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Metabolomic analysis using porcine skin: a pilot study of analytical techniques

Julie Wu BS,[1] Oliver Fiehn PhD,[2] April W Armstrong MD MPH[1]

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[1]University of California Davis, Department of Dermatology, Sacramento, CA, USA

[2]University of California Davis Genome Center, Department of Molecular and Cellular Biology, Davis, CA, USA

Correspondence:

April W. Armstrong, MD, MPH
Vice Chair of Clinical Research
Director, Dermatology Clinical Research Unit
Director, Teledermatology
University of California, Davis
3301 C St., Suite 1400
Sacramento, CA 95816
Questions regarding manuscript content: aprilarmstrong@post.harvard.edu
Reprints: dermatology@ucdmc.ucdavis.edu

Abstract

Background: Metabolic byproducts serve as indicators of the chemical processes and can provide valuable information on pathogenesis by measuring the amplified output. Standardized techniques for metabolome extraction of skin samples serve as a critical foundation to this field but have not been developed.

Objectives: We sought to determine the optimal cell lysage techniques for skin sample preparation and to compare GC-TOF-MS and UHPLC-QTOF-MS for metabolomic analysis.

Methods: Using porcine skin samples, we pulverized the skin via various combinations of mechanical techniques for cell lysage. After extraction, the samples were subjected to GC-TOF-MS and/or UHPLC-QTOF-MS.

Results: Signal intensities from GC-TOF-MS analysis showed that ultrasonication ($2.7x10^7$) was most effective for cell lysage when compared to mortar-and-pestle ($2.6x10^7$), ball mill followed by ultrasonication ($1.6x10^7$), mortar-and-pestle followed by ultrasonication ($1.4x10^7$), and homogenization (trial 1: $8.4x10^6$; trial 2: $1.6x10^7$). Due to the similar signal intensities, ultrasonication and mortar-and-pestle were applied to additional samples and subjected to GC-TOF-MS and UHPLC-QTOF-MS. Ultrasonication yielded greater signal intensities than mortar-and-pestle for 92% of detected metabolites following GC-TOF-MS and for 68% of detected metabolites following UHPLC-QTOF-MS.

Conclusion: Overall, ultrasonication is the preferred method for efficient cell lysage of skin tissue for both metabolomic platforms. With standardized sample preparation, metabolomic analysis of skin can serve as a powerful tool in elucidating underlying biological processes in dermatological conditions.

Keywords: metabolomics; dermatology; cell lysage; GC-MS; LC-MS; skin tissue

Abbreviations

GC-TOF-MS: gas chromatography time-of-flight mass spectrometry

UHPLC-QTOF-MS: ultrahigh performance liquid chromatography quadrupole-time-of-flight mass spectrometry

PC: phosphatidylcholine

HILIC: Hydrophilic Interaction Liquid Chromatography

ESI: electrospray ionization

MeOX: methoxyamine hydrocloride

MSTFA: N -methyl-N-(trimethysilyl)-trifluoroacetamide

FAMEs: fatty acid methyl esters

Introduction

The emerging field of metabolomics is the systematic study of the metabolome, a collection of low molecular mass metabolites of a biological cell, tissue, or organism [1]. Although it has been explored in toxicology, stem cell differentiation, and fertility research, its application in analyzing skin samples has not yet been studied [2-5]. Modern advances in analytical technologies have led to the rapid growth and widespread adoption of the “omics” fields, which includes genomics [6], transcriptomics [7], proteomics [8], and metabolomics as the most recent.

Metabolic byproducts are produced at the transcriptional and translational levels, which may serve as indicators of the chemical processes that have taken place [9]. Because metabolites are the end products of cellular processes, the study of a biological system’s metabolome is most closely relevant to the observed phenotype [10]. Metabolomics studies provide valuable information on the physiology of a system by measuring the amplified output in response to genetic and environmental perturbations [1]. Traditionally, metabolomic analyses have been performed using biofluids such as urine, plasma, or serum to evaluate the overall physiological state of the system [11-13]. However, such analyses do not allow for efficient investigation at the tissue or organ-level. In comparison, when the investigators use tissue biopsies to examine the intracellular metabolites, these metabolomics analysis could yield direct signatures of the cellular processes at the active target tissue or organ. For example, tumor tissues have been used to study energy metabolism in breast and ovarian cancer [14,15]. Owing to the relative ease of obtaining skin tissue samples compared to other tissues for analysis, metabolomics is highly suitable for studying disease pathogenesis of dermatological conditions.

Metabolomics studies of the skin will enable us to determine the biological pathways involved with dermatologic diseases. Furthermore, such findings have implications for identification of novel biomarkers for early diagnosis, disease progression monitoring, and personalized medicine development [10]. In order to conduct these studies, standardized sample preparation procedures need to be developed to determine the optimal methods for obtaining the skin metabolome in humans. However, studies in the cutaneous metabolome are scarce and no standardized procedures exist for the cell lysage of skin prior to extraction. Although the methodology for extraction of biofluids has been well developed, the methodology for extraction of skin tissue samples has not yet been assessed [16]. In this study, we compared several different pulping techniques in order to determine the optimal cell lysage method for skin tissue sample extraction. In addition, we assessed two different metabolomics platforms for the analysis of skin tissue: gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and ultrahigh performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS).

Materials and Methods

Sample Preparation and Extraction

Cell lysage

Porcine skin samples were used as a model for human skin tissue for metabolomic analysis. Owing to the methodological nature of this study, the porcine skin was obtained from a local market and, therefore, IRB approval was deemed unnecessary. The

porcine skin samples were biopsied and subsequently quenched in liquid nitrogen for one minute. In preparation for extraction, several different techniques for efficient breakage of the cells were evaluated:

(1) Mortar-and-pestle: A liquid nitrogen-cooled mortar was used for varying amounts of time until tissue was fully ground manually.

(2) Ultrasonication: After the addition of 1 ml of the extraction solution (acetonitrile/isopropanol/water (3:3:2, v/v/v)) to the porcine skin tissue, the sample was ultrasonicated for ten minutes using a VWR 50HT sonicator.

(3) Mortar-and-pestle/ultrasonication: The sample was first ground by using a mortar-and-pestle and subsequently ultrasonicated for ten minutes.

(4) Ball mill/ultrasonication: The sample was ground with a Retsch MM301 Ball Mill at 1800 rpm and then ultrasonicated for ten minutes.

(5) Homogenizer: An IKA Ultra turrax T10 Basic disperser was used until the tissue was homogenous. This technique was repeated twice (trial 1 and trial 2).

Extraction

The extraction solvent (acetonitrile/isopropanol/water (3:3:2, v/v/v)) was degassed with nitrogen and pre-chilled at -20 degrees Celsius using the ThermoElectron Neslab RTE 740.

To extract metabolites and precipitate proteins, the skin tissue homogenate (20mg) was mixed with 1 ml of extraction solvent, vortexed for 10s (MiniVortexer), and subsequently shaken for 5 min at 4 degrees Celsius using the Orbital Mixing Chilling/Heating plate. After centrifugation for 2 min at 14000rcf (Eppendorf 5415 D), the supernatant was removed and concentrated to complete dryness using a cold trap vacuum concentrator (Labconco Centrivap) at room temperature for 4 hours. The dried aliquot was then re-suspended with 500uL of degassed 50% aqueous acetonitrile to remove most of the complex lipids, vortexed for 10s, and centrifuged for 2 min at 14000rcf. After the supernatant was evaporated to complete dryness, the dried metabolic extract was either submitted to LC-MS or submitted to derivatization for GC-MS.

[We performed GC-MS on techniques 1-5. We then performed GC-MS again and LC-MS on techniques 1 and 2 using six replicates for each technique. Below we describe both analysis techniques.]

GC-TOFMS Analysis

GC-TOFMS Derivatization

The dried metabolic extract was derivatized by the addition of 10ul of 40mg/ml methoxyamine hydrocloride (MeOX) dissolved in pyridine, shaken at 30 degrees Celsius for 90 min. Methoximation was applied to protect carbonyl functions and to prevent sugar ring cyclization. Following methoximation, 90 ul of N-methyl-N-(trimethysilyl)-trifluoroacetamide (MSTFA) and 1 ul of a mixture of internal retention index markers were added to the sample, which was then shaken at 37 degrees Celsius for 30 minutes. The mixture of internal retention index markers was prepared by dissolving fatty acid methyl esters (FAMEs) in chloroform. The MSTFA is a silylating agent that exchanges all acidic protons against a trimethylsilyl group to enhance the volatility of polar metabolites. For injection on GC, the reaction mixture was transferred to a 2 ml autosampler glass vial with a glass microinsert.

GC-TOFMS Data Acquisition

The derivatized samples were injected using a Gerstel automatic liner exchange-cold injection system, operated in split-less mode. An Agilent 6890 gas chromatograph was used with a 30 m long, 0.25mm internal diameter Rtx-5Sil (95% dimethey/5% diphenyl polysiloxane flim) MS column for separation (Restek). Pure helium (99.9999%) as carrier gas was maintained at a constant flow rate or 1 ml min^{-1} during the analysis. The GC oven temperature was initially held at 50°C for 1 min, then ramped up to 330°C at 20°C min^{-1} and held constant for 5 min. For mass spectrometry, the GC was coupled to a Leco Pegasus IV time-of-flight mass spectrometer. Electron impact ionization was employed at 70 eV, with an ion source temperature of 250°C, to ionize species as they elute from the GC column. Mass spectra were acquired with a scan range of 85 to 500 *m/z* and a scan rate of 20 spectra per second.

Metabolite Identification and Data Analysis

Retention index and mass spectra were the two independent parameters used for metabolite identification. Leco ChromaTOF software version 2.32 was employed for data pre-processing, automatic mass spectral deconvolution, and peak detection. The mass spectra were exported and further processed using the BinBase algorithm for metabolite identification. Signal intensities were reported as peak heights and a quantification report table was generated (SetupX database).

UHPLC-MS Analysis

UHPLC-MS Data Acquisition

Following extraction, liquid chromatography was performed on an Agilent 1200 series UHPLC system equipped with a Waters Acuity 1.7um BEH HILIC 2.1x150mm column for separation. The mobile phases were solvent A (5mM ammonium acetate with 0.2% acetic acid) and solvent B (9:1 acetonitrile:water with 5mM ammonium acetate and 0.2% acetic acid). The samples were resuspended in 100ul of solvent B for injection. The gradient program was applied as follows: 0-4 min, isocratic 100% B; 4-12 min, linear gradient 100%-45% B; 12-20 min, isocratic 45% B. The column was then re-equilibrated with 100% B for 20 minutes. For mass spectrometry, the LC eluents were analyzed with an Agilent G6530A accurate-mass QTOF, equipped with an Agilent ESI Jet Stream ion source. Data was collected in positive ESI mode. The capillary voltage was 3000V; the source gas temperature was set to 350°C and the gas flow rate was set to 10 L/min. Mass spectra were acquired with a mass range of 50-1700 Da at 4 scans per second.

Metabolite Identification and Data Analysis

The resulting raw data were deconvoluted using the Molecular Feature Extraction algorithm in the Mass Hunter Qualitative Analysis Software (Agilent). Mass Profiler Professional (Agilent) was used for data analysis and alignment. The resulting mass spectral data was searched against the METLIN, NIST MS, and LipidBLAST libraries for metabolite identification.

Results

Comparison of Preparatory Methods for Sample Extraction

We evaluated the efficiency of cell lysage techniques through measuring total chromatographic peak intensities. The sum of the signal intensities of 493 metabolite peaks detected by a preliminary GC-MS run was used for comparison of these pulverization methods. In descending order, ultrasonication alone resulted in a total signal intensites of $2.7x10^7$; mortar-and-pestle, $2.6x10^7$; ball mill/ultrasonication, $1.6x10^7$; homogenizer (trial 2), $1.6x10^7$; mortar-and-pestle/ultrasonication, $1.4x10^7$; and homogenizer (trial 1), $8.4x10^6$.

The evaluation of the various preparatory techniques for extraction showed that ultrasonication alone and mechanical grinding through the use of mortar-and-pestle yielded superior results compared to the other methods. Therefore, these two methods were selected for further evaluation based on the highest total peak intensities.

To identify the single most optimal pulverization method, ultrasonication and lysage using mortar-and-pestle were each applied to six additional porcine skin samples. All samples were subsequently analyzed by GC-TOF-MS and UHPLC-QTOF-MS. To determine the optimal cell lysage method, a signal-intensity ratio was calculated for each detected metabolite using the formula: mean signal intensities from ultrasonicated samples divided by mean signal intensities from mortar-and-pestle samples. A signal-intensity ratio greater than one indicates that ultrasonication resulted in greater signal intensities than mortar pestle for that particular metabolite. After GC-TOF-MS analysis, the signal-intensity ratio was >1 for 235 out of 255 total metabolites (92%), which indicated that ultrasonication was the favored method for these metabolites. Specifically, from the GC-TOF-MS analysis,162 metabolites showed significantly higher signal-intensity ratio favoring ultrasonication ($p< 0.05$) (**Table 1**). From UHPLC-QTOF-MS, the signal-intensity ratio was >1 for 15 out of 22 total metabolites (68%), which indicated the majority of the metabolites were better captured with the ultrasonication method. From UHPLC-QTOF-MS, three metabolites (Elaidiccarnitine, PC (phosphatidylcholine) 16:0, Butyryl-L-carnitine) showed significantly greater signal-intensity ratio favoring ultrasonication ($p< 0.05$), and one metabolite (Methylbutyroylcarnitine) displayed signal-intensity ratio favoring lysage using mortar-and-pestle ($p< 0.05$) (**Table 2**).

Comparison of GC-TOF-MS and UHPLC-QTOF-MS/MS for Sample Analysis

We then compared GC-TOF-MS and UHPLC-QTOF-MS/MS for metabolite analysis of skin samples to determine the most optimal analytical platform. Following the extraction, the porcine skin samples were either derivatized and subjected to GC-TOF-MS or subjected to UHPLC-QTOF-MS for metabolomic analysis. For GC-TOF-MS, a total of 256 metabolites, including 120 of which are not yet annotated, were reported by BinBase (**Table 1**). For UHPLC-QTOF-MS, 22 metabolites were detected by METLIN, NIST MS, and LipidBLAST (**Table 2**).

Table 1a. Annotated metabolites detected by GC-TOF-MS

Metabolite (BinBase Name)	Signal-Intensity Sonication/Mortar Pestle	P-value	Metabolite (BinBase Name)	Signal-Intensity Sonication/Mortar Pestle	P-value
Adenosine	9.7	<0.001	1-methylhydantoin TMS	2.3	<0.001
Guanosine	7.5	<0.001	Valine	2.2	<0.001
Cysteine	6.5	<0.001	Isoleucine	2.2	<0.001
Lysine	6.2	<0.001	Oxoproline	2.2	<0.001
Phosphoethanolamine	6.2	0.02	Linolenic acid	2.2	0.03
Pipecolic acid	4.8	0.20	Fucose + rhamnose	2.2	<0.001
Guanine	4.6	<0.001	2-monopalmitin	2.2	<0.001
3-phosphoglycerate	4.4	0.01	Methionine sulfoxide	2.2	<0.001
Sucrose	4.1	<0.001	N-methylalanine	2.1	<0.001
Phosphoric acid	3.8	<0.001	Linoleic acid	2.1	0.07
Putrescine	3.7	<0.001	Lauric acid	2.1	<0.001
Aspartic acid	3.6	<0.001	Isothreitol	2.1	<0.001
Creatinine	3.5	<0.001	Glyceric acid	2.0	0.03
Glutamic acid	3.5	<0.001	Methylhexadecanoic acid	2.0	<0.001
Cytidine-5'-diphosphate	3.4	<0.001	Xylitol	2.0	<0.001
Pseudo uridine	3.3	<0.001	Thymine	2.0	<0.001
Aminomalonic acid	3.3	0.02	Isolinoleic acid NIST	2.0	0.06
GABA	3.3	0.48	2-hydroxyglutaric acid	2.0	0.02
2-aminoadipic acid	3.3	0.02	Cholesterol	1.9	<0.001
Palmitoleic acid	3.2	0.02	2-hydroxybutanoic acid	1.9	<0.001
Myristic acid	3.2	0.01	Pantothenic acid	1.9	<0.001
5-methoxytryptamine	3.2	0.21	Fructose	1.9	0.02
Tryptophan	3.1	<0.001	Monomyristin NIST	1.9	0.01
Uracil	3.0	<0.001	Isothreonic acid	1.9	<0.001
Malic acid	3.0	0.01	Oleic acid	1.8	0.07
Nicotinamide	3.0	0.33	2-ketoisocaproic acid	1.8	0.02
Glycerol	2.9	<0.001	4-hydroxybutyric acid	1.8	<0.001
Trans-4-hydroxyproline	2.9	<0.001	N-acetyl-D-mannosamineor	1.8	0.01
N-acetylglycine NIST	2.9	<0.001	Ribitol	1.8	0.28
Hypoxanthine	2.8	<0.001	Xanthosine	1.8	0.04
Beta-alanine	2.8	<0.001	FAD	1.8	0.05
1,2-anhydro-myo-inositol NIST	2.8	<0.001	Cystine	1.8	0.08
Xanthine	2.8	<0.001	Stearic acid	1.7	0.01
Serine	2.7	<0.001	Pyruvic acid	1.7	0.16
Conduritol-beta-epoxide	2.7	<0.001	2-deoxytetronic acid NIST	1.7	<0.001
Inosine	2.7	<0.001	Maltose	1.7	0.02
4-hydroxyproline	2.7	<0.001	Ribose	1.7	<0.001

Urea	2.6	<0.001	Ribonic acid	1.7	<0.001
Leucine	2.6	<0.001	Elaidic acid	1.6	0.23
Inositol myo-	2.6	<0.001	Taurine	1.6	0.02
Proline	2.6	<0.001	3-hydroxybutanoic acid	1.6	0.20
Glucose	2.6	<0.001	Asparagine	1.6	0.03
Methionine	2.6	<0.001	Dodecane	1.6	0.10
1-monopalmitin	2.6	<0.001	Arachidic acid	1.5	<0.001
Erythritol	2.6	<0.001	2-monoolein	1.5	0.01
Glycine	2.5	<0.001	Hydroxycarbamate NIST	1.5	0.02
Ethanolamine	2.5	<0.001	Indole-3-lactate	1.5	0.06
Tyrosine	2.5	<0.001	Glutamine	1.4	0.16
Phenylalanine	2.5	<0.001	Nonadecanoic acid	1.4	0.15
1-monoolein	2.5	<0.001	Lactic acid	1.3	0.37
Succinic acid	2.5	<0.001	Benzoic acid	1.3	0.03
Salicylic acid	2.5	<0.001	Capric acid	1.3	0.07
Gluconic acid	2.5	<0.001	1-hexadecanol	1.3	0.17
Threonic acid	2.5	<0.001	Arabitol	1.3	0.01
Threonine	2.4	<0.001	2-hydroxyvaleric acid	1.2	0.37
1,5-anhydroglucitol	2.4	<0.001	Mannitol	1.2	0.63
Mannose	2.4	<0.001	Octadecanol	1.2	0.26
Glycerol-alpha-phosphate	2.4	<0.001	1-monostearin	1.2	0.70
Glycerol-3-galactoside	2.4	<0.001	2-oxogluconic acid NIST	1.2	0.22
Ornithine	2.4	0.01	Hydroxylamine	1.1	0.86
Pyrophosphate	2.4	0.02	Zymosterol	1.0	0.86
Alanine	2.3	<0.001	Pelargonic acid	0.9	0.83
Palmitic acid	2.3	0.01	Adipic acid	0.9	0.60
Arachidonic acid	2.3	<0.001	Glycolic acid	0.8	0.63
Fumaric acid	2.3	0.01	Cyclohexylamine NIST	0.6	0.28
Threitol	2.3	<0.001	Uridine	0.6	0.42
Fucose	2.3	<0.001	Malonic acid	0.3	0.28
Thymidine	2.3	<0.001			

Table 1b. Un-annotated metabolites detected by GC-TOF-MS

Metabolite (BinBase Name)	Signal-Intensity Sonication/Mortar Pestle	P-value	Metabolite (BinBase Name)	Signal-Intensity Sonication/Mortar Pestle	P-value
233824	6.2	<0.001	465393	1.8	0.09
446628	5.8	<0.001	349922	1.8	<0.001
200484	5.4	0.02	213735	1.8	0.03
232722	5.3	<0.001	415114	1.7	0.15
203221	3.9	<0.001	221579	1.7	0.01
279691	3.8	<0.001	438101	1.6	0.40
208557	3.8	0.01	288808	1.6	0.27
199786	3.7	<0.001	356925	1.6	0.02
373802	3.6	<0.001	402237	1.6	0.08
213130	3.5	0.01	445906	1.6	0.23
228249	3.4	<0.001	234717	1.6	0.03
438057	3.3	<0.001	437266	1.6	0.01
200471	3.2	0.03	241218	1.6	0.05
321685	3.1	<0.001	308073	1.6	0.19
227352	3.1	<0.001	367932	1.5	0.03
213185	3.0	<0.001	232534	1.5	0.04
216834	3.0	<0.001	237761	1.5	0.08
310052	3.0	<0.001	223597	1.5	0.02
470307	2.8	<0.001	352756	1.5	0.06
221585	2.8	0.01	204344	1.4	0.09

470290	2.8	0.01	212022	1.4	0.04
443309	2.7	<0.001	415158	1.4	0.04
232154	2.7	<0.001	268313	1.4	0.03
213697	2.7	<0.001	288822	1.4	0.31
307966	2.6	<0.001	232539	1.4	0.34
487231	2.6	0.01	483487	1.3	0.21
211946	2.6	0.05	204448	1.3	0.03
267682	2.6	<0.001	429880	1.3	0.03
487430	2.6	0.02	356938	1.3	0.09
213964	2.6	0.03	300379	1.3	0.12
438058	2.5	0.02	268579	1.3	0.20
223675	2.5	<0.001	204425	1.3	0.24
289052	2.5	0.02	408731	1.2	0.14
223629	2.5	0.02	470289	1.2	0.42
206604	2.4	<0.001	437786	1.2	0.18
470298	2.4	0.04	408490	1.2	0.16
367991	2.3	0.07	444613	1.2	0.59
470304	2.3	0.05	353747	1.2	0.44
242128	2.3	0.11	201887	1.2	0.18
299091	2.3	0.01	330991	1.2	0.46
220021	2.2	0.01	242675	1.1	0.67
229203	2.2	0.01	200477	1.1	0.78
207223	2.2	<0.001	269146	1.1	0.78
218964	2.2	0.01	322652	1.1	0.84
199553	2.2	<0.001	444410	1.1	0.67
203295	2.1	0.01	238243	1.1	0.34
224843	2.1	<0.001	225539	1.1	0.78
211972	2.0	0.02	229969	1.0	0.95
216816	2.0	<0.001	274174	0.9	0.77
241090	2.0	<0.001	224794	0.9	0.65
218512	2.0	0.01	408852	0.9	0.76
213253	1.9	0.04	357045	0.8	0.43
267742	1.9	0.03	273925	0.8	0.29
241087	1.9	0.02	233200	0.8	0.57
270594	1.9	0.02	221571	0.8	0.72
223871	1.9	0.01	231850	0.8	0.56
455340	1.9	<0.001	216428	0.7	0.49
199942	1.8	0.01	309540	0.6	0.59
305055	1.8	0.04	271063	0.6	0.10
362130	1.8	0.04	201042	0.4	0.40

Table 2. Metabolites detected by UHPLC-QTOF-MS

BinBase Name	Signal Intensity Sonication/Mortar Pestle	P-value
Elaidiccarnitine	22.0	0.02
PC (phosphatidylcholine) 16:0	7.0	0.02
PC (phosphatidylcholine) 18:2	6.6	0.49
Creatinine	5.4	0.15
PC (phosphatidylcholine) 38:6	4.3	0.09
Butyryl-L-carnitine	3.2	0.03
PC (phosphatidylcholine) 32:1	3.1	0.23
PC (phosphatidylcholine) 40:6	2.6	0.09
PC (phosphatidylcholine) 36:4	2.2	0.24
PC (phosphatidylcholine) 40:5	2.0	0.25
DL-Stearoylcarnitine	2.0	0.22
PC (phosphatidylcholine) 36:3	1.8	0.31
PC (phosphatidylcholine) 38:4	1.5	0.48
PC (phosphatidylcholine) 36:5	1.3	0.53
PC (phosphatidylcholine) 38:5	0.8	0.72
PC (phosphatidylcholine) 34:2	0.8	0.55
Carnitine	0.8	0.76
Propionyl-L-carnitine	0.4	0.56
Valine	0.2	0.05

Methylbutyroylcarnitine	0.1	<0.001
Phenylalanine[a]		
L-Octanoylcarnitine[b]		

[a]Phenylalanine was only detected in samples prepared by the mortar-and-pestle technique.

[b]L-Octanoylcarnitine was only detected in samples prepared by the sonication technique.

Discussion

Metabolomics studies can provide important insights into disease processes by measuring the amplified chemical output from a type of tissue or an organism [1]. In dermatology, the field of metabolomics analysis is yet to be explored. Accurate metabolite characterization of tissues begins with sample preparation. Thus, the evaluation of optimal sample preparation methods is critical to developing protocols in dermatology for metabolomics research.

The reliability and accuracy of sample preparation is paramount because it affects the downstream interpretation of metabolites [17]. Sample preparation consists of sampling, quenching, and metabolite extraction. Although such procedures have been described for biofluids [16,18,19] these methodologies have not been evaluated for skin tissue for metabolite analysis. Skin sample preparations differ from those of biofluids because additional steps of cell pulverization are required prior to metabolite extraction. This prerequisite is crucial because efficient cell breakage allows for a more comprehensive and unbiased collection of metabolites.

This study is the first comprehensive investigation systematically evaluating the various cell lysage techniques in dermatology for metabolomic analysis. Based on the total peak intensities of all detected metabolites, the study results indicate that mechanical breakage via ultrasonication alone or grinding using a liquid nitrogen-cooled mortar was more effective for cell lysage than the other methods--the use of a homogenizer, a combination of grinding using the mortar-and-pestle followed by ultrasonication, and a combination of using a ball mill followed by sonication.

Specifically, ultrasonication using a VWR 50HT sonicator was the most efficient method for capturing metabolite signal, whereby cell disruption is induced by cavitation microstreaming. Cavitation microstreaming is created by high intensity sound waves generating microscopic air bubbles that produce hydraulic shear gradients [20,21]. Cell disintegration by sonication is advantageous in that the process itself overlaps with the extraction method because the same solvents are used. Limitations to ultrasonication include potentially reduced reproducibility from variability in treatment time and sample viscosity.

Cell disintegration by the classical means of mechanical grinding using mortar-and-pestle is achieved by inflicting either tensile or shear stress at points of strain to disrupt the cell membrane [21]. This approach may produce variable results as efficiency is dependent upon the nature of the sample, the skills of the operator, and the time spent on manual grinding [20]. In this study, we found that ultrasonication was superior to the mortar-and-pestle method for metabolite extraction for skin tissue. In comparison, a study analyzing mycobacterial metabolome found that a combination of mechanical grinding followed by sonication was most optimal [20]. More studies specific to skin tissue are necessary to determine and validate optimal cell breakage method in dermatologic metabolomic analyses.

In this study, two different analytical platforms were evaluated for metabolomic analysis of skin tissue—mass spectrometry coupled with either gas chromatography or liquid chromatography. It is unclear in the literature which analytical technology is more widely used and could serve as the gold standard for metabolomics [18,22]. From this study, GC-MS resulted in the detection of an extensive cutaneous metabolome, which included the detection of primary metabolites less than 550 Da. Moreover, GC-MS allows for the collection of quantitative information and is highly efficient, sensitive, and reproducible. In particular, the standardized electron ionization energy (70ev) generates reproducible fragmentation patterns for identification, which enables the sharing of data and the establishment of mass spectral libraries [23]. The main drawback to using GC-MS for detection of global metabolites is that only thermally stable, volatile compounds or compounds made volatile following derivatization are amenable to this platform of analysis [18,23].

Similar to GC-MS, LC-MS is quantitative and highly sensitive. Unlike GC-MS, LC-MS is advantageous in that it is not limited to thermally stable and volatile compounds. Thus, sample preparation without the derivatization step is simplified [24]. Owing to the diverse range of analyte polarities present in a sample, one LC run does not offer adequate coverage and several combinations of column chemistries (reverse-phase, normal phase, HILIC) and ESI modes (positive and negative) are required. Although this renders LC-MS quite impractical for large-scale studies, it allows tailoring of the separation for specific classes of compounds [22,25]. Selecting a separation procedure is dependent upon the proposed hypothesis. GC-MS is suitable for the detection of fatty acids, steroids, and flavonoids, whereas LC-MS is suitable for the detection of lipids, peptides, and nucleotides [25]. Because of

the complexity of the metabolome, there is no single analytical technology capable of the comprehensive analysis of all metabolites in a given biological sample to date, and the use of multiple combined analytical platforms has been documented to achieve greatest metabolome coverage [24].

In dermatology, a few studies have explored urine and serum metabolomics in atopic dermatitis and lepromatous leprosy, respectively [26,27]. Other investigators have employed metabolomic platforms to identify volatile signatures from melanoma as well as study the effects of ionizing radiation [28-30].

To our knowledge, this study exemplifies the first evaluation of preparatory methods involved with extraction of porcine skin tissue, which serves as a model in the preparation of human skin tissue samples for future metabolomic studies. Our findings indicate that, with the proper sample preparation, metabolomic analysis of skin tissue is a feasible and worthwhile task. We selected porcine skin tissue as a model for human skin tissue owing to their histological and physiological similarities [31,32]. Ongoing efforts are underway to evaluate these methods with human skin samples. At the current time, metabolomic analysis of skin samples remains a relatively unexplored field in dermatology. Analyzing the metabolome of skin tissue creates an exciting means to elucidate underlying biological processes for various dermatological diseases.

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