mass range 85–500 m/z using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co.).

Data processing was performed in accordance with the methods described in previous reports [19,20]. Briefly, the MS data were exported in netCDF format, and peak detection and alignment were performed using MetAlign software (Wageningen UR, The Netherlands). The resultant data were then exported in CSV format and analyzed using in-house analytical software. For semi-quantification, the peak height of each ion was calculated and normalized to the peak height of 2-isopropylmalic acid as an internal standard. Names were assigned to each metabolite peak based on the method described in a previous report [20].

Statistical analysis

Data are expressed as median and range. Levels of serological markers and metabolites between the cohorts with and without cachexia were compared using the Mann-Whitney U test or Wilcoxon Signed-Rank test. Presence of intra-day variances in metabolite levels was then determined using Kruskal-Wallis multiple comparison (z-test) with bonferroni correction to compare values for each metabolite across all four time points assessed. Survival time was estimated using the Kaplan-Meier method. All statistical analyses were performed using NCSS 2007 software (NCSS, LLC. Kaysville, UT, USA).

Results

Patients' characteristics

From December 2009 to July 2011, 21 patients were enrolled in this study: 9 with cachexia and 12 without (Figure 1). Clinical characteristics are summarized in Table 1. Median body weight loss rates over 6 months in the cohorts with and without cachexia were 13.4% and 2.5%, respectively ($p = 0.0001$). Of the 21 patients enrolled, 19 had no history of prior treatment. No significant differences were noted in levels of tumor markers, LDH, CRP, or HbA1c between the cohorts. However, levels of total cholesterol and LDL cholesterol were significantly lower in the cohort with cachexia. Eight patients in the cohort with cachexia and 8 in that without were male in the present study. Median ages in the cohorts with and without cachexia were 66.5 (range, 36 to 77) and 68.5 (39 to 76) years, respectively ($p = 0.98$). No severe adverse events or unintended effects were noted among any participants in the present study.

Table 1. Patient characteristics.

		With Cachexia	Without Cachexia	p-value
		n = 9	n = 12	
Age	(years)	72 (39–76)	64.5 (36–77)	0.57
Gender	Male/Female	8/1	8/4	0.25
PS	0/1/2/3	0/5/3/1	4/8/0/0	0.005
Stage	IVA/IVB	5/4	5/7	0.54
BW loss rate	(%)	13.4 (10.2–30.8)	2.5 (−5.2–4.6)	0.0001
Body weight	(kg)	55.6 (45.0–77.0)	57.5 (44.6–77.3)	0.48
Body mass index		22.6 (18.2–25.8)	19.7 (16.5–26.1)	0.15
Anorexia	Gr 0/1/2/3	0/8/0/1	5/7/0/0	0.02
Prior treatment	(No/Yes)	8/1	11/1	0.83
Laboratory data				
WBC	(/μL)	5100 (4100–17600)	5850 (3000–111600)	0.97
Hb	(g/dL)	11.9 (9.3–15.2)	13.7 (11.3–14.6)	0.20
TP	(g/dL)	6.4 (4.8–6.8)	6.7 (6.0–7.5)	0.15
Alb	(g/dL)	3.2 (2.1–4.7)	3.9 (3.5–4.4)	0.14
LDH	(U/L)	169 (125–228)	179 (127–340)	0.27
TChol	(mg/dL)	131 (99–167)	187 (136–205)	0.001
LDL	(mg/dL)	72 (45–91)	100 (71–143)	0.02
HDL	(mg/dL)	42 (22–73)	53 (29–93)	0.20
TG	(mg/dL)	90 (44–112)	112 (62–159)	0.09
CRP	(mg/dL)	1.9 (0.1–2.64)	0.55 (0.1–6.97)	0.89
HbA_{1c}	(%)	5.7 (4.8–6.5)	6.4 (4.6–7.3)	0.11
Tumor marker				
CEA	(ng/mL)	5.0 (1.4–29.2)	4.7 (2.0–883.7)	1.00
CA19-9	(U/mL)	271 (5–1974)	379 (1–80673)	0.43
DUPAN-2	(U/mL)	560 (33–230000)	605 (25–140000)	0.89

Abbreviations: PS, Eastern Cooperative Oncology Group performance status; BW loss rate, body weight loss rate over 6 months; TChol, total Cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; CRP, C-reactive protein.

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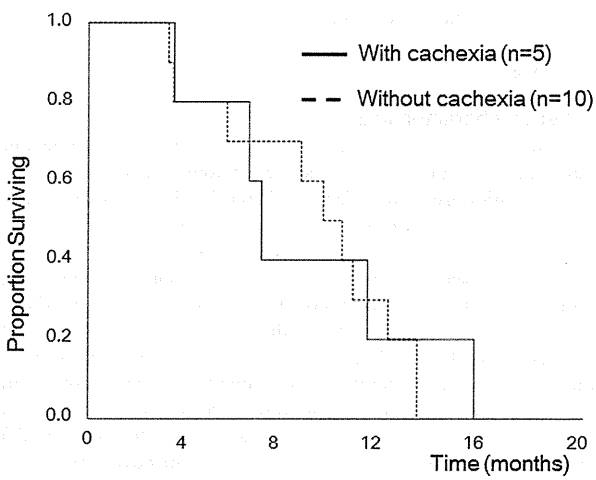

Figure 2. Kaplan-Meier curve for overall survival in patients who had no prior treatment.

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Treatment and survival

After enrolling in this study, all 19 patients who had had no prior treatment subsequently received chemotherapy (n = 15) or chemoradiotherapy (n = 4). Regimens used in the 15 patients treated with chemotherapy were gemcitabine monotherapy (n = 13) and combination therapy of gemcitabine plus TS-1, an oral fluoropyrimidine prodrug (n = 2). In the four patients treated with chemoradiotherapy for locally advanced cancer, the regimens used (as part of another clinical study) were concurrent chemoradiotherapy with gemcitabine plus TS-1 (n = 2) and heavy-particle radiotherapy with gemcitabine (n = 2). The remaining 2 patients who had had prior treatment received best supportive care after enrolling in this study.

We analyzed survival in the 19 patients who had received no prior treatment. The median survival time in patients with stage IVA (n = 9) and stage IVB (n = 10) was 15.6 and 7.0 months (logrank test, $p<0.001$), respectively, while that in patients treated with chemotherapy (n = 15) and chemoradiotherapy (n = 4) was 9.5 and 19.7 months (logrank test, $p = 0.004$) respectively. Although this study was too small to detect survival difference, the median survival time in patients who received chemotherapy was 7.0 months with cachexia (n = 5) and 9.5 months without (n = 10) (logrank test, $p = 0.85$), and 1-year survival rates were 20.0% and 30.0%, respectively (Figure 2).

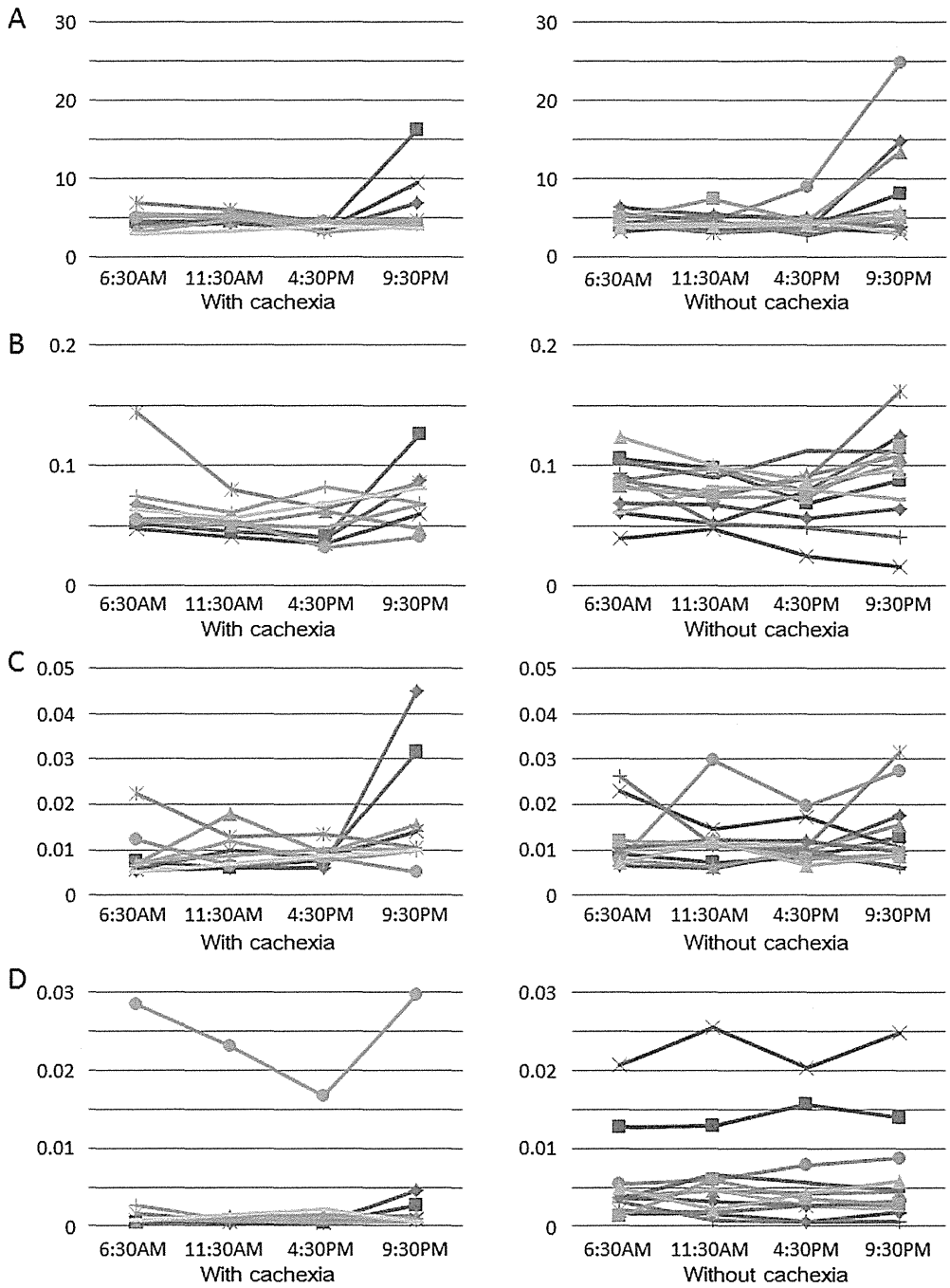

Figure 3. Intra-day variation in A) lactic acid, B) alanine, C) catechol, and D) paraxanthine.
doi:10.1371/journal.pone.0113259.g003

Metabolomics

In our GC/MS-based metabolomics analysis system, which mainly targeted water-soluble metabolites, 124 metabolites were detected in subjects' serum samples (Table S1). Of these 124 metabolites, 1 metabolite, namely 2-isopropylmalic acid, was used as an internal standard, and 8 were probably extracted from non-serum source, for example from eppendorf tubes. These nine metabolites were therefore excluded from subsequent analyses.

Kruskal-Wallis multiple comparison (z-test) with bonferroni correction was used to evaluate changes in metabolite levels at four different time points throughout the day. These analyses showed that the levels of 60 of the 115 evaluated metabolites differed significantly between those means at any 1 of 4 time points. Univariate analysis of the 115 metabolites identified considerable inter-individual variability of levels of some metabolites throughout a single day. Figure 3 describes representative metabolites,

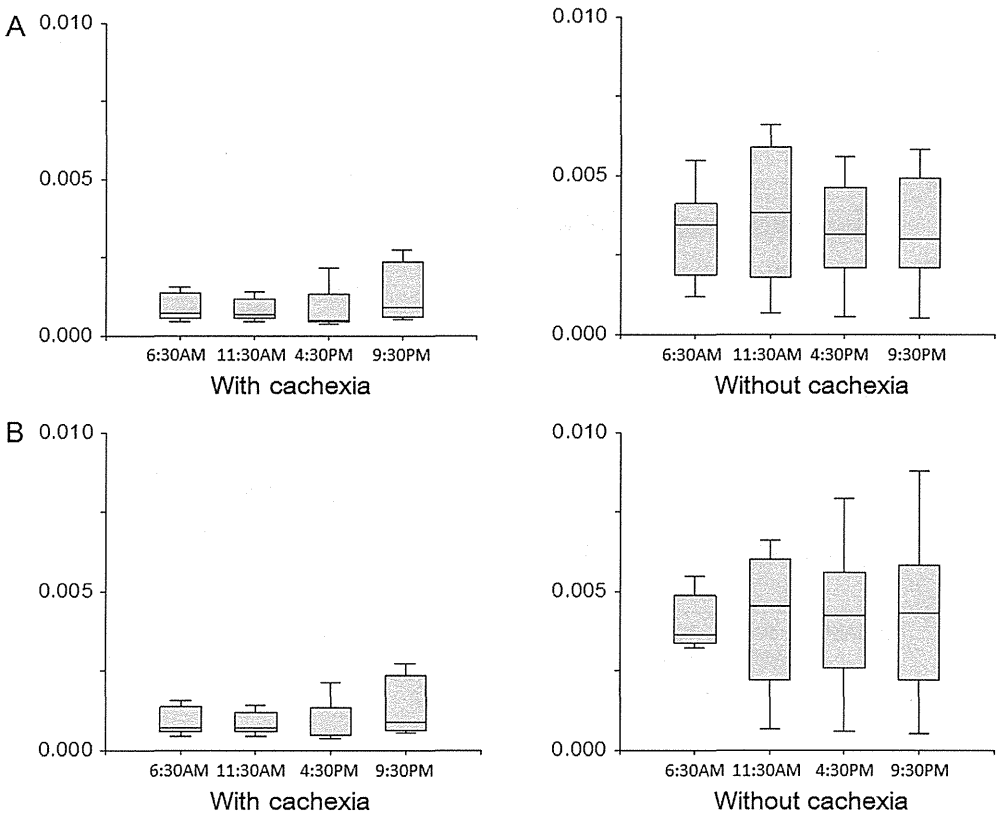

Figure 4. Intra-day variation in paraxanthine. A. In all patients. At 6:30 AM: 0.75×10^{-3} (95% CI, $0.28–2.7\times10^{-3}$) in patients with cachexia vs. 3.61×10^{-3} ($1.91–5.48\times10^{-3}$) pg/ml in those without cachexia, p = 0.35; TNF-α: 7.1 (1.2 to 30.2) vs. 3.3 (1.2 to 30.0) pg/ml, p = 0.27; leptin: 2.4 (1.0 to 9.8) vs. 4.0 (2.0 to 9.9) ng/ml, p = 0.27, respectively. B. In male patients. IL-6: 15.2 (range, 2.6 to 23.4) in patients with cachexia vs. 9.4 (1.4 to 28.7) pg/ml in those without cachexia, p = 0.57; TNF-α: 12.4 (1.2 to 29.0) vs. 3.2 (1.4 to 28.6) pg/ml, p = 0.29; leptin: 2.3 (1.0 to 8.8) vs. 3.45 (2.0 to 2.8) ng/ml, p = 0.34, respectively.
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including lactic acid, alanine, catechol, and paraxanthine. Metabolites with significant differences in intra-day levels on univariate analysis were (fold change in levels in the cohort with cachexia to that without, p-value): at 6:30 AM, catechol (0.70, p = 0.04) and paraxanthine (0.21, p = 0.006); at 11:30 AM, valine (0.68, p = 0.01), proline (0.66, p = 0.049), p-hydroxybenzoic acid (0.79, p = 0.03), and paraxanthine (0.16, p = 0.006); and at 4:30 PM, valine (0.62, p = 0.02) and paraxanthine (0.13, p = 0.007). Although differences in levels of paraxanthine were not statistically significant (0.26, p = 0.08) at 9:30 PM, the levels were clearly lower in the cohort with cachexia at all time points (Figure 4). Of note, the female patient with outlying paraxanthine values in the cohort with cachexia was the study's only heavy drinker of coffee, which is a metabolic substrate of paraxanthine (Figure 3 D).

Serological markers

Median levels of IL-6, TNF-α, and leptin at 6:30 AM didn't differ significantly between the cohorts with and without cachexia, and the observed trends in level fluctuations were similar to those previously reported (Figure 5A) [7] [9]. Because serum serological marker levels in healthy volunteers vary substantially depending on gender and age [21], we analyzed values in men. In the 16 male patients, median levels of IL-6, TNF-α, and leptin at 6:30 AM didn't differ significantly between the cohorts with and without cachexia (Figure 5B). Leptin concentrations during the day remained lower in the cohort with cachexia than in that without (Figure 6). However, median leptin levels did show substantial intra-day variation; 2.4 (range, 1.0 to 9.8) ng/ml and 1.1 (0.8 to 4.4) ng/ml at 6:30 AM and 11:30 respectively for patients with cachexia (p = 0.003), and 3.95 (2.0 to 9.9) ng/ml and 3.1 (1.5 to 7.8) ng/ml at 6:30 AM and 11:30 respectively for those without cachexia (p = 0.03).

Discussion

Cancer cachexia is a multifactorial syndrome characterized by body weight loss and muscle and adipose tissue wasting and inflammation, and is often associated with anorexia. Abnormalities commonly associated with cachexia include alterations in carbohydrate, lipid and protein metabolism. A substantial amount of research is currently focused on determining the mechanism behind cachexia development, with several factors considered to be putative mediators of cancer anorexia, including hormones (e.g. leptin), neuropeptides, cytokines (e.g. IL-1, IL-6, and TNF-α), and neurotransmitters (e.g. serotonin and dopamine). Here, we examined levels of typical serological factors, such as IL-6, TNF-α, and leptin, and used metabolomics to identify metabolites associated with cachexia in patients with pancreatic cancer and investigate intra-day variations in levels of these metabolites. To our knowledge, this is the first study to analyze cancer cachexia

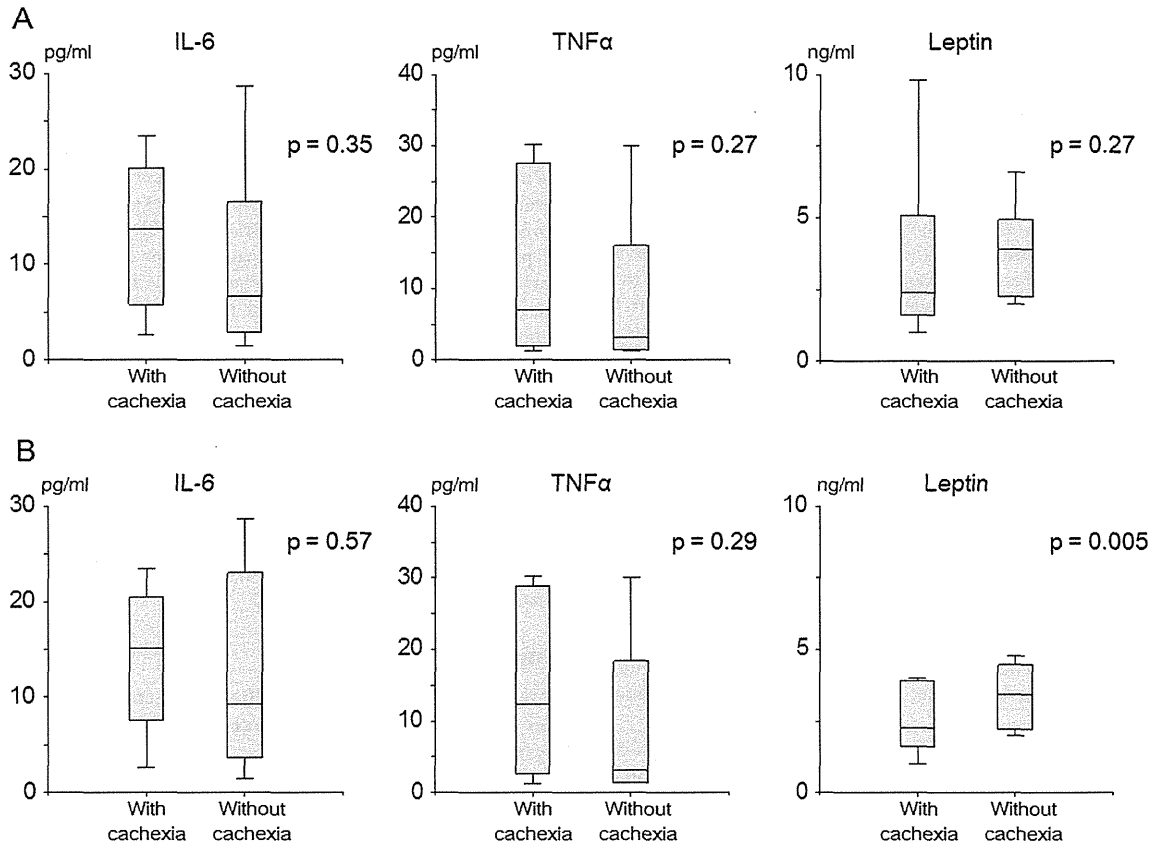

Figure 5. Serological markers between patients with cachexia and those without cachexia. A. In all patients. IL-6: 13.8 (range, 2.6 to 23.4) in patients with cachexia vs. 6.8 (1.4 to 28.7) pg/ml in those without cachexia, p = 0.35; TNF-α: 7.1 (1.2 to 30.2) vs. 3.3 (1.2 to 30.0) pg/ml, p = 0.27; leptin: 2.4 (1.0 to 9.8) vs. 4.0 (2.0 to 9.9) ng/ml, p = 0.27, respectively. B. In male patients. IL-6: 15.2 (range, 2.6 to 23.4) in patients with cachexia vs. 9.4 (1.4 to 28.7) pg/ml in those without cachexia, p = 0.57; TNF-α: 12.4 (1.2 to 29.0) vs. 3.2 (1.4 to 28.6) pg/ml, p = 0.29; leptin: 2.3 (1.0 to 8.8) vs. 3.45 (2.0 to 2.8) ng/ml, p = 0.34, respectively.
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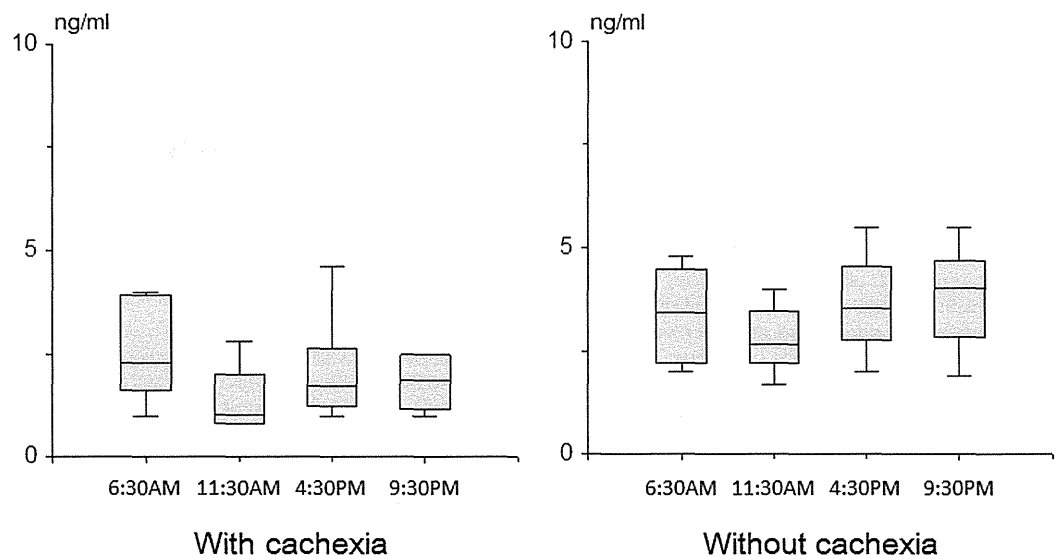

Figure 6. Intra-day variation in leptin.
doi:10.1371/journal.pone.0113259.g006

with consideration to the influence of intra-day variation of metabolite levels.

Metabolomics analysis identified some metabolites whose levels differed significantly in patients with cachexia from levels in those without. While speculating as to why levels of a substantial number of metabolites differed markedly by time of sample collection is difficult in the present study, we did find that levels of paraxanthine remained lower in the cohort with cachexia than in that without throughout the day. Paraxanthine, the dimethyl derivative of xanthine, is the preferential path of caffeine metabolism in humans. Paraxanthine cannot be synthesized as a natural plant product and is seen only as a natural metabolite of caffeine in animals. Because paraxanthine, like caffeine, is a psychoactive central nervous system stimulant and increases energy expenditure and lipid turnover, it may be related to the pathogenesis of cancer cachexia or anorexia [22,23]. It remains unclear that the reduction in the levels of paraxanthine is associated with regulation in order to maintain homeostasis of lipid mobilization.

While paraxanthine concentrations were not detectable using the common HPLC assay under fasting conditions in a previous study [23,24], we were able to measure values using a GCMS-QP2010 Ultra in the present study. We did not strictly control caffeine intake in subjects but recognized on post-hoc survey that one patient with outlying values in the cohort with cachexia was a heavy drinker of coffee. The association between paraxanthine level and cachexia should be confirmed in further validation studies.

In agreement with the results of previous studies [9,21,25,26], median IL-6 and TNF-α levels in the cohort with cachexia were nearly double those in the cohort without, and leptin levels in the cohort with cachexia were around half those in the cohort without, albeit without statistical significance. Serum leptin levels in healthy volunteers vary substantially by gender (women tend to have higher values than men), age (elderly tend to have higher values than younger individuals), nutritional status, and body mass index [21]. Wallace et al. demonstrated a significant positive correlation between body fat loss and increase in leptin levels of healthy subjects and cancer patients ($r = 0.731$) [27]. The large inter- and intra-individual variation in levels in the present study suggests that, although leptin is indeed involved in the pathophysiology of cachexia, it cannot serve as a useful marker at one time. Intra-day variation in leptin suggests that leptin level is associated with anorexia and eating behavior in patients with cancer cachexia, as well as amount of body fat [28,29].

Several pharmacological and nutritional approaches to the treatment of cancer cachexia have been evaluated. When nutritional strategies alone were found insufficient for improving cachectic syndrome, pharmacological approaches, such as steroids, methylprogesterone, and ghrelin agonists, to counteract metabolic changes were tried, albeit without success [8]. Development of a successful treatment method will likely require a better understanding of the pathogenesis of cancer cachexia and identification of a dynamic metabolic surrogate marker of pharmacological intervention.

We explored the intra-day variation in metabolites in cancer cachexia by means of metabolomics. Although a major limitation of this study is the small sample size of cachectic patients with pancreatic cancer, a cohort which included only one female, prominent intra-day variance in serum metabolite levels was demonstrated in pancreatic cancer patients, regardless of cachexia. Metabolomics may be a useful tool in identifying the dynamic metabolic response. However, many metabolites identified by metabolomics showed large intra- and inter-day variations in levels. We therefore believe that metabolomics should be evaluated while taking into account those variations [30]. Although the level of paraxanthine remained significantly lower in the cohort with cachexia throughout the day unrelated to the intra-day variation, we consider that a time-matched method may be necessary to minimize influence of intra-day variation of values due to circadian rhythm in metabolomic research. These findings should be confirmed in further validation studies. The identification of metabolites involved in cancer cachexia will leads to the elucidation of its pathophysiology.

In conclusion, we demonstrated here that levels of some serological markers and metabolites were affected by cancer cachexia. We found that many metabolites exhibited substantial intra- and inter-day variation, suggesting the potential need to pay attention to intra-day variation in these levels in metabolomics research.

Supporting Information

Table S1 List of metabolites detected in subjects' serum using our GC/MS-based metabolomics analysis system.
(DOCX)

Checklist S1 TREND Statement Checklist.
(PDF)

Protocol S1 Clinical Study Protocol (Japanese version).
(DOC)

Protocol S2 Clinical Study Protocol (English version).
(DOC)

Statement from Their Ethics Committee or Institutional Review Board S1.
(PDF)

Acknowledgments

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Author Contributions

N/A. Conceived and designed the experiments: YF TK SN TA MY HM. Performed the experiments: YF TK NC YI MT NK TM SN. Analyzed the data: YF TK MY HM. Contributed reagents/materials/analysis tools: TK SN TA MY. Wrote the paper: YF TK MY HM.

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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.

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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

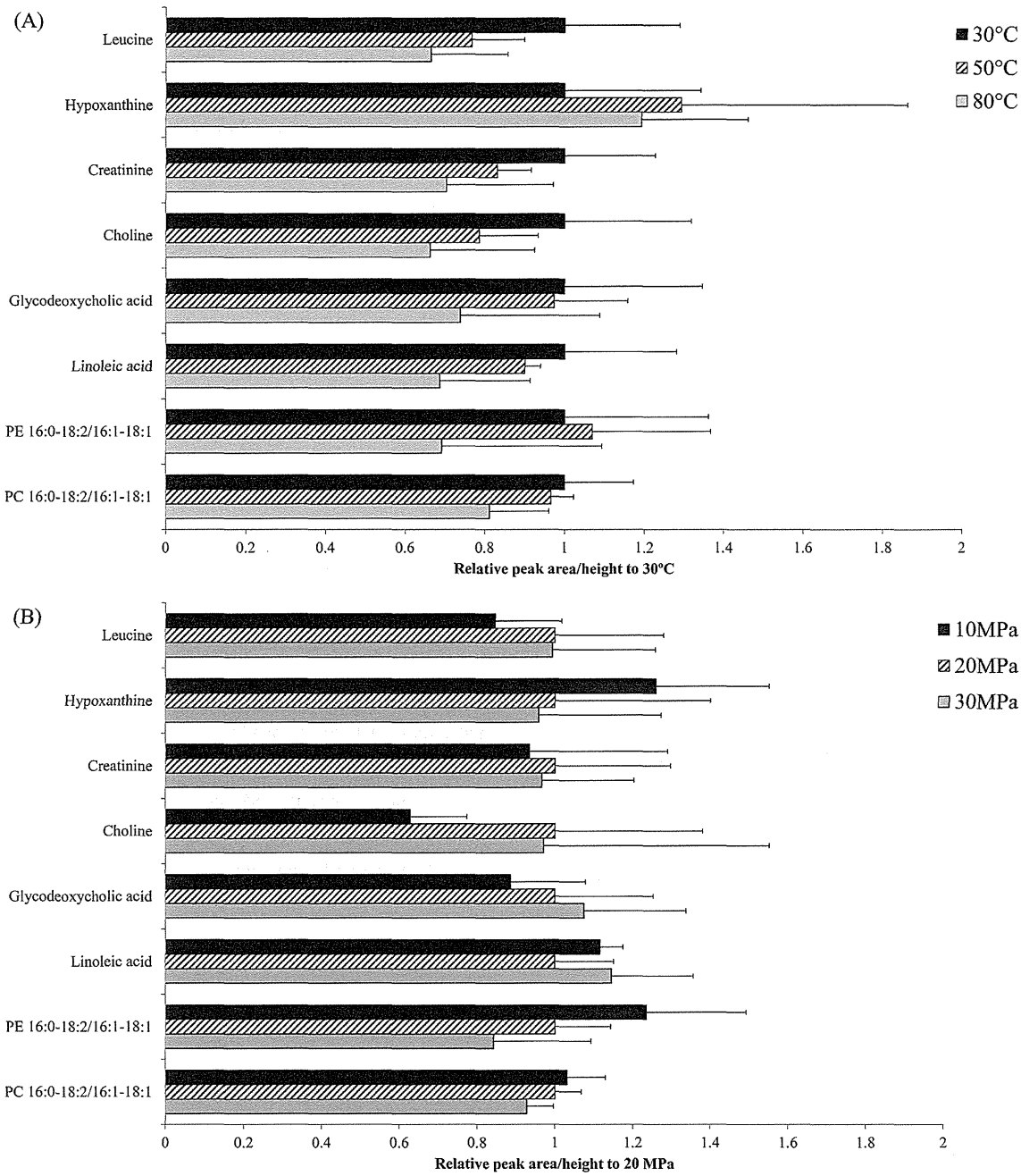

Fig. 1. Effects of temperature (A) and pressure (B) for SFE of various metabolites in dried serum spots (DSS). DSS ($n = 5$) were extracted under conditions of various temperatures (30 °C, 50 °C, 80 °C) and pressures (10 MPa, 20 MPa, 30 MPa), and then analyzed by LC/MS/MS. The value is shown as the relative 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

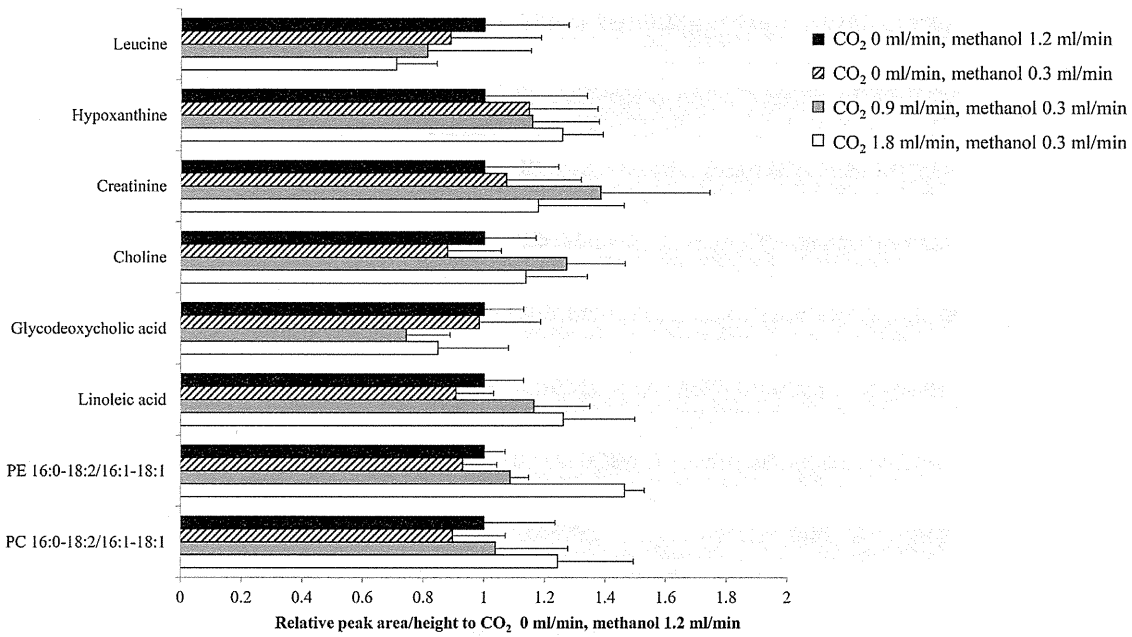

Fig. 2. Effect of solvent constitution for SFE of various metabolites in dried serum spots (DSS). DSS ($n=5$) were extracted under various $SCCO_2$/methanol compositions (0.3 ml/min methanol, 1.2 ml/min methanol, 0.9 ml/min $SCCO_2$ and 0.3 ml/min methanol, 1.8 ml/min $SCCO_2$ and 0.3 ml/min methanol) 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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