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Phospholipid capture combined with non-linear chromatographic correction for improved serum metabolite profiling

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Serum analysis with LC/MS can yield thousands of potential metabolites. However, in metabolomics, biomarkers of interest will often be of low abundance, and ionization suppression from high abundance endogenous metabolites such as phospholipids may prevent the detection of these metabolites. Here a cerium-modified column and methyl-tert-butyl-ether (MTBE) liquid–liquid extraction were employed to remove phospholipids from serum in order to obtain a more comprehensive metabolite profile. XCMS, an in-house developed data analysis software platform, showed that the intensity of existing endogenous metabolites increased, and that new metabolites were observed. This application of phospholipid capture in combination with XCMS non-linear data processing has enormous potential in metabolite profiling, for biomarker detection and quantitation.

KEY WORDS: phospholipid; metabolite profiling; liquid chromatography; mass spectrometry; serum.

1. Introduction

Biofluids such as serum are complex matrices, comprising tens of thousands of components. This complexity requires sophisticated separation techniques in order to obtain comprehensive spectral information about individual compounds. One of the most commonly employed techniques in metabolite profiling is liquid chromatography mass spectrometry (LC/MS) (Weckworth, 2003; Guttman *et al*., 2004; Shen *et al*., 2005a, b; Wilson *et al*., 2005; Yang *et al*., 2005). This approach can reduce ion suppression by decreasing the number of competing analytes entering the mass spectrometer ion source at any one time (Siuzdak, 2003).

However, the high serum concentration of readily ionizable compounds such as the phospholipids, highly polarized molecules with an ionic headgroup, causes significant ionization suppression of lower abundance species (Bennett and Van Horne, 2003; Meng and Bennett, 2004; Bennett and Liang, 2004; Van Horne *et al*., 2004; Shen *et al*., 2005a, b). Phospholipids are a key part of membrane bilayers and phosphatidylcholine is the principal phospholipid circulating in human plasma, where it is an integral component of the lipoproteins (Phillips and Dodge, 1967). In fact, endogenous phospholipids such as lysophosphatidylcholine dominate the LC chromatogram of methanol-extracted hum an serum (figure 1). Phospholipid metabolites include lysophospholipids such as lysophosphatidylcholine and lysophosphatidic acid, which stimulate cell signaling and influence cell proliferation, migration and survival and so are important pathophysiologically in humans and other animals (Moolenaar *et al*., 2004; Sigal *et al*., 2005). However, despite being known biomarkers for certain diseases (Sasagawa *et al*., 1998; Xu *et al*., 2003; Mills and Moolenaar, 2003; Sutphen *et al*., 2004; Walter *et al*., 2004), phospholipids and their metabolites are not always desirable in the metabolite profile, as they could mask less abundant metabolites that may be potential biomarkers. A key challenge therefore is removing this class of molecules, whilst leaving the rest of the metabolite profile intact.

Common sample preparation techniques in metabolite profiling involve protein precipitation through the addition of an organic solvent such as methanol or acetonitrile, liquid–liquid extraction (LLE) or acid protein precipitation (Khan *et al*., 1992; Vanholder *et al*., 1992; Janson and Rydâen, 1998; Creighton, 1983; Van Oss, 1989; Sivaraman *et al*., 1997; Daykin *et al*., 2002; Polson *et al*., 2003; Want *et al*., 2006). However, these approaches are not particularly effective at removing phospholipids. In order to determine its applicability to metabolite profiling, we investigated the removal of phospholipids (here termed phospholipid capture or PLC) from human serum and the resulting effect on the detection of endogenous metabolites. Chemical sorbents containing lanthanide metal active surface elements have been demonstrated to be successful at removing most endogenous phospholipids (Meng and Bennett, 2004). Van Horne *et al*. have shown that almost 99% of phospholipids were removed from serum using cerium-modified columns, while

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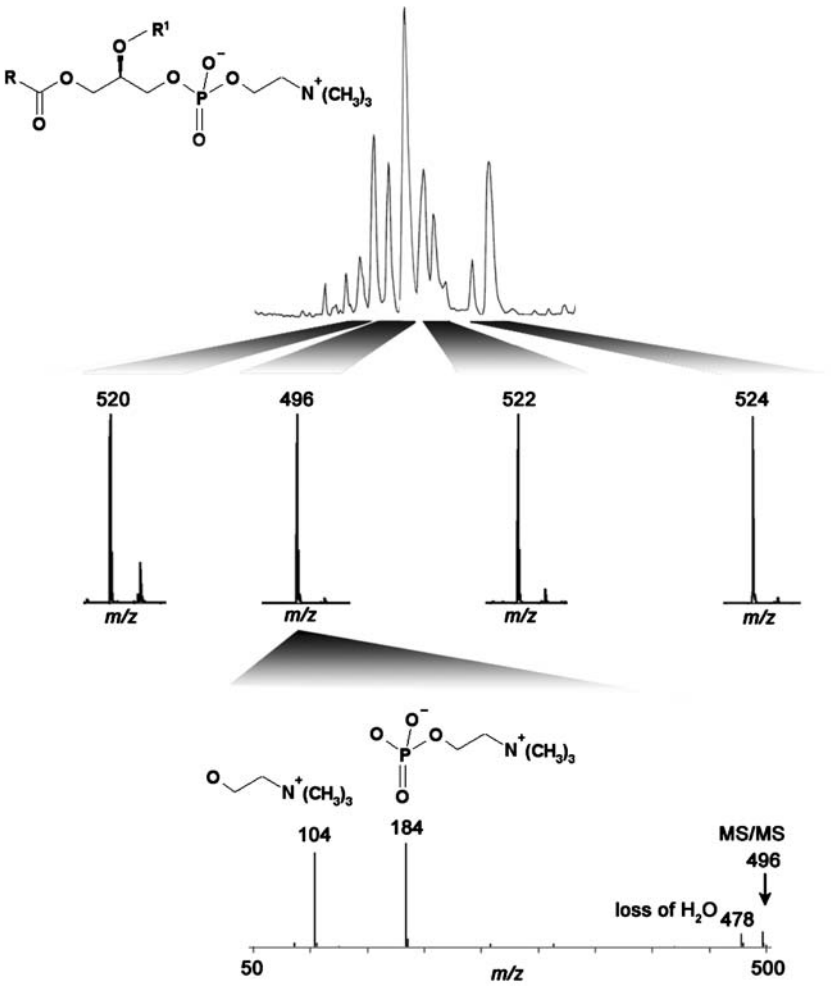

Figure 1. (*Top*) Phospholipids such as the lysophosphatidylcholines dominate the LC chromatogram of methanol-extracted human serum. The most abundant is 1-palmitoylglycerophosphatidylcholine. These highly ionizable endogenous metabolites may mask the presence of lower abundance molecules. The four most abundant phospholipids were identified using MS/MS and FTMS. (*Bottom*) MS/MS data for 1-palmitoylglycerophosphatidylcholine (m/z 496) obtained on a Micromass Q-TOF instrument. Highlighted are prominent fragments at m/z 104 and m/z 184 and a loss of water at m/z 478.

concurrently giving high recoveries of specific spiked drug analytes (Van Horne and Bennett, 2003; Bennett and Van Horne, 2003; Meng and Bennett, 2004; Bennett and Liang, 2004; Van Horne *et al*., 2004), the latter being the main focus of this group. In our study, this lanthanide sorbent was employed, harnessing the high oxophilicity of lanthanide metal centers (Tandem Labs). This silica-based propoyl sulfonic acid column modified with inorganic cerium salt (Van Horne and Bennett, 2003) was used in conjunction with 10 protein precipitation and LLE sample preparation methods in order to determine its effectiveness toward improving the metabolite profile.

2. Materials and methods

2.1. Chemicals and materials

Human serum (male, H-1388) was obtained from Sigma Chemical Co. (St. Louis, MO). Nanopure water was generated by a Nanopure Infinity Ultrapure water system. All organic solvents used in sample preparation were Optima grade from Fisher Scientific (Los Angeles, CA). All solvents, including the water for the LC/MS studies were HPLC grade (J. T. Baker, Phillipsburg, NJ). The proprietary cerium-modified columns were kindly provided by Tandem Labs (Salt Lake City, UT).

2.2. Identification of the major serum phospholipids

In order to evaluate the phospholipid capture (PLC) approaches, four major serum phospholipids belonging to the family of lysophosphatidylcholines were monitored, 1-palmitoylglycerophosphatidylcholine (m/z 496), 1-lineoylphosphatidylcholine (m/z 520), oleoyl lysophos phatidylcholine (m/z 522) and 1-steroyl-sn-glycero-3-phosphocholine (m/z 524) (figure 1). These phospholipids had been identified previously through the application of FTMS and MS/MS to fractions collected from LC/MS analyses of human serum (Want *et al*.,

2006). The chemical characterization of the most abundant phospholipid in human serum after organic solvent extraction [1-palmitoylglycerophosphatidylcholine (*m*/*z* 496)], is described here. Analysis by ESI-FTMS corresponded to a molecular formula of $C_{24}H_{51}NO_7P$, with a relative error of 0.10 ppm (parts per million). The calculated isotope pattern for $C_{24}H_{51}NO_7P$ was consistent with the elemental composition. Further, the MS/MS spectrum (figure 1) of *m*/*z* 496 from serum LC/MS analysis was identical to that of commercially purchased 1-palmitoylglycerophosphatidylcholine. Data obtained from ESI-FTMS and MS/MS analyses enabled the identification of three other lysophosphatidylcholines (not shown).

2.3. Sample preparation

Serum samples were prepared using 10 solvent systems, divided into two groups (polar and non-polar solvents), following the procedure detailed in tables 1 and 2. The starting volume of serum was 100 μL and all solvent to serum ratios were 3:1 (v:v). After PLC using the cerium-modified columns, samples were dried in a speedvac and resuspended in 5:95 acetonitrile:water for LC/MS analysis.

2.4. Polar solvents

Systems 1–5 employed protein precipitation using (1) 100% methanol, (2) 50:50 acetonitrile:methanol (3) 50:50 acetone:methanol, (4) 50:50 acetone:acetonitrile and (5) 50:50 MTBE:methanol. This approach resulted in a single supernatant fraction for each sample (termed the organic fraction). For protein precipitation, after adding reagents and vortexing, the serum samples were incubated at 4 °C for 20 min. After centrifugation at 13,200×*g* for 10 min the supernatants were collected and passed through the cerium-modified column at a rate of 100 μL/min.

2.5. Non-polar solvents

Systems 6–9 employed liquid-liquid extraction (LLE) using (6) 100% methyl-tert-butyl-ether (MTBE), (7) 100% MTBE acidified to pH2, (8) 100% ethyl acetate (EA) and (9) 100% dichloromethane (DCM). This approach resulted in two fractions for each sample (termed the organic and aqueous fractions). The organic fraction was studied in most detail. In system 10, the serum sample was subject to protein precipitation with methanol, but supernatant was dried down and resuspended in MTBE, which was then passed through the column. For LLE, serum samples were shaken at room temperature for 10 min, centrifuged at 3000 rpm for 10 min, before passing the organic layer through the cerium-modified column. System 10 is included in this group as it was the MTBE that was passed through the column.

2.6. Analysis of cerium-captured metabolites

In order to determine the degree to which the phospholipids had bound to the column, after the organic phase had been passed through, all cerium-modified columns were washed with 100 μL 100% MEOH, followed by 100 μL 50% MEOH and then 100 μL 100% H_2O. These eluents were combined, dried down and resuspended in 5:95 acetonitrile:water for LC/MS analysis.

2.7. Control samples

After protein precipitation or LLE, duplicate (control) serum samples were not passed through the column, but dried in a speedvac and resuspended in 5:95

Table 1
Preparation of serum samples prior to PLC using 10 solvent systems

Solvent system	Protein precipitation
Polar solvents	
Methanol	Vortexed 30s
Acetonitrile:MeOH (1:1)	Incubated at 4 °C for 20 min
Acetone:MeOH (1:1)	Centrifugation at 13,200×*g* for 10 min
Acetonitrile:Acetone (1:1)	Supernatant passed through cerium-modified column (100 μL/min)
MTBE:MeOH	
	LLE
Non-polar solvents	
MTBE	Shaken at room temperature for 10 min
MTBE pH2	Centrifuged at 3000 rpm for 10 min
Ethyl acetate	Organic layer passed through cerium-modified column (100 μl/min)
Dichloromethane (DCM)	Aqueous layer split into two portions. One passed through cerium-modified column (100 μl/min).
Methanol, MTBE*	

The starting volume of serum was 100 μl and all ratios of solvent to serum were 3:1.
*Methanol, MTBE. Serum sample was subject to protein precipitation with methanol, but supernatant was dried down and resuspended in MTBE, which was then passed through the column. It is included in the non-polar group as it was the MTBE that was passed through the column.

Table 2
Fractions analyzed from each solvent system

Solvent system	Organic		Aqueous		Eluent
	With	Without	With	Without	
Methanol	Y	Y	–	–	Y
Acetonitrile:MeOH (1:1)	Y	Y	–	–	Y
Acetone:MeOH (1:1)	Y	Y	–	–	Y
Acetonitrile:acetone (1:1)	Y	Y	–	–	Y
MTBE:methanol	Y	Y	–	–	Y
MTBE	Y	Y	Y	Y	Y
MTBE pH2	Y	Y	Y	Y	Y
Ethyl acetate	Y	Y	Y	Y	Y
Dichloromethane (DCM)	Y	Y	Y	Y	Y
Methanol, MTBE	Y	Y	–	–	Y

The eluent fraction contained the 'cerium-captured' metabolite features, meaning those that bound to the column.

acetonitrile:water for LC/MS analysis. This was to enable the evaluation of metabolite features before and after PLC using the cerium-modified columns. In addition, each of these solvent systems were passed through the cerium-modified columns in the absence of serum, to serve as further controls in determining whether any new m/z values were produced from the solvent or the column itself.

2.8. LC/MS analysis

Serum metabolite profiles with and without cerium-modified column clean-up were compared using reverse-phase chromatography and mass detection with an Agilent 1100 LC/MSD SL single quadrupole system (figure 2). For each run, 10 μl of the serum metabolite extract was injected onto the same C_{18} column (Symmetry® Column, 2.1× 100 mm, 3.5 μm particle size, Waters, Milford, MA) and eluted under gradient conditions of 5–90% acetonitrile (0.1% formic acid) over 60 min at a flow rate of 250 μL/min. Mass spectral data from 100 to 1000 m/z were collected in the positive ionization mode. Each solvent system was analyzed in duplicate and then each sample was analyzed in triplicate (totaling six replicates).

2.9. LC/MS data analysis

Changes in the serum metabolite profiles after PLC were evaluated using XCMS data analysis software, which can be downloaded freely as an R package from the Metlin Metabolite Database (http://www.metlin.scripps.edu) (Smith *et al*, 2005). This software approach employs peak alignment, matching and comparison (Smith *et al*., 2006), and allows for the determination of fold changes in peak intensities as well as the generation of extracted ion chromatograms (EICs) for easy visual comparison of peaks of interest. A metabolite feature was defined as a mass spectral peak in the mass region of 100–1000 with a signal-to-noise ratio of greater than 10:1. Isotopic and adduct peaks were treated as individual metabolite features. The intensities of the most abundant phospholipids, with m/z at 496, 520, 522 and 524 were calculated before and after PLC, allowing the percentage removal of these four phospholipids to be estimated. In the same way, metabolite features over the whole chromatogram, with particular focus on the retention time region of the phospholipids (1500–2100 s), were compared before and after PLC in order to establish any potential endogenous metabolite feature enrichment.

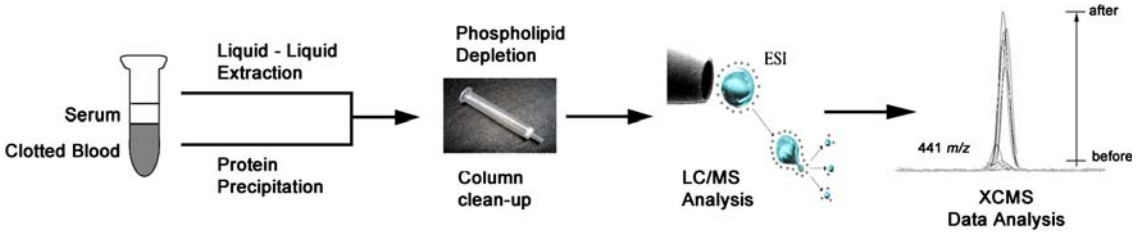

Figure 2. Overview of the sample processing steps employed for PLC using cerium-modified columns. Serum samples were prepared as shown in tables 1, 2 and analyzed using LC/MS. Serum metabolite profiles were compared using XCMS data analysis in order to evaluate the effects of different solvent systems on phospholipid capture.

3. Results and discussion

PLC from serum using the cerium-modified columns was evaluated using ten different solvent systems (tables 1, 2). The first four systems were chosen based on their efficiency of protein removal and metabolite recovery (Want *et al*., 2006), while the latter, less polar solvents, were chosen based on work regarding the use of these cerium-modified columns for phospholipid removal (Van Horne and Bennett, 2003; Bennett and Van Horne, 2003; Meng and Bennett, 2004; Bennett and Liang, 2004; Van Horne *et al*., 2004).

The aim of this study was to optimize both phospholipid removal and metabolite enrichment. Thus the cerium-modified columns were evaluated in conjunction with each solvent system for their ability to remove phospholipid from serum, the nature of additional cerium-captured metabolite features and, more importantly, the resulting enrichment of metabolite features. This enrichment was measured in terms of the number of newly detected metabolite features after PLC as well as the number of conserved metabolite features increasing in intensity after PLC. The number of metabolite features detected at each stage, and the distribution of metabolite features based on hydrophobicity were investigated. All data were generated using an in-house developed software package, XCMS (Smith *et al*., 2006), which enabled the accurate comparison of samples through peak alignment and matching. A metabolite feature was defined as a mass spectral peak with a signal-to-noise ratio of greater than 10:1.

3.1. Enrichment of Metabolite Features

The use of XCMS showed that many new metabolite features were detected after serum PLC with each solvent system. Additionally, some metabolite features detected prior to PLC were no longer observed, many of which can be attributed to phospholipids, their isotopes, adducts and perhaps fragments. Furthermore, many conserved metabolite features were observed to increase in intensity after PLC. These observations are discussed in relation to the solvent system used.

3.2. Detection of new metabolite features

The total number of metabolite features detected after PLC varied widely with each solvent system used, from 2100 (DCM and ethyl acetate) to 4000 (methanol). In some cases, this number was dramatically different to the number of features seen before PLC. The number of unique features within each method from both before and after PLC was also calculated. Simply, this is the number of different metabolite features that were seen from the analysis of both these fractions. All approaches produced >2800 unique metabolite features.

The number of new metabolite features detected after PLC was expressed as a percentage of the total number of metabolite features detected after PLC (table 3). This varied from 21% for the most polar solvent (MEOH) to 56% for the least polar solvent (ethyl acetate), and the general trend seemed to be that the higher the number of features seen after PLC, the lower the proportion of new features. Among the most polar solvents, the addition of acetonitrile or MTBE to methanol did not affect the number of new metabolite features detected, but the addition of acetone to methanol did. The combination of acetone and acetonitrile also increased the number from that obtained with methanol alone. In addition, with these solvents, the proportion of features not detected after PLC varied widely, from 18% to 61%.

When serum was first extracted with methanol but then resuspended in MTBE for PLC (termed MEOH, MTBE), the proportion of newly detected metabolite features increased dramatically from that obtained using either methanol or MTBE alone, but the number of original features no longer detected was twice that of MTBE.

The least polar solvents, ethyl acetate and dichloromethane produced $>45\%$ new metabolite features after

Table 3
Performance of each solvent system with regards to metabolite enrichment, shown as % new features detected after PLC and increase in intensity of conserved features

Solvent system	# Features *before* PLC	# Features *after* PLC	Total # unique features	% New features detected after PLC	% Original features not detected after PLC	Of original features, % showing >2-fold intensity increase after PLC	Av %RSD
MTBE	2641	2622	3502	33	33	40	25
MEOH, MTBE	3626	2159	4601	45	67	40	24
Ethyl acetate	4906	2421	6273	56	79	35	26
DCM	4696	2142	5654	45	75	43	20
MTBE pH 2	2209	2421	2805	25	17	23	24
ACE:MEOH	3801	3221	5844	41	29	15	23
MTBE:MEOH	3315	3287	4043	22	23	14	47
ACN:MEOH	3801	3558	4664	24	29	15	24
ACN:ACE	5142	3168	6306	37	61	56	40
MEOH	3816	3976	4662	21	18	18	25

Also shown is the total number of unique features from the combination of before and after PLC and the average % RSD of feature intensity.

PLC, but >70% of the metabolite features detected before PLC were no longer observed. In fact, the number of metabolite features observed after PLC halved with these solvents. Analysis of the cerium-captured components showed highest numbers of metabolite features with these solvents. MTBE is more polar than ethyl acetate and dichloromethane, and with this solvent, of the ~2600 individual metabolite features resulting from PLC with MTBE, 33% of the metabolite features were newly detected. The distribution of these metabolite features is shown in figure 3. Additionally, 33% of the ~2600 metabolite features detected before PLC were no longer observed.

The distribution of these new metabolite features based on hydrophobicity was also investigated. Within the more polar solvent systems, many of the newly detected features were in the region of the phospholipids, which indicates that even with incomplete phospholipid depletion, new metabolite features could still be detected. However, the majority were very polar, eluting in the first 500 s of the LC run. Additionally, the MTBE:MEOH combination showed many new metabolite features between 500 and 1000 s, which none of the other solvent systems did. This is in fact a very similar profile to that obtained using MTBE alone, whereas MTBE pH2 shows a more similar profile to the protein precipitation methods. Overall, the profiles obtained for the less polar solvent systems (LLE plus the MEOH extraction resuspended in MTBE), were more varied.

3.3. Increased intensity of conserved metabolite features

Between the solvent systems, there was much variation in the conserved metabolite features that were observed to increase >2-fold in intensity after PLC (~15–55%). The MTBE:MEOH combination showed the lowest percentage of increased intensity features and ACE:ACN the highest, coincidentally these two systems showed the greatest variation in reproducibility.

In general though, the intensity increase in metabolite feature intensity was solvent dependent, with the more polar solvent systems showing high metabolite feature yields (~3000), but the smallest intensity increases (<30%). Addition of acetonitrile, acetone or MTBE did not affect these observations. Serum treated with acetonitrile:acetone showed a >2-fold intensity increase in ~50% of the metabolite features after PLC, but the number of features not detected after PLC was ~60%.

When serum was first extracted with methanol but then resuspended in MTBE for PLC (MEOH, MTBE), the proportion of increased intensity metabolite features rose dramatically from that obtained using methanol and was similar to MTBE alone.

Among the less polar solvents, MTBE treated serum showed a >2-fold intensity increase in 40% of the metabolite features after PLC, with >2600 features observed, particularly in the "mid-eluting range" of 1500–2100 s, as illustrated by XCMS-generated representative EICs (figure 4). Such metabolite enrichment was also noted to a lesser degree within the phospholipid "later-eluting range" of 2600–3000 s. By comparison with controls, it was established that metabolite features in these regions of the chromatogram did not arise from the solvent or the column.

The enrichment of potential endogenous serum metabolites after MTBE treatment and PLC is also illustrated in the 3-D plot (figure 5), where intensity changes can be observed. From comparison with controls, it is likely that some of the early eluting features (<1000 s), particularly of the lower m/z values

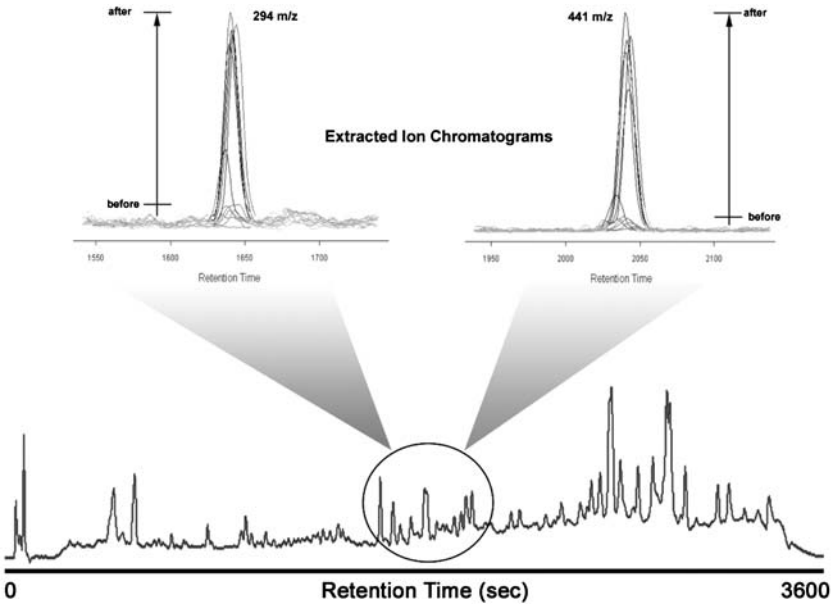

Figure 3. Distribution of newly detected metabolite features after MTBE treatment and PLC using the cerium-modified column.

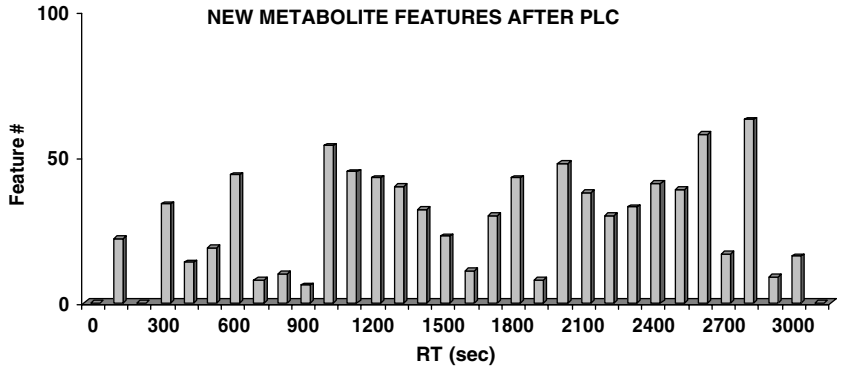

Figure 4. Two representative EICS generated by XCMS illustrating that metabolite feature intensities increased after MTBE treatment and PLC using the cerium-modified column.

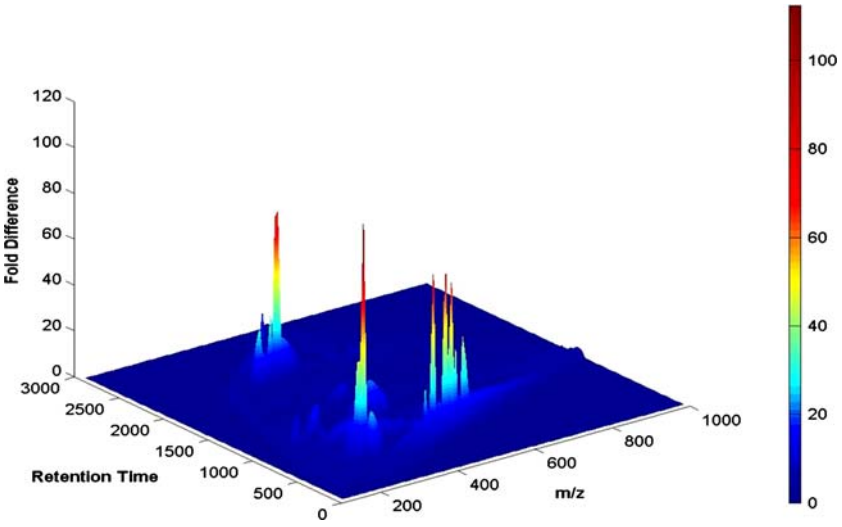

Figure 5. 3D plot showing the increase in metabolite feature intensities of potential endogenous serum metabolites after MTBE treatment and PLC using the cerium-modified column.

(< 400 m/z), arose from the column. However, those between 400 and 600 m/z are believed to be genuine endogenous metabolite features.

The distribution of increased intensity metabolite features by hydrophobicity was also investigated. The more polar solvents showed a general small increase over the whole LC run, with a slightly higher increase in the polar region. ACE:ACN and MTBE:MEOH also showed a large increase in the 1000–2000s region, where the phospholipids elute. Within the less polar solvents, MTBE and MTBE pH 2 showed a general increase over whole run, while ethyl acetate and dichloromethane showed a larger increase of very hydrophobic metabolite features.

3.4. Cerium-captured metabolites

With all solvent systems, the distribution of observed cerium-captured features correlated well with the features observed before but not after PLC. For example, the most polar solvents, whose PLC profiles revealed a high number of conserved metabolite features (those present both before and after PLC), showed a low number of captured metabolite features. The addition of any solvent to methanol increased number of captured metabolite features, with MTBE being most effective in this respect. The more hydrophobic solvents e.g. ethyl acetate, dichloromethane and MTBE, resulted in the largest number of captured features and the lowest number of conserved features. When the metabolite feature profiles after PLC were compared with the cerium-capture metabolite feature profiles, there were features that were present in both. These were components that only bound in part to the column, such as the phospholipids in the cases where low depletion was achieved. The number of captured metabolite features that were not present after PLC (i.e. were found before PLC and on the column, but not after PLC) decreased with solvent system polarity, indicating that the lower the solvent polarity, the more effectively components bound to the column.

The distribution of captured metabolite features showed that, with all solvent systems, the highest number were found in the most polar region of the chromatogram. Comparison with controls showed that many of these features were from the column itself. However, with the more polar solvents, and also with MEOH:MTBE, many bound metabolite features were found between 1000 and 2000s region of the phospholipids.

3.5. Aqueous fraction produced by systems 6–9 (LLE)

When LLE was performed, with MTBE, MTBE pH2, ethyl acetate and dichloromethane, both the aqueous and organic fractions were analyzed. While serum treated with the more polar solvents retained more phospholipid from human serum, with LLE, many of the phospholipids partitioned into the aqueous phase. When this fraction was passed through the cerium column, most of the phospholipid (>90%) did not bind to the column.

3.6. Reproducibility of metabolite feature intensity

The average percent relative standard deviation (Avg. %RSD) for the intensity of all detected metabolite features from each method is detailed in table 3. In this study, a feature is defined as a mass spectral peak whose signal to noise ratio exceeds 10:1 (see Section 2). Individual metabolite feature intensity %RSD was obtained from six replicates, where Avg. % RSD reports the average value for all detected features within each method. All solvent systems showed variation in feature intensity of <26%, except for MTBE:MEOH and ACE:ACN, at 47% and 40% respectively. The reason for this is unknown, but it is important to emphasize that as this is an average of several thousand metabolite features, individual metabolite feature %RSD values can vary greatly within each method.

3.7. Determination of the optimum PLC system

Serum treated with more polar solvents retained more phospholipid from human serum initially, probably due to increased solubility in these solvents. Subsequent PLC using the cerium-modified column ranged widely, with 12–60% of the most abundant phospholipids being removed from the serum. This is shown in table 4, where the amount of phospholipid in each sample is expressed as the % contribution to the chromatogram signal (by intensity) before and after PLC. With these more polar solvents, about 20% of the chromatogram signal was due to phospholipid before PLC, with the contribution still being 17–22% after PLC. This correlates with the relatively low degree of phospholipid depletion from these samples.

Conversely, the less polar solvents, MTBE, ethyl acetate and DCM retained fewer of the phospholipids after LLE and proved overall to be more efficient at PLC (>97% of the most abundant phospholipids removed). These less polar solvents varied more in the amount of phospholipid detected before PLC, with between 1 and 11% of the chromatogram signal due to phospholipids. However, after PLC, <1% of the signal was due to phospholipids, again correlating well with the degree of phospholipid depletion from these samples. Interestingly, MTBE pH 2 extracted much less phospholipid from the serum initially than MTBE pH 6, and the degree of PLC was less. This was different to observations by Bennett and Liang (2004), who showed that MTBE at pH 2 was more effective for PLC than at higher pH, removing >35 times more phospholipid. However, we report that for metabolite profiling, the application of MTBE at pH 6 resulted in ~10% more metabolite features overall, and ~20% more metabolite features with an increase of >2-fold after PLC than MTBE pH 2.

The combination of MTBE and methanol resulted in the initial extraction of more phospholipid from the serum (19% chromatogram contribution) than MTBE alone (11%), and only slightly less than with methanol alone (22%). The percentage of phospholipid removal however, was only 27%. However, when serum was first treated with methanol, the supernatant dried down and resuspended in MTBE for PLC (MEOH, MTBE), the effectiveness of PLC increased to 99%. In this case, the contribution of phospholipids before PLC was 22%, the same as with methanol alone. This is an interesting observation, because although the phospholipids were not particularly soluble in MTBE alone, when extracted with methanol first, they redissolved well in MTBE. Importantly, the initial phospholipid amount was similar to that observed using methanol alone, so the degree of PLC was substantial. Importantly, this finding shows that the solvent that is applied to the column is crucial to

Table 4
Performance of each solvent system with regards to % phospholipid removal and also the contribution of phospholipids to the chromatogram signal before and after PLC

	% Phospholipid removal	% Contribution of phospholipids to chromatogram signal	
		Before PLC	After PLC
MTBE	99	11	<1
MEOH, MTBE	99	22	<1
Ethyl acetate	99	10	<1
DCM	98	4	<1
MTBE pH 2	83	<1	<1
ACE:MEOH	60	21	17
MTBE:MEOH	27	19	17
ACN:MEOH	22	22	22
ACN:ACE	14	21	18
MEOH	12	22	21

the degree of phospholipid binding and perhaps the binding of other serum components.

Overall, MTBE and ethyl acetate PLC with the cerium-modified column achieved the highest depletion, retaining at least 99% of the most abundant phospholipids. However, MTBE resulted in 7% more metabolite features overall and ~5% more features with an increase of >2-fold after PLC than ethyl acetate.

Van Horne and colleagues report the use of MTBE for serum PLC in order to enhance the detection of drug analytes, resulting in >95% phospholipid removal and concomitant improvement in the precision of analyte quantitation of >5% (Van Horne *et al.*, 2004). When evaluating its applicability to metabolite profiling, MTBE treatment and PLC proved the most effective approach, resulting in >2800 metabolite features, with 40% showing a ≥ 2 fold increase in intensity.

Figure 6 shows representative chromatograms of the MTBE treatment of human serum (a) after LLE alone and (b) after LLE followed by PLC using the cerium-modified column. The EICs of the three most abundant "mid-eluting" phospholipids show the almost complete removal of the phospholipids after clean up.

When evaluating a system such as this for metabolite profiling, it is important to determine which of the factors discussed (number and distribution of metabolite features before and after PLC as well as metabolite feature enrichment and captured metabolite features) is most important for the study in mind. If all factors are to be taken into consideration, it would be prudent to use a combination of solvents in order to achieve the most comprehensive metabolite profile. Whilst the more polar solvents extracted the largest number of metabolite features from serum initially, they are not ideally suited for PL removal, although there were some new metabolite features detected. Conversely, the less polar solvents extract less metabolite features from serum but are more effective at PLC.

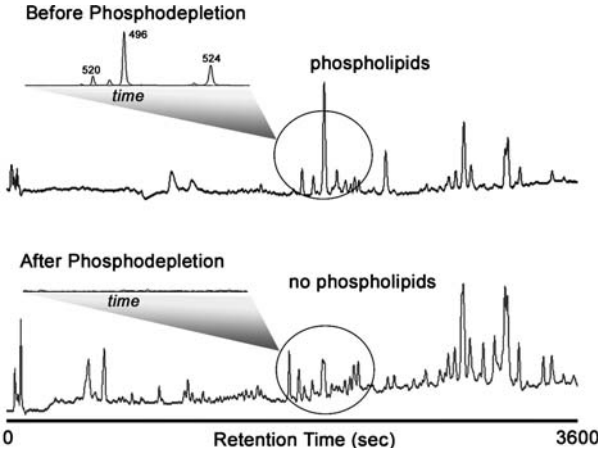

Figure 6. Chromatograms of MTBE extracted serum with (A) no PLC and (B) PLC using a cerium-modified column. Inset, the EICs of the major phospholipids at m/z 496, 520 and 524 show that they have been depleted after treatment. New metabolite features can be seen throughout the chromatogram.

4. Conclusion

The aim of metabolomics is to monitor as many endogenous metabolites as possible within a specific biofluid. When dealing with complex matrices such as serum, the possibility of ion suppression from highly abundant endogenous metabolites, such as phospholipids, must be considered. We conclude that MTBE is an effective solvent for preparing serum for the removal of phospholipids. Concomitantly with the removal of phospholipids, enrichment of endogenous potential metabolites was observed. MTBE treatment followed by PLC using the cerium-modified column removed 99% of the four most abundant phospholipids and resulted in ≥ 2-fold intensity increase in 40% of detected metabolite features. Furthermore, treatment of serum with MTBE resulted in different m/z values to those observed after methanol extraction, which in itself may be beneficial, increasing the breadth of the serum metabolite profile. The more polar solvent systems resulted in the recovery of the largest number of metabolite features, but were the least effective at removing the phospholipids from the serum with the cerium-modified column. As methanol is not a suitable solvent for this phospholipid removal system, it may be advantageous to use this MTBE treatment, or perhaps MEOH, MTBE approach in addition to a more 'traditional' protein precipitation method. This increase in potential serum biomarker detection has important applications in metabolite profiling for disease diagnosis and detection.

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