Article

JOURNAL OF THE CHINESE CHEMICAL SOCIETY

A Comparative Study of Pentafluorophenyl and Octadecylsilane Columns in High-throughput Profiling of Biological Fluids

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(Received: December 20, 2016; Accepted: March 25, 2017; DOI: 10.1002/jccs.201600873)

In high-throughput metabolomic profiling, chromatographic separation is crucial because a well-performed chromatographic separation may reduce signal suppression from complex biological matrices and improve the discoverability of low-abundance metabolites. We compared the performance of pentafluorophenyl (PFP)- and octadecylsilane (ODS)-based columns in profiling biological fluids. Peak resolutions and consistencies were acquired using several reversed-phase columns and were evaluated. Total and extracted ion chromatograms demonstrated that the PFP column achieved better analyte separations than the ODS column. Low relative standard deviations on peak areas and retention times (<10.2 and <0.9%, respectively) acquired using the PFP column evidenced the high reproducibility and consistency of this column. In our study, a PFP column was used for profiling metabolomes extracted from urine and serum samples. Metabolomic study revealed a metabolome difference in normal and overweight participants. In total, 26 lipid species were significantly perturbed and further identified. Choline-containing lipids were the most abundant perturbed lipidome in overweight participants, followed by sphingolipids and various phospholipids. We recommend the use of PFP columns in high-throughput metabolomic analysis to promote the development of basic biological and clinical research in the future.

Keywords: Biological fluids; Metabolomics; Octadecylsilane; Pentafluorophenyl; Partial least squares-discriminant analysis.

INTRODUCTION

Metabolomics is the study of endogenous low-weight metabolites (typically <1500 Da) for clarifying the functional information in biological system states.[1,2] It is widely applied in the study of various diseases,[3,4] biomarker discovery,[1] drug efficacy and toxicity screening,[5] nutrition[6] and environmental exposure.[7,8] Over the decades, researchers have attempted to explore specific low-weight molecules that can serve as diagnostic markers for monitoring and predicting disease progression and therapeutic inspection.[9,10] Up to 40% of the genes in the human genome are hypothetical and their functions remain unknown,[11] of which some may be identified as orphan genes. Therefore, functional omics, including transcriptomics, proteomics, and metabolomics, are gaining much attention in academia. Metabolomics is advantageous because it serves as a direct signature of the investigated biochemical activity, enabling the simple correlation of phenotypes and activities. Through biofluid (blood and urine) metabolite profiling, metabolomics summarizes the physiological status of an individual and therefore has high potential in personalized medicine[12] because different individuals metabolize compounds at different rates.

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most commonly used methodologies in metabolomics. Several studies have highlighted the benefits of combining multiple platforms while analyzing biofluids and extracts. However, because of the limitations due to cost and instruments,

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laboratories tend to use a single analytical platform. NMR-based metabolomics has the advantage of simple sample preparation methods and is nondestructive to the analytes. However, the low resolution of NMR limits its applicability to nonpolar compounds, such as long-chain lipid detection and structural profiling and determination.[1] Gas chromatography (GC) is widely applied in metabolomics because of its low equipment and maintenance costs and accessible databases. However, GC has major drawbacks; in particular, the extracted metabolites have high tendency to undergo significant alterations with time at high temperatures,[13] thus possibly yielding erroneous results.

Utilization of liquid chromatography in metabolomics has grown rapidly over the last decade.[14] Among the liquid chromatography methods, hydrophilic interaction liquid chromatography (HILIC) is used for analyzing anionic, cationic, uncharged, and zwitterionic compounds.[15] HILIC has been used extensively and optimized for analyzing targeted metabolites. However, its peak retention time drift renders untargeted metabolite profiling difficult.[16] Moreover, it requires a longer equilibration time compared to reverse-phase liquid chromatography (RPLC).[17] Therefore, untargeted profiling studies are performed mainly using a reverse-phase octadecylsilane (ODS) column because it generates the reproducible data required for high-throughput studies.[18] However, the major drawback of the ODS column, particularly the C_{18} column, is that polar compounds from the extracts tend to be eluted at or close to the void volume,[19] whereas pentafluorophenyl (PFP) column is able to separate polar isomeric compounds.[20]

In high-throughput metabolomics, the successful acquisition of metabolites within the sample depends on the quality of the peak shape of the analytes. Distorted peaks often jeopardize correct peak integration and reduce analyte sensitivities and resolutions,[21] which may lead to inaccurate results. Several researchers have suggested multiple factors, such as mobile phase, analyte concentration, and appropriate column selection, that affect the peak quality.[21] We compared the performance of three RPLC columns, namely the commonly used two ODS (including C_{18} and T3) columns and one (PFP) column, in the chromatographic separation of polar and nonpolar metabolites extracted from the biological fluids of normal and overweight participants at positive and negative ionization modes.

RESULTS AND DISCUSSION

In high-throughput analysis, the analysis speed is critical because faster analyses may reduce the analysis cost and increase the throughput. Shortening the analytical column reduces the analytical run but increases the risk of co-elution, which affects the resolution of the analyte peaks.[22] Reducing the column particle size (to as low as 1.7–1.9 μm) improves the separation affinity because it increases the absorption surface while shortening the column length. This approach is challenging because a column with smaller particles can easily accumulate LC backpressure throughout; therefore, an LC system that strongly resists high backpressures is essential. Later, the development of core–shell technology strongly reduced the backpressure of the column while maintaining a high separation efficiency.[23]

C_{18} and T3 columns are commonly used for universal metabolomic profiling of both polar and nonpolar compounds extracted from urine[24] and blood.[25] The efficacy of separating nonpolar and moderately polar analytes depends on the density of the C_{18} alkyl chains, the accessibility of silanol, and the bonding groups between the silica beads and C_{18} alkyl groups within the ODS column.[26] An organofluorinated-silica-based stationary phase was introduced for analyzing polar analytes over the last decade.[27] The PFP ring attached to silica in a propyl chain exhibits both reversed- and normal-phase retention and offers different selectivity for polar and nonpolar compounds compared to traditional alkyl and HILIC phases.[27,28] Moreover, Wong *et al.* reported the high efficiency of PFP in the chromatographic separation and quantification of various chemical homologs.[29] However, the PFP phase has limited applications in both qualitative and quantitative metabolomics.[30] We compared the performance of commonly used ODS and PFP phase columns in polar and nonpolar high-throughput metabolite profiling.

Chromatographic performance

Figures 1 and 2 present the total ion chromatograms (TICs) obtained using different RP columns of the urine and serum extracts, respectively. The number of acquired metabolites from the urine samples profiled using F5 (Figure 1(a)) is comparable to that obtained using T3 (Figure 1(b)) and C_{18} (Figure 1(c)). The total compound chromatogram and spectra generated from the acquired TIC evidenced the higher efficiency of F5

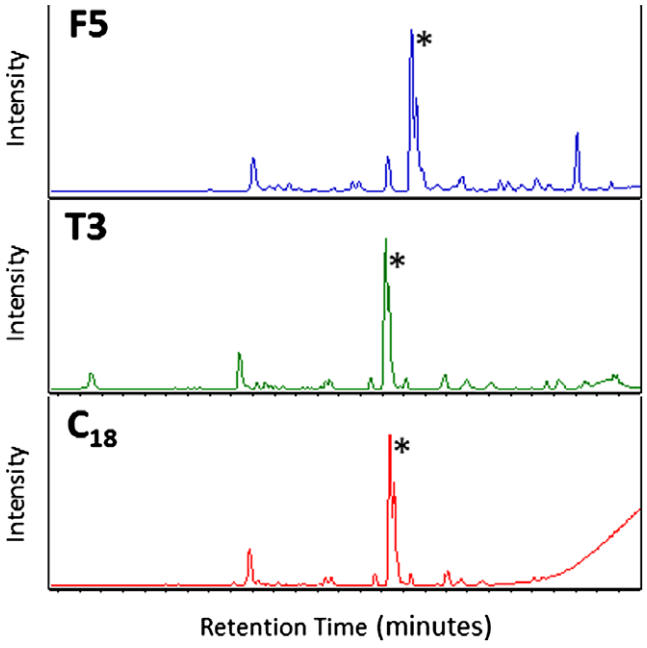

Fig. 1. Total ion chromatogram acquired from urine samples by using F5, T3, and C_{18} columns. Asterisks (*) show the peaks that were further extracted from the total compound chromatogram (Figure 2).

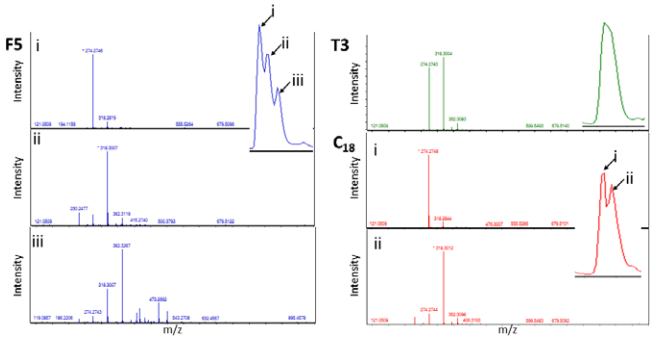

Fig. 2. Spectra extracted from the selected peak (*) in Figure 1. Peaks were acquired using F5, T3, and C_{18} columns. Well-resolved peaks can be observed from the peak acquired using F5; the spectra demonstrate less suppression from the matrices. Co-elution of analytes occurred in T3, and a slight separation of the analytes was realized using the C_{18} column.

in separating co-eluted metabolites (Figure 2); the TIC acquired from serum-extracted polar metabolites was similar (Figure 3). We deduced that the PFP phase exhibited both reverse- and normal-phase retention and offered higher selectivity for polar compounds

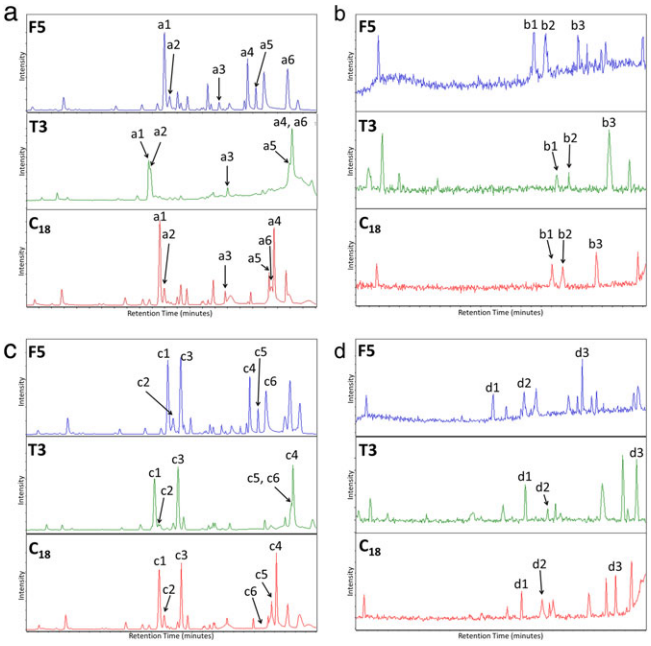

Fig. 3. Total ion chromatogram profiled from polar (a and b) and nonpolar (c and d) extracts from serum samples by using F5, T3, and C_{18} columns at the positive (a and c) and negative (b and d) ionization modes. a1, C16 sphinganine; a2, hydroxystearic acid; a3, lucidenic acid; a4, docosanedioic acid; a5, L-methionine; a6, unknown; b1, myoinositol; b2, linoleic acid; b3, *N*-palmitoyl proline; c1, monoglycerol (12:0); c2, sphingosine (t18:0); c3, ceramide (t28:0); c4, MG(P-20:0); c5, lysophosphotidylcholine (LPC) (18:2); c6, LPC (16:0); d1, fatty acid D1; d2, fatty acid D2; d3 lysophosphatidic acid (20:4).

compared with ODS.[28] In addition, dipole–dipole, hydrogen-bonding, π–π,[27,31] and ion-exchange interactions[29] in the PFP offer larger capacity factors for aromatic and polycyclic aromatic hydrocarbons because of the formation of a donor–acceptor complex. Moreover, the fluorinated phase inherent in the compound shape and size selectivity[32] enhances the separation of metabolite matrices. Organofluorines of the PFP stationary phase show high electronegativity, low polarizability, and strong lipophobicity and hydrophobicity,[29] offering binding possibilities of various nonpolar compounds. These unique characteristics of the PFP column enable the good chromatographic separation of polar metabolite extracts. Figures 3(c) and (d) present the TIC acquired using F5 in the positive and negative ESI modes, respectively. The number of nonpolar

metabolites acquired using the PFP column was comparable to that acquired using ODS C_{18} (Figures 3(c iii) and (d iii)).

Peak quality evaluation

We extracted the ion chromatogram of selected metabolites from the TIC. Table 1 lists some of the contemporaneous *m/z* profiled from the serum sample by using the F5, T3, and C_{18} columns. Choi and Row proposed that a good and well-resolved peak should have a near-zero asymmetry, narrow peak width, and positive kurtosis (leptokurtic features)[33] (Table S1, Supporting information, peak shape category). We observed that metabolite peaks acquired using F5 generally exhibited satisfactory peak quality. However, several peaks (mostly from nonpolar extracts profiled in the negative ionization mode) presented with negative kurtosis. When zoomed in, these peaks were marginally flat-topped[34] (platykurtic) without any peak tailing or broadening. These outcomes are attributable to multiple factors such as excessive sample load, detector overrange, on-column analyte reaction, and mobile phases.[35–37] The relative standard deviation (RSD, %) of the extracted ion chromatograms with a peak area of <10.2% and retention time acquired using F5 was relatively low compared to other columns (Table 2). This proved that F5 was highly stable and consistent in the intraday (n = 5) and interday (n = 15) analyses. Chromatographic performance of the ODS column was dependent on the interaction of the analytes with the alkyl chains or the free silanol.[22] Most peaks acquired using C_{18} or T3 columns were unsatisfactory, as they exhibited peak tailing or fronting because of their peak asymmetry features. In addition, their peak area RSD ranged from 0.4% to 25% (Table 2), suggesting inconsistencies during the batchwise analysis of samples. Such results are not tolerable because high-throughput studies involve hundreds or thousands of samples, and inconsistencies can jeopardize the eventual outcomes and mislead the overall study.

Application of the F5 column for analyzing the metabolome perturbation in overweight participants

Obesity is defined as the medical condition in which excessive body fat accumulates to such an extent that it may negatively affect health.[38] Growing incidence of obesity is a major public health concern worldwide.[39] Obesity is one of the most crucial risk factors contributing to the overall burden of diseases including heart diseases, type 2 diabetes mellitus, obstructive sleep apnea, cancer, and osteoarthritis.[38] Using the body mass index (BMI) formula $\frac{\text{weight (kg)}}{[\text{height (m)} \times \text{height (m)}]}$, people are clinically classified as normal (BMI, 18.5–24.9), overweighed (25–29.5), and obese (≥30). Our study participants were healthy volunteers (both sexes; age between 25 and 40) not under any medication. The participants were enrolled into two groups: normal (n = 6, BMI = 18.5–24.9) and overweight (n = 6, BMI ≥ 25). Blood and urine samples were collected from all participants after 4 h of fasting. The samples underwent preparation and extraction according to the methods described in this paper.

The biofluids of overweight and normal participants were injected alternately during the analysis. One blank sample (8 μL of methanol) was injected after every sixth sample during sequencing; no significant carryover of polar or nonpolar extracts was observed. All acquired raw data were preprocessed using MZmine 2 and subjected to multivariate analyses. Partial least squares-discriminant analysis (PLS-DA) was performed by using 12 samples from the overweight and normal groups (six biological replicates each). From the visualization data, the PLS-DA score plots enabled us to discriminate the properties of each sample: samples (symbols) that were close together shared similar properties, whereas those far apart were dissimilar.[40]

Figure 4 shows the results of the PLS-DA analysis of nonpolar serum extracts at both positive and negative ionization modes. In total, 12 symbols were assigned into the normal and overweight groups along component 1 with the following established components: R^2Y = 0.9869, Q^2 = 0.2627 and R^2Y = 0.9913, Q^2 = 0.6436 for nonpolar extracts analyzed using positive (Figure 4(a)) and negative (Figure 4(b)) ionization modes, respectively. From these figures, the clustering of the normal participants was localized to the negative region of component 1, whereas that of the overweight participants was localized in the positive region of component 1. To inspect the contribution variables (nonpolar metabolites) and to distinguish between the groups in our PLS-DA model, variable importance projection (VIP) was conducted with a threshold of 1.5.

Table 1. Peak quality evaluation on metabolites extracted from extracted ion chromatogram profiled using different RP columns

Fraction	*Ionization*	*Matched metabolites*[a]	*Width*			*Asymmetry*			*Kurtosis*		
			F5	*T3*	*C_{18}*	*F5*	*T3*	*C_{18}*	*F5*	*T3*	*C_{18}*
Polar	Positive	Dihydrothymine									
		Tetradecanedioic acid									
		C16 Sphinganine									
		Isovalerylglucuronide									
		Unknown (318.3017)									
		Docosanedioic acid									
		Unknown (429.3680)									
		Chlorogenin									
		Lucidenic acid									
		Unknown (485.4317)									
		Unknown (679.5103)									
		Unknown (737.5632)									
	Negative	Tetradecyl isobutyrate									
		Unknown (311.1706)									
		Unknown (325.1853)									
		N-palmitoyl proline									
		N-oleoyl glutamine									
Non-Polar	Positive	Sph (14:0)									
		Sph (16:0)									
		Sph (d16:1)									
		MG (12:0)									
		Sph (t18:0)									
		MG (18:0)									
		Unknown (371.3159)									
		LysoSM (t14:0)									
		Cer (d26:0)									
		MG (26:5)									
		Unknown (485.4315)									
		LPC (18:2)									
		SM (d26:0)									
		PC (36:3)									
	Negative	FA Chain length 10-16									
		Unknown (265.1479)									
		Unknown (283.2646)									
		LPA (10:0)									
		LPA (12:0)									
		LPA (16:0)									
		LPA (20:4)									
		LPA (20:3)									
		PA (38:9)									
		PI (26:1)									
		PS (36:0)									

[a] Each metabolite was matched with the available databases.

Each extracted peak was evaluated on the basis of the acquired peak quality, measured in terms of peak width, peak asymmetry, and kurtosis. The peak quality is color scaled:

Least satisfactory Most satisfactory (refer Table S1 for detailed information).

Subsequently, the VIP scores were cross-validated with statistical analysis by using the *t*-test; $p < 0.05$ was considered significant. Thus, the nonpolar metabolites that led to clustering of both groups at different ionization modes were successfully identified (Table 3). A multivariate analysis was conducted using the acquired dataset of urine samples. Using the established model, a negative Q^2 (data not shown) was presented, which indicated that the model lacked a predictive value. Hence, the model was discarded.

Table 2. Intra-batch and inter-batch relative standard deviations of the metabolite peak area and retention time

			RSD (%) area						RSD (%) retention time					
			Intra-batch (n = 5)			Inter-batch (n = 15)			Intra-batch (n = 5)			Inter-batch (n = 15)		
Fraction	Polarity	Metabolites	F5	T3	C18	F5	T3	C18	F5	T3	C18	F5	T3	C18
Polar	Positive	Dihydrothymine	0.28	3.95	8.82	4.59	5.83	8.68	0.14	0.38	0.33	0.15	0.38	0.36
		Tetradecanedioic acid	0.74	2.86	16.15	6.38	4.84	13.66	0.14	0.38	0.33	0.15	0.38	0.36
		C16 Sphinganine	0.14	3.46	2.71	4.94	4.29	2.15	0.45	0.10	0.89	0.68	0.34	1.07
		Isovalerylglucuronide	0.44	2.57	1.40	1.96	3.25	6.80	0.86	0.09	1.53	0.95	0.16	1.41
		Unknown (318.3017)	0.50	3.52	4.02	3.96	5.00	13.22	0.43	0.09	0.83	0.64	0.35	1.03
		Docosanedioic acid	5.52	13.82	19.91	8.33	10.00	17.88	0.14	0.39	0.28	0.15	0.38	0.36
		Unknown (429.3680)	0.70	6.58	17.61	7.67	11.4	20.28	0.15	0.39	0.31	0.15	0.36	0.36
		Chlorogenin	1.23	13.61	24.89	7.53	21.53	25.24	0.15	0.44	0.35	0.15	0.55	0.37
		Lucidenic acid	0.56	10.28	12.97	4.02	17.35	18.22	0.12	0.23	0.19	0.15	0.26	0.33
		Unknown (485.4317)	0.34	12.44	23.38	8.98	15.37	20.44	0.15	1.06	2.15	0.17	0.70	0.62
		Unknown (679.5103)	0.23	2.55	1.47	8.04	6.38	4.79	0.91	0.04	2.54	0.73	0.09	1.80
		Unknown (737.5632)	0.45	2.62	0.54	4.51	8.10	10.86	0.91	0.05	2.53	0.73	0.09	1.82
	Negative	Tetradecyl isobutyrate	4.00	5.08	10.21	8.50	13.30	7.06	0.40	0.26	0.06	0.27	0.26	0.19
		Unknown (311.1706)	7.84	3.49	8.92	11.73	9.34	10.27	0.15	0.11	0.11	0.30	0.14	0.22
		Unknown (325.1853)	3.00	7.22	4.31	3.58	7.39	5.08	0.23	0.10	0.10	0.19	0.16	0.21
		N-Palmitoyl proline	0.84	7.25	8.04	5.84	6.07	6.52	0.35	0.33	0.14	0.23	0.28	0.19
		N-Oleoyl glutamine	2.73	6.30	24.05	5.46	4.18	20.26	0.39	0.27	0.07	0.28	0.27	0.20
Nonpolar	Positive	Sph (14:0)	2.38	7.18	4.09	4.26	5.93	2.97	0.57	0.11	0.40	0.60	0.27	0.79
		Sph (16:0)	4.03	6.06	6.58	7.10	12.10	11.90	0.18	0.45	0.27	0.16	0.38	0.25
		Sph (d16:1)	1.64	7.53	11.15	3.31	5.92	6.10	0.49	0.10	0.24	0.55	0.25	0.74
		MG (12:0)	2.89	5.49	6.03	5.39	4.58	3.74	0.59	0.12	0.45	0.62	0.26	0.82
		Sph (t18:0)	4.90	6.64	9.55	6.11	9.64	6.51	0.56	0.16	0.40	0.59	0.24	0.80
		MG (18:0)	4.62	12.39	11.92	7.87	10.53	14.93	0.19	0.46	0.29	0.17	0.37	0.24
		Unknown (371.3159)	2.17	3.67	19.65	3.21	12.44	16.45	0.18	0.45	0.27	0.16	0.37	0.25
		LysoSM (t14:0)	1.98	6.90	20.43	2.05	16.88	25.16	0.18	0.62	0.15	0.19	0.57	0.29
		Cer (d26:0)	2.98	6.19	10.27	4.94	10.63	14.42	0.18	0.45	0.26	0.16	0.37	0.25
		MG (26:5)	2.12	4.66	10.69	8.77	17.65	20.29	0.20	0.11	0.17	0.18	0.21	0.22
		Unknown (485.4315)	2.48	9.05	24.95	7.47	7.14	25.30	0.18	0.58	1.45	0.19	0.56	0.32
		LPC (18:2)	0.29	3.22	7.58	5.77	5.11	7.05	0.17	0.41	0.28	0.16	0.38	0.24
		SM (d26:0)	2.26	10.18	9.27	4.25	10.91	6.15	0.20	0.05	0.17	0.19	0.18	0.25
		PC (36:3)	2.57	1.72	8.12	4.43	5.80	8.19	0.19	0.46	0.29	0.17	0.38	0.24
	Negative	FA chain length 10-16	3.13	0.48	17.55	4.62	14.82	18.17	0.15	0.22	0.23	0.33	0.22	0.40
		Unknown (265.1479)	4.77	1.61	2.84	6.57	7.12	3.11	0.61	0.09	0.43	0.90	0.09	0.45
		Unknown (283.2646)	5.16	8.00	2.43	5.32	16.58	7.32	0.19	0.23	0.22	0.36	0.22	0.42
		LPA (10:0)	0.48	3.99	3.89	3.11	6.00	11.08	0.19	0.32	0.39	0.39	0.24	0.37

Table 2. Continued

Fraction	Polarity	Metabolites	RSD (%) area						RSD (%) retention time					
			Intra-batch (n = 5)			Inter-batch (n = 15)			Intra-batch (n = 5)			Inter-batch (n = 15)		
			F5	T3	C18	F5	T3	C18	F5	T3	C18	F5	T3	C18
		LPA (12:0)	3.81	2.39	12.68	2.94	3.40	8.66	0.15	0.26	0.28	0.31	0.22	0.38
		LPA (16:0)	4.35	3.72	7.57	6.51	5.54	7.51	0.22	0.27	0.22	0.38	0.24	0.44
		LPA (20:4)	1.62	3.60	4.19	1.83	17.41	10.12	0.18	0.22	0.22	0.34	0.21	0.42
		LPA (20:3)	4.16	3.87	5.87	9.44	15.64	4.19	0.46	0.11	0.52	0.68	0.17	0.55
		PA (38:9)	10.24	6.54	5.45	12.42	17.82	11.44	0.51	0.07	0.87	0.50	0.10	0.88
		PI (26:1)	4.44	3.39	3.64	8.49	15.17	8.26	0.63	0.08	0.87	0.62	0.11	0.72
		PS (36:0)	8.16	5.82	7.95	11.19	19.73	10.95	0.18	0.07	0.88	0.59	0.10	0.73

Overweightness dysregulates lipid metabolism

Twenty-six significantly perturbed lipid species were observed in the overweight participants (Table 3). These lipids included choline-containing lipids (phosphatidylcholine [PC] and sphingomyelin [SM]), sphingolipids (sphingosine and ceramides), glycerol (diacylglycerol and triacylglycerol), and polar phospholipids (phosphatidylglycerol [PG] and phosphatidylserine [PS]). Figure 5 shows the deduced interrelationship among the various classes of lipids in the overweight participants. Studies have highlighted that phosphorylcholine-containing lipids play a critical role in regulating cellular membrane fluidity and antioxidant functioning and facilitating membrane fusion, energy storage, and release of polyunsaturated and saturated fatty acids.[41] Studies have also reported a strong correlation between the phosphorylcholine-containing lipids in obese patients[42] via ceramides. Ceramide is formed following the catalysis of sphingomyelin by sphingomyelinase[40] or a *de novo* synthesis following the condensation of serine and palmitoyl CoA, in which multiple enzymes are involved.[43] Several proinflammatory cytokines are secreted and upregulated in the adipose tissues of obese individuals. Such upregulation potentially dysregulates sphingolipids (including sphingosine and ceramide), further triggering subsequent inflammatory reactions.[43] In addition, lysophospholipid, a single-carbon-chain lipid molecule with a polar head group and a bioactive lipid molecule,[44] was perturbed in our study. These bioactive lipid molecules had been previously reported to inherit unknown functions in the development of various cancers[45–47] and stroke.[48] Thus, the high susceptibility of obese patients to cancer and stroke may be due to dysregulated lysophospholipids. Moreover, perturbation of triacylglycerides, which serve as key clinical diagnostic criteria for obesity,[49] correlated positively with the participants' blood biochemistry tests in this study.

CONCLUSIONS

We found the performance of the PFP column superior to that of the ODS columns. Metabolites in the urine sample profiled using the PFP column exhibited high resolution (peak separations). The peak area acquired using the PFP column during intra-batch and inter-batch analysis (serum) evidenced that the RSD of the PFP column was relatively low compared to that of the ODS columns. This result indicates that the PFP

Table 3. Discriminating variables (metabolites) highlighted by the PLS-DA following variable importance projection (VIP) and cross-validation using the *t*-test

Ionization mode	Detected *m/z*	Subclass	Matched metabolites	VIP[1]	*t*-Test[2] (*p*-value)
Positive	660.4963	Choline-containing lipid	LPC (28:2)	•••	*
	756.5538		PC (34:3)	••	**
	780.5538		PC (36:5)	••	**
	838.6321		PC (38:5)	••	*
	806.5695		PC (38:6)	•••	*
	808.5851		PC (38:6)	••	*
	842.6634		PC (40:4)	•	*
	866.6385		PC (42:4)	•••	*
	856.5851		PC (42:9)	••	**
	892.6790		PC (44:5)	•••	**
	992.7103		PC (52:11)	••	*
	1080.8355		PC (58:9)	••	*
	589.4340		SM (d26:2)	••	**
	675.5436		SM (d32:1)	•••	*
	731.6062		SM (d36:1)	••	**
	777.6480		SM (t38:0)	••	**
	246.2428	Sphingolipids	Sph (d14:0)	••	*
	298.2741		Sph (d18:2)	•••	**
	432.2796		HexSph (d16:2)	•••	*
	658.4889		HexCer (t30:2)	••	*
	527.5034	Glycerol	DAG (O-30:0)	•••	*
	651.5922		TAG (P-38:0)	•••	***
Negative	745.5489	Phosphatidylglycerol	PG (34:2)	••	*
	775.5494		PG (36:1)	•	*
	826.4664	Phosphatidylserine	PS (40:10)	•••	*
	854.4977		PS (42:10)	••	*

[1] VIP scores for the constructed PLS-DA model.
[2] $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ determined using the *t*-test.

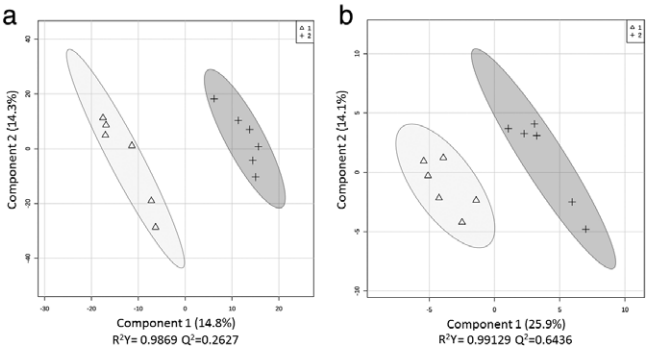

Fig. 4. Partial least squares-discriminant analysis score plots for nonpolar extracts analyzed at (a) positive and (b) negative ion modes in samples extracted from overweight (Δ) and normal (+) participants. Eclipses are the mean ± standard deviation of the sample.

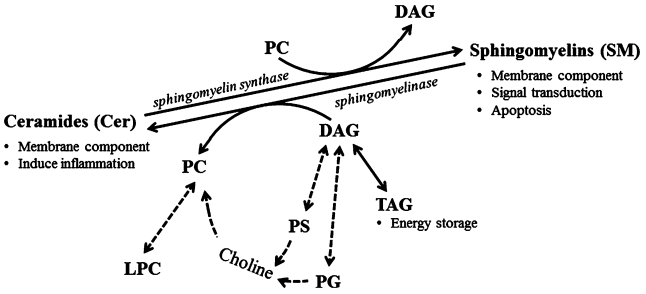

Fig. 5. Dysregulation and metabolic pathway of sphingomyelin (SM), ceramide (Cer), phosphotidylcholine/lysophosphotidylcholine (PC/LPC), phosphatidylserine (PS), phosphatidylglycerol (PG), diacylglycerol (DAG), and triacylglycerol (TAG) in overweight participants.

column has higher reproducibility and enhances the discoverability of low-abundance metabolites. Through statistical analyses, we identified the differences between overweight and normal participants; 26 potential lipids with significant ($p < 0.05$) changes were identified in samples extracted from overweight participants, and the results concurred with those of previous studies. Therefore, we recommend the use of a PFP column in high-throughput metabolomics to promote the development of basic biological and clinical research in the future.

EXPERIMENTAL

Chemicals and reagents

Betaine and hexakis(1*H*,1*H*,3*H*-perfluoropropoxy)-phosphazene (HP-0921) (which served as the internal standard) were purchased from Apollo (Graham, NC, USA). American Chemical Society (ACS)-grade or LC-MS-grade methanol, acetonitrile, and chloroform were obtained from J. T. Baker (Philipsburg, NJ, USA). Sodium chloride (NaCl) was purchased from Merck (Darmstadt, Germany). The water used in the LC system was purified using a Milli-Q system (Millipore, Milford, MA, USA) at a resistivity of >18.2 MΩ·cm. LC-MS grade ammonium acetate (NH_4OAc) and formic acid (HCOOH) were acquired from Sigma–Aldrich (St. Louis, MO, USA).

Biological material and sample preparation

This study was approved by the Ethics Review Board of University Sabah Malaysia (REF: JKEtika 1/15 (7)). Blood and urine samples were obtained from deidentified donors after informed consent was obtained. Urine samples were collected and treated as described by Contrpois *et al*. but with a marginal modification.[15] Briefly, 200 μL of the urine sample was immediately desalted using 800 μL of methanol and centrifuged at 15000 *g* for 10 min at 4°C. The supernatant was transferred and evaporated using a Speedvac concentrator system. The dried sample was stored at −80°C. Before analysis, the dried urine sample was reconstituted using 400 μL of methanol admixed with the internal standards.

Serum was prepared using whole blood coagulated at a low temperature (4°C). The coagulated blood was centrifuged at 2500 *g* for 15 min at 4°C. The supernatant was collected for extraction. The extraction protocol for serum was a modified version of the Bligh and Dyer extraction protocol.[1,50] Briefly, the supernatant was relocated into glass tubes and treated with a chloroform/methanol/NaCl (0.9%) in water (1:1:1 v/v/v) solvent mixture for polarity-based metabolite extraction. The upper and lower layer of the mixtures were transferred to separate tubes and evaporated to dryness. The dry extracts were reconstituted with methanol, admixed with the internal standards, and stored at −80°C until further analysis. These internal standards served to correct variations in the signal response during analysis and aided signal normalization to adjust the variations between each sample run.

LC-QTOF acquisition

The extracted urine and serum samples were analyzed using an Agilent 1260 infinity HPLC system coupled with an Agilent 6520 Q-TOF MS system. A 1.5-μL sample was injected into the following three analytical columns separately: Kinetex C_{18}, Kinetex F5 (2.1 mm × 100 mm × 2.6 μm; Phenomenex, Torrance, CA, USA), and Acquity HSS T3 (2.1 mm × 100 mm × 3.0 μm; Waters, Milford, MA, USA). All analytical columns used in this study were RP columns. Therefore, the same gradient program was applied for all columns. The columns were maintained at 35°C at a flow rate of 350 μL/min during analysis. The mobile phases were composed of solvent A (H_2O–0.1% HCOOH–1% 10 mM NH_4OAc) and solvent B (acetonitrile/methanol [6:4 v/v] – 1% of 0.1% HCOOH–1% 10 mM NH_4OAc). The gradient elution program was initiated from 1% to 70% solvent B in 7 min, followed by 100% solvent B from 7.1 to 10 min and maintained for 3 min. Later, the columns were conditioned with the initial gradient for 3 min before the next sample was injected.

The data acquisition was set between an *m*/*z* of 100 and 1500. Positive and negative heated electrospray ionization (ESI) were deployed at 3500 and −3500 V, respectively. The ion source conditions were set as follows: gas temperature of 325°C, drying gas flow at 4 L/min, and nebulizer flow at 45 psig. The mass spectrometer was calibrated with Tune Mix (Sigma–Aldrich, St Louis, MO, USA) before each batch analysis. Internal mass calibration standards betaine and hexakis (1*H*,1*H*,3*H*-tetrafluoropropoxy) phosphazine were introduced during the runs. In the positive ion mode, the internal mass calibration standards were *m*/*z* of

121.0509 and 922.0098, respectively, whereas in the negative ion mode the corresponding values were *m/z* of 112.9856 and 1033.9881 (TFA adducted HP-0921).

Data analysis

Köfeler *et al*. considered every spectrum in a TIC to be a single event with unique matrix effects and solvent composition; this caused difficulties in standardizing metabolite quantitation.[51] The quality of the metabolite peaks acquired from the three RP columns was evaluated using the criteria proposed by Contrpois *et al*. with modifications. Briefly, metabolic features (characterized by a unique *m/z* and retention time) were extracted using the MassHunter Qualitative Analysis Software B 05.00 (Agilent Technologies, Santa Clara, CA, USA), followed by evaluation of the peaks that corresponded to peak quality, including peak width, asymmetries, kurtosis, and overall spectral signal intensities. Intraday precision and accuracy were measured for the selected metabolites by analyzing five extracted samples on the same day (n = 5), whereas the interday values were determined in triplicate per sample on three consecutive days (n = 15).

Metabolomics was conducted for comparing the metabolites extracted from participants with normal BMI and overweight participants. The metabolic features of the participants were extracted using MZmine 2 to compensate for retention times drift during each analysis run.[52] These preprocessed chromatograms were exported as a peak list table in the comma separated values format. The acquired datasets were further analyzed through multivariate methods by using MetaboAnalyst 3.0.[53] Principal component analysis (PCA), an unsupervised–supervised pattern recognition technique, was carried out on all log-transformed and Pareto-scaled datasets to obtain an overview of the data distribution and to identify potential outliers.[40] Through PCA, the direction of the variances in the dataset could be identified without relating to the class labels (i.e., it summarized the original variables to fewer variables on the basis of weighted averages).[40] PLS-DA, a supervised pattern recognition technique, was performed for inspecting the internal relationships between the matrix X variables and response matrix Y. The quality of the PLS-DA model was evaluated using residuals (R^2), and the model predictability parameter (Q^2) was determined using MetaboAnalyst 3.0. Throughout the analysis, VIP was conducted for estimating the weightage of each variable in the projection used in the selected PLS-DA model, and the score threshold was set to 1.5; the variables with values higher than this threshold were selected for additional analysis. The *t*-test was performed using Statistical Package for Social Sciences (SPSS; IBM corporation, Armonk, NY, USA). The putative features of the identified *m/z* were further fragmented and matched against METLIN with a mass accuracy of ±5 ppm.

Each class of polar lipids exhibits a unique fragmentation pattern. Throughout molecular fragmentation, we could identify the lipids' classes. During metabolite matching, molecular fragmentation was conducted on significantly ($p < 0.05$) perturbed metabolites (polar lipids), and the fragmentation patterns were matched with the Lipid Metabolites and Pathway Strategy (LIPIDMAPS) at a mass accuracy of ±5 ppm.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Higher Education, Malaysia (FRGS0428-SG-1/2015 and TRGS0006-SG-2/2014), and Universiti Malaysia Sabah (SBK0180-SG-2014).

Supporting information

Additional supporting information is available in the online version of this article.

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