A Mass Spectrometric Study for Comparative Analysis and Evaluation of Metabolite Recovery from Plasma by Various Solvent Systems

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A solvent system that extracts a maximum number of metabolites belonging to diverse chemical classes from complex biofluids, such as plasma, may offer useful inputs to understand the metabolic and physiological state of an individual. The present study compared seven solvent systems for extraction of metabolites from plasma. The extracts were analyzed by mass spectrometry (MS) and MS/MS (MS2) using a quadrupole time-of-flight liquid chromatography/MS system in positive and negative modes of ionization. Metabolites with molecular mass below 400 were identified using Human Metabolome Database MS2 and MS search interfaces. The acetone/isopropanol (2:1) system yielded promising results in positive ionization mode, as the maximum number of MS and MS2 features was detected in the extract. It was found to be superior in extraction of various classes of metabolites, especially organic acids, nucleosides and nucleoside derivatives, and heterocyclic molecules. Glycerophosphocholines in the mass range of 400–700 were found to be efficiently extracted by the methanol/chloroform/water (8:1:1) system. In negative mode as well, the maximum number of MS2 features was detected in methanol/chloroform/water and acetone/isopropanol extracts. The fingerprints of molecular features obtained in the negative and positive modes differed from each other to a significant extent.

KEY WORDS: Q-TOF LC/MS, differential extraction by solvents, Human Metabolome Database, phospholipids, negative ionization, organic acids

INTRODUCTION

Small molecules typically below 1500 daltons comprise the metabolome of the biofluids of a tissue.¹ As the end products of cellular regulatory processes, levels of these metabolites reflect the response of cells to genetic and environmental influences. NMR spectroscopy is being used effectively for metabolic fingerprinting. As sensitivity of this technique is low in comparison with mass spectrometry (MS),² liquid chromatography coupled to MS (LC/MS) has gained momentum as a technique of choice in the recent past for analysis of the metabolites in complex biological samples.

The enormity of the metabolome remains a subject of debate, as numbers ranging from a few thousand to tens of thousands of small molecules have been proposed to exist in blood.³ As it may not be necessary to determine the individual level of every single endogenous metabolite in a

biofluid, a “fingerprint” of a biofluid may provide the necessary information. As a result of the large differences in the chemical and physical properties of metabolites, selection of the appropriate extraction solvent needs to be given prime importance. As a possibility of metabolites sharing an almost-identical mass-to-charge ratio (m/z) exists, it is often necessary to use a MS/MS (MS2)-based approach for identification of the metabolites. Gas chromatography (GC)/MS2 has enabled the acquisition of very detailed fragmentation information and a higher level of molecular specificity and selectivity. Most naturally occurring metabolites lack volatility and hence, are not amenable to direct analysis by GC/MS.⁴ LC/MS offers a more comprehensive coverage of polar, less-polar, and neutral metabolites. Although, normal-phase and reversed-phase (RP) matrices have been used in metabolomics, RP matrices, such as C18 and C8, are used most extensively.⁵ MS, in positive and negative mode, may further increase the coverage of metabolites.⁶

Plasma contains a wide array of chemically diverse compounds present over a broad range of concentration, and of these, many are known to be noncovalently bound to proteins.⁷ The extraction solvent may play an important

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TABLE 1

Solvent Systems Used for Extraction of Metabolites		
Extraction system	Solvent composition	Ratio
A	Acetone/isopropanol	2:1
B	Methanol	1
C	Methanol/chloroform/water	5:2:2
D	Methanol/acetone/water	7:7:2
E	Methanol/chloroform	1:2
F	Methanol/chloroform/water	8:1:1
G	ACN/water	7:3

role in stripping off the metabolites from its bound form. Review of literature provides a wide array of extraction solvent mixes that have been used for extraction of metabolites from biofluids and tissues.^{8–13} The present work used seven different solvent systems for extraction and analyzes the metabolites recovered from plasma.

MATERIALS AND METHODS

Collection of Sample

Fresh EDTA anticoagulated blood collected from four healthy male donors was centrifuged at 3000 rpm for 10 min at 4°C. The plasma samples were pooled and stored at –80°C, and the work was carried out at the Department of Biotechnology, Manipal Life Sciences Centre, Manipal, Karnataka, India. All of the subjects involved have given informed consent.

Sample Preparation

Solvent systems used for extraction of metabolites are shown in Table 1. To 60 µl plasma, 500 µl chilled, sonicated extraction solvent was added. The mixture was sonicated for 1 min and incubated in ice for 30 min. In addition to its role in deaeration, sonication is found to increase the efficiency of extraction.⁴ Ultrasonic waves have also been reported to induce aggregation of proteins, which may aid in deproteinization of the sample.¹⁴ After centrifugation at 13,000 rpm for 10 min at 4°C, the deproteinized solution (organic layer in case of Solvent C) was collected and concentrated to dryness. The dried extract was stored at –80°C until use. Blanks were prepared by concentrating 500 µl of the respective solvents to dryness. For MS analyses, dried mixtures were reconstituted in 30 µl 50% acetonitrile (ACN; sonicated just before use) containing 0.1% formic acid and immediately loaded for a MS run.

Chromatographic and MS Conditions

MS and MS2 analyses were performed by LC electrospray ionization time-of-flight (LC-ESI-TOF) MS and LC ESI

quadrupole TOF (Q-TOF), respectively, with the use of the Accurate-Mass Q-TOF LC/MS (Agilent 6520) system. Chromatographic separation of the analytes was done using C18 Agilent Zorbax, 3 µM (4.6×150 mm) column. For the positive ion mode, the mobile phases used were as follows: A (water with 0.1% formic acid) and B (90% ACN with 0.1% formic acid). MS analyses in the negative ionization mode were performed with 10 mM ammonium acetate and 90% ACN as mobile phases A and B, respectively. The gradients were set as given in Fig. 1.

Collision energy was provided at the rate of 6 V/100 Da (offset –2). The run was performed at a rate of 500 µl/min and infused into the ion spray source held at 3.5 kV. Nitrogen was used as the drying gas at 350°C at a flow of 10 l/min. Nebulizer pressure and fragmentor volt were maintained at 35 psig and 110 V, respectively. Spectral peaks in the mass range of 50–900 were acquired for MS and MS2 studies. Nitrogen was used for collision-assisted dissociation (CID) of the precursor ions. Precursors for CID were selected on the basis of abundance. A maximum of four precursors was selected/cycle and fragmented at energy of 4 V/100 Da (offset –2). Each run was separated by a blank run serving to wash and re-equilibrate the column prior to the next run.

Bioinformatics Analysis of MS Data

The spectral data were acquired using the Agilent Mass Hunter acquisition software, and the features were extracted from raw data files using the molecular feature extraction algorithm of the Mass Hunter qualitative analysis software. It was observed that some compounds present in blank were not getting removed from the final compound list by the blank subtraction feature present in the Qualitative Analysis Software.

These base peaks were not eluting throughout the run and hence, were not being identified and subtracted by the software. Using R script, we were able to subtract these compounds, and the final compound list was obtained for each run.

The MS2 spectra obtained by the Mass Hunter data

FIGURE 1

Gradient program used for liquid chromatography.

TABLE 2

Solvent Systems from Researchers around the World					
Solvent system	A	B	C	D	E
Reference	10	12,17	8,9	18	11,19

acquisition software was exported in the m/z data format (mzdata) and converted to Mascot Generic Format (mgf) using the mzdata-to-mgf convertor downloaded from the website of the Centre of Excellence in Computational Systems Biology at The University of Western Australia (<http://www.ce4csb.org/software.shtml>). A perl script was used to match the precursor ion list obtained from XCMS2^{ten} against the mgf format data to obtain the list of precursor ions with an abundance of >2000 and their corresponding fragment ions. Considering the percentage abundance as 100% for the fragment ion exhibiting maximum abundance, the perl script was used to calculate the relative abundance percentage for each of the fragment ions. MS2 data file for the precursor ion found to be common in replicate files was uploaded into the online Human Metabolome Database (HMDB) MS2 search interface for metabolite identification.^{15,16} Tolerance levels of ± 0.04 Da were permitted for parent and fragment ions. For MS search in HMDB interface (http://www.hmdb.ca/search/spectra?type=ms_search), a MW tolerance of ± 0.02 Da was used. The metabolite with H^+ as an adduct ion was considered for identification. MS search in Mass Scripps Metlin Database (<http://metlin.scripps.edu>) in simple or batch was also performed to gain additional information.

RESULTS

Researchers across the globe have reported various solvent systems for the extraction and study of small molecules from biological samples (Table 2).

Reports on extraction of nucleotides, drugs, their derivatives, and other metabolites from biological specimens using 50–70% ACN are available.^{13,20–22} Methanol/chloroform/water (8:1:1) is being used in our laboratory for the study of amino acids in plasma (unpublished results). All of these solvent systems were used, and the resultant extracts were subjected to MS and MS2 analyses.

Table 3 represents the results acquired for triplicate MS analyses in positive mode.

The acetone/isopropanol system (Solvent A) extracted a maximum number of molecular features, followed by methanol/chloroform (Solvent E). Although elemental analysis based on accurate m/z determination by single MS has been used for identification of probable metabolites in body fluids, tandem MS provides a more reliable and accurate characterization of the metabolome in a sample.^{17,23,24} The extracts were subjected to MS2 in positive ionization mode, wherein the precursor ions were selected based on abundance for CID. As shown in Table 3, acetone/isopropanol extract proved its superiority by recovering a maximum number of features. Solvent C, i.e., methanol/chloroform/water (5:2:2), showed an average of only 425 MS features, indicating poor recovery of metabolites in the organic (nonpolar) layer. Aqueous layer was not studied, as it was slightly hazy in appearance. The acetone/isopropanol system effectively extracted small molecules in the m/z range of 100–300. Cholesterol derivatives, especially steroid hormones, play significant roles in human physiology, and most of them have molecular masses in the range of 300–400. Solvents A and B showed good recovery of metabolites in this range. A scan through HMDB reveals that most of the phospholipids (PLs; i.e., diacylated PLs) possess masses >600 , predominantly above 700 Da. The maximum number of metabolites with m/z values >700 was recovered by Solvents E and C.

It was found that a number of features common to

TABLE 3

Comparative Analysis of MS Features and Distribution of MS2 Features Extracted by Various Solvents							
Solvent	Number of Features						
	A	B	C	D	E	F	G
MS	1474 $\pm$ 93	1156 $\pm$ 87	425 $\pm$ 76	1194 $\pm$ 131	1381 $\pm$ 67	1274 $\pm$ 70	1193 $\pm$ 12
MS2, m/z							
50–100	1.5 $\pm$ 0.7	1.5 $\pm$ 2.1	2 $\pm$ 1.4	2.5 $\pm$ 0.7	1.5 $\pm$ 0.7	2.0	3.5 $\pm$ 0.7
100–200	88 $\pm$ 1.4	71.0	77.5 $\pm$ 2.1	85.5 $\pm$ 4.9	72.5 $\pm$ 7.7	72.5 $\pm$ 0.7	76 $\pm$ 1.4
200–300	73 $\pm$ 2.8	72 $\pm$ 5.6	59.5 $\pm$ 2.1	66 $\pm$ 1.4	53 $\pm$ 2.8	62.5 $\pm$ 0.7	63.5 $\pm$ 4.9
300–400	54.5 $\pm$ 0.7	50.5 $\pm$ 7.7	45.5 $\pm$ 4.9	38 $\pm$ 0	41.5 $\pm$ 2.1	42 $\pm$ 2.8	44 $\pm$ 1.4
400–500	56 $\pm$ 5.6	43.5 $\pm$ 3.5	40 $\pm$ 1.4	47.5 $\pm$ 2.1	37.5 $\pm$ 3.5	48 $\pm$ 1.4	45 $\pm$ 1.4
500–700	57.5 $\pm$ 0.7	47.5 $\pm$ 10	38 $\pm$ 2.8	38 $\pm$ 1.4	30 $\pm$ 4.2	47.5 $\pm$ 2.1	41 $\pm$ 1.4
700–900	38.5 $\pm$ 4.9	24 $\pm$ 4.2	48.5 $\pm$ 3.5	22 $\pm$ 0	53.5 $\pm$ 0.7	24.5 $\pm$ 3.5	40.5 $\pm$ 2.1
Total	369 $\pm$ 15.6	310 $\pm$ 29.7	311.5 $\pm$ 12	299.5 $\pm$ 7.8	289.5 $\pm$ 16	299 $\pm$ 7.1	313 $\pm$ 8.5

replicate files decreased with increasing m/z . Sixty-three percent to 83% of the features with $m/z < 400$ were common to replicate files (Table 4). However, only 30–42% of the features possessing $m/z > 400$ were found to replicate in the files. A fragment ion list obtained for each of the common features with m/z below 400 was therefore manually entered into the online HMDB MS2 search interface for metabolite identification. The identification was done by correlating the m/z value and abundance of the fragment ions against the MS2 spectra of the metabolites available in the open source HMDB, a comprehensive metabolomics database that integrates quantitative, chemical, physical, and biological data of thousands of endogenous human metabolites.²⁵ A total of 63–72% of the metabolites could be identified by a MS2 search in the various extracts, with the exception of Solvent C, from which ~57% of the metabolites were identified. More than 65% of these metabolites were identified with the Fit% of > 0.7 (similar results were observed for RFit% and Purity%). Fit score indicates how well the masses and intensities of the library spectrum agree with those found in the acquired spectrum, and RFit signifies the agreement of the masses and intensities of the acquired spectrum with those found in the library spectrum. Purity score is based on

the agreement of library spectrum and the acquired spectrum with each other. As the MS2 spectrum for not all plasma metabolites is available in the repository, identification of the unidentified MS2 features was done by a MS search. Precursor ions of m/z 204.122, 218.14, and 342.26, identified as L-acetylcarnitine, propionyl carnitine, and trans-2-dodecenoylcarnitine by MS search, showed predominant presence of a fragment ion of m/z 85.03 (60–100% intensity) in a MS2 scan, which is characteristic of carnitine,²⁶ and hence, these molecules were included in the list of compounds identified by MS2 search. Tyrosine was also included in the list, as the MS2 scan for the precursor ion of m/z 182.08 yielded a strong signal (100% intensity) for the immonium ions at m/z 136 and a comparatively weak signal (22% intensity) for m/z 91, characteristic of a tyrosine molecule. Fragmentation profiles of tyrosine and phenylalanine standards were comparable with the respective features identified in plasma. A list of the identified compound from the various extracts is shown in Supplemental Table 1. The identified compounds were grouped into different super classes as per the HMDB metabocard. The summary of the categorization is displayed in Table 4. Solvent A could recover metabolites belonging to all of the classes to a satisfactory extent. Its

TABLE 4

Summary of Metabolite Identification by MS2 Search in Positive Mode

Solvent	Number of MS2 Features ($m/z < 400$)						
	A	B	C	D	E	F	G
Total extracted (average)	217	195	185	192	169	179	187
Common to replicate files, <i>CF</i>	160	122	153	125	112	131	122
Super class							
Amino acids and conjugates	36	36	32	36	31	38	34
Organic acids	18	13	18	17	16	16	15
Nucleosides and conjugates	11	4	6	7	5	4	3
Carbohydrates and conjugates	7	7	6	3	4	5	5
Alcohols	5	6	7	6	6	7	6
Ketones and aldehydes	6	3	5	4	3	3	5
Cholesterol and derivatives	5	7	5	6	3	5	5
Heterocyclic molecules	8	8	4	4	6	7	6
Amines	1	0	1	0	0	0	2
Miscellaneous	4	4	3	3	3	4	4
MS2 features identified							
by MS2 search, <i>MF</i>	101	88	87	86	77	89	85
% Identified by MS2 ^a	63.1	72.1	56.9	68.8	68.8	67.9	69.7
LogP hydrophobicity (average) ^b	−0.59	−0.5	−0.58	−0.53	−0.53	−0.59	−0.41
Polarity index of extract ^c	5.16	5.5	↓ ^d	5.45	4.92	5.78	6.99

^aCF is the number of features common to replicate files and MF is the number of features identified by MS2 search; ^a(MF/CF) × 100. ^bSum of predicted LogP hydrophobicity values of metabolites identified by MS2 search/MF. ^cCalculated by values adapted from The HPLC Solvent Guide, Wiley-InterScience, 2002 (<http://www.perfinity.com/downloads/Solvent%20Miscibility%20and%20Polarity%20Chart.pdf>). ^dOrganic phase of methanol/chloroform/water (5:2:2) extract will be rich in chloroform and hence, is relatively less polar.

superior extractive efficiency is especially evident in the extraction of organic acids, nucleosides and derivatives, heterocyclic molecules, and carbohydrates and conjugates. Compounds belonging to the class amino acids and conjugates were identified maximally in the extracts obtained using Solvents F, A, and B. Cholesterol and derivatives include several steroid hormones of clinical significance. Solvent B showed a maximum number of features classified into this group. It is interesting to note the distinctive difference in extractive abilities of Solvents A and G (ACN extract) in the recovery of nucleosides and nucleoside conjugates. The maximum number of these molecular species was identified in Solvent A, and the least number was found in ACN extract. The predicted LogP hydrophobicity values were found from HMDB for all of the metabolites identified by the MS2 search. The average value of hydrophobicity for the metabolites extracted by each solvent and the polarity of each solvent sample system are shown in Table 4. Solvent G was the most polar of the solvents used, and Solvents A, B, D, and F are moderately polar. Extracts obtained using Solvents C and E have low polarity. Theoretically, it would be expected that Solvent G would recover hydrophilic metabolites to a great extent. Our results, however, showed that relatively more hydrophobic metabolites were extracted in Solvent G. ACN is known to disturb hydrophobic interactions and strip the small molecules bound to albumin.²⁷ This could perhaps be one of the reasons for the observed results. Metabolites on the lower side of hydrophobicity scale were identified to a greater extent in the extracts of A, C, and F. Many small molecules bind reversibly to plasma proteins. The nature of the carrier proteins and the interactions involved in binding may influence the recovery of such metabolites. Consequently, the extractability of the molecule in its free form may differ from that of the bound form. Thus, it appears that extractability of the metabolites cannot be predicted based solely on their hydrophobicity values. It does, however, appear that the solvent systems, which exhibit moderate polarity, are better extractants of plasma metabolites having $m/z > 400$. Overall, Solvent A was found to be the best solvent. Its excellent extractive capability was especially evident in the case of nucleosides and nucleoside derivatives.

We observed precursor ions above m/z 400, eluting predominantly after 8.5 min of the run when %ACN in the mobile phase reached above 75%. MS search (multiple MWs entered in one search) in HMDB showed that the MW of the majority of the features matched with lipid derivatives, especially glycerophospholipids (GPLs). The majority of these GPLs in the range of m/z 400–700 were

phosphocholine (PC) and phosphoethanolamine (PE) derivatives. PCs are the predominant form of PLs found in blood. A positively charged choline group in glycerophosphocholines (GPCs) renders them ionizable in positive mode, and hence, PCs are preferentially detected to a great extent in positive mode when compared with the PLs, which are relatively more acidic.²⁸ CID of GPCs is known to typically yield product ions of m/z 104 and 184, representing $C_5H_{14}NO^+$ and $C_5H_{15}NPO_4^+$ fragments, respectively.^{26,29} Fragment ion of m/z 86 corresponding to $C_5H_{12}N^+$ has also been reported in the MS for GPCs.^{30,31} Precursor ions with m/z above 400, yielding ions diagnostic of GPCs, eluted predominantly after 13.8 min of the LC run. PCs generally possess molecular mass > 700 . LysoPCs are the PCs that have lost one of the two O-acyl chains, and they possess molecular masses in the range of 400–650. The majority of the features yielding fragment ion of 104 was in the m/z range of 400–700 (Fig. 2). MS2 for LysoPCs typically exhibiting the mass fragment of 104 have been reported.^{30,32} Solvent F appeared efficient in extracting these molecular species. One must be cautious while interpreting results based on a single mass fragment. Hence, the files were analyzed for features exhibiting fragment ions of m/z 184 and 86. Distribution of the number of features yielding these product ions between the two mass ranges (400–700 and > 700) was not significantly different, and hence, the overall number of these features is presented in Fig. 2. Solvent F appeared to extract the GPCs efficiently, as the extract showed the presence of a maximum number of precursor ions yielding product ions characteristic of GPCs, followed by Solvents G and A. GPLs, especially PLs, are highly hydrophobic. It is surprising that Solvents G and F, which are relatively more polar, were able to extract PCs efficiently. The am-

FIGURE 2

Extraction of precursor ions showing GPC-specific fragmentation. Precursor ions with i) m/z 400–700 yielding fragment 104.1; ii) m/z > 700 yielding fragment 104.1; iii) m/z > 400 yielding fragment 184; iv) m/z > 400 yielding fragments 104.1 and 184; and v) m/z > 400 yielding fragments 86 and 104.1 and 184.

FIGURE 3

Comparative analysis of MS and MS2 features analyzed in negative mode of ionization. Number of features with m/z between 50 and 900 extracted by (A) LC-MS and (B) LC-MS2.

phipathic nature of the PCs probably aids in their extraction by these solvents. Various chemical modifications of the PLs occur in tissues. These modifications may alter the chemical nature of the molecules, thereby affecting their extractability. One must also remember that GPLs in circulation are in free form or are bound to proteins. The solvent system should be able to disrupt the interactions between the bound GPLs and the proteins and further extract them into the soluble phase. A MS2 scan of C and E extracts showed the lowest number of ions fragmenting to yield GPC-specific fragmentation. A MS search of the MS2 features ($m/z > 700$) acquired in Extracts C and E showed that of the metabolites identified, $>80\%$ matched to the masses of PLs, i.e., PCs, phosphatidylglycerolphosphates (PGPs), PEs, phosphatidylinositols (PIs), and others. Interestingly, the MS2 files of these extracts showed presence of 20–24 precursor ions possessing m/z 700–850, continuously being eluted with retention time ranging between 8.5 and 9.3 min. These features showed poor fragmentation. Anionic PLs, such as PGPs and PIs, may fragment to a limited extent as a result of their poor ionization efficiency during the run in positive mode. It should also be noted that C and E extracts showed the presence of a maximum number of features possessing $m/z > 700$ (Table 3). As these extracts showed the least number of fragments char-

acteristic of GPCs, the possibility of the relatively acidic PLs being recovered to a greater extent in these extracts cannot be ruled out.

A critical role played by solvents in extraction of compounds from complex tissues is well known.^{33,34} Many metabolites, especially sugars and organic acids, may still remain unionized and may not get detected in the positive mode. Ionization in negative mode may help in the detection of these molecular species.³⁵ Hence, further efficacy of extraction systems was investigated by subjecting the extracts to MS and MS2 analysis in the negative mode. As shown in Fig. 3, an acetone/isopropanol mixture extracted a maximum number of MS features. A MS2 scan showed very poor fragmentation at the collision energy of 4 V/100 Da (offset -2). Fragmentation was hence performed at 6 V/100 Da (offset -2). Solvents F, i.e., methanol/chloroform/water (8:1:1) that had been developed in the laboratory for study of amino acids in blood (unpublished results), and A (acetone/isopropanol mixture) showed a maximum number of MS2 features. The data were extracted from these raw files (Solvents A and F), and the fragmentation data of 20 precursors ($m/z < 400$) showing satisfactory fragmentation, i.e., more than two fragments with at least one fragment exhibiting $>10\%$ intensity from each file, were randomly selected for metabolite identification by MS2 search in the negative mode of ionization. The MW of the parent molecule was obtained by adding a molecular mass of 1 Da to the m/z of a precursor ion. Out of the 40 ions selected, only 20 could be identified. The results are as given in Table 5. None of these metabolites was present in the list of features identified in the positive mode. Of these, 16 compounds belonged to the class of organic acids as per HMDB. This compelled us to compare the molecular masses of the MS2 features acquired for each extract in negative and positive modes. Eighty-seven percent to 92% of the molecular masses in the negative mode

TABLE 5

Metabolites Identified by MS2 Search in Negative Mode	
Adipic acid	3-Mercaptopyruvic acid
Atrolactic acid	Stearic acid
4-Hydroxybenzoic acid	Succinic acid
2-Hydroxy-3-methylbutyric acid	Tetradecanedioic acid
Caprylic acid	Vanillic acid
Gentisic acid	Indoxyl sulfate ^a
L-Lactic acid	Dehydroascorbic acid ^b
Myristic acid	Allose ^b
Trans-2-octenoic acid	Homovanillic acid ^c
Orotic acid	Homoveratric acid ^c

^aHeterocyclic molecules; ^bcarbohydrates; ^calcohols.

files could not find similar matches in the respective positive mode files.

DISCUSSION

Fiehn and Kind¹⁰ used acetone/isopropanol as the extraction system for profiling of plasma metabolites by the GC-MS method. The volatility of the polar metabolites needs to be enhanced by derivatization, prior to GC analysis. Derivatization of metabolites in a biofluid may add to the complexity of the sample rendering the analysis very challenging. The LC/MS method therefore serves as a powerful alternative. There is no unique extraction and analytical protocol for plasma or serum metabolome analysis that will cover all of the metabolites present in the sample. A combination of techniques, including LC-MS2, GC-MS, TLC-GC-flame-ionization detection, direct infusion MS2, and NMR, could identify tentatively 4229 from human serum.⁵ When complex samples are subjected to LC-MS2 analysis, a large number of molecules tend to coelute from the LC column. The molecules are ionized, and these ions enter into the first mass analyzer and migrate based on their *m/z*. A defined number of precursor ions are selected/cycle based on abundance for CID, and hence, the metabolites of high abundance may mask the detection of metabolites of lower abundance. In precursor-rich regions, a significant number of features may remain undetected. This issue can be addressed to an extent by changing the gradient conditions or by extending the run time, especially in the regions where an extracted ion chromatogram displays broad, prominent peaks. In proteomics studies, the coverage can be improved by choosing an alternative option, wherein precursors can be selected based on their various charge states. Although laborious and time-consuming, multiple LC-MS2 runs based on alternative principles of separation can maximize the metabolome coverage.

For any of the analytical strategies to give optimum results, it is essential that the sample represents the tissue of its origin to the maximum possible extent. In the present investigation, our main goal was to find an extraction system that will extract the maximum number of molecules encompassing various classes of the metabolites. The acetone/isopropanol mixture proved its efficacy as an extraction solvent for LC-MS studies in positive and negative modes in MS as well as MS2 modes. From the huge plethora of small molecules present in plasma, we could uncover only a small fraction of features using the defined conditions. Few of the metabolites, such as orotic acid, which was identified in the negative mode in the acetone/isopropanol extract at a Fit% >0.95, are of metabolic significance, as their level is reportedly elevated in many inborn errors of the urea cycle disorders and other disorders

involving arginine metabolism.³⁶ Similarly, succinyl acetone, detected in the positive mode with a Fit% >0.9, is a pathognomonic marker for hepatorenal tyrosinemia if found in elevated levels.³⁷ Nine different fatty acyl carnitines were identified in the MS2 scan, and all of the solvents were able to extract seven to eight of these molecules. An altered level of carnitines is implicated in fatty acid disorders.³⁸ A newborn screening program by MS2 generally uses triple-quadrupole analyzers, wherein target molecules are selected and analyzed.^{39,40} ESI-Q-TOF of the affected samples may further provide information about additional metabolites for gaining useful inputs about the disease. A GC-MS-based study in pathogenic *Corynebacterium* species⁴¹ and other reports highlight the clinical importance of plasma PL fingerprints in liver and cardiovascular disorders.^{42,43} Our study showed that Solvent F extracted GPCs very efficiently.

The molecular masses obtained in negative mode differed significantly from that acquired in positive mode. This emphasizes the need for analyses in both of the modes to get a broader picture and significant perspective of the metabolome.

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