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Human plasma metabolite profiling: the impact of chromatography and data acquisition methods on metabolites detected by LC-MS

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1. Introduction

- Comprehensive untargeted endogenous metabolite profiling requires consideration to the diverse number of compound classes present in samples such as serum or plasma. Sample preparation, LC separation and MS data acquisition all determine the number of compounds detected and influence the success of identification.
- Protocol optimisation was performed on NIST standard reference material (SRM-1950), consisting of human plasma. This was to ensure the best possible method was used before undertaking analysis of critical low volume samples. Optimisations were made to both sample preparation and LC separation to ensure compounds injected onto the column were separated and eluted.
- Data analysis required several key elements, the first being ion feature and detection software in order to distinguish between chemical noise and genuine ion signals. Chromatographically resolved features needed to be detected to distinguish between consistently detected ion signals that appeared in the background. Furthermore, blank sample analysis enabled identification of peaks appearing at low levels that were not sample related. Finally, the mass range chosen for sample analysis also greatly influenced the number of metabolites detected but also the data interpretation.
- Compounds can naturally exhibit neutral loss of part of the molecule meaning the ion measured may not represent the protonated positive ion [M+H]+ but instead [M-X+H]+. Commonly loss of water can occur however in the case of some lipid species such as monoacylglycerophosphocholines, diacylglycerophosphocholines and triacylglycerols the neutral loss can be larger. These phenomenon, in addition to ion adduct formation, demonstrated the need for an untargeted ion feature detection irrespective of metabolite identification by accurate mass. Equally, metabolite IDs require confirmation preferably with MS/MS supporting data to ensure IDs are correct.
- These experiments describe a work flow that aimed to optimise untargeted metabolite profiling designed to maximise ion feature detection and eventual identification by accurate mass and supporting MS/MS data.

2. Methods

Aliquots of the NIST SRM-1950 metabolites in human plasma were prepared by protein precipitation in methanol. Following centrifugation, supernatants were dried down then reconstituted with an equal volume of re-suspension solvent (water / methanol 1:1). An optimised binary gradient (Fig.1) using 20 mM ammonium formate with 0.1% formic acid (Pump A) and methanol (Pump B) was used with a Phenomenex Kinetex C18 column (1.7 um, 100 × 2.1 mm) with a flow rate of 0.4 mL/min and maintained at 50°C (LC system: Shimadzu Prominence XR).
A hybrid ion trap-time of flight mass spectrometer, LCMS-IT-TOF (Shimadzu Corporation, Kyoto, Japan) was used for analysis. Data acquisition was by ESI using continuous polarity switching in order to ionize a full range of analytes in positive and negative mode in a single run. Data-dependent MS/MS and MS3 was used with appropriate dynamic and static exclusion lists and triggering thresholds. The sample was analysed with five replicates for statistical analysis. Data processing was completed using either XCMSOnline (Scripps Center for Metabolomics, La Jolla, Calif.) or Shimadzu Profiling Solution software combined with the statistical program SIMCA-P (Umetrics, Umeå, Sweden).

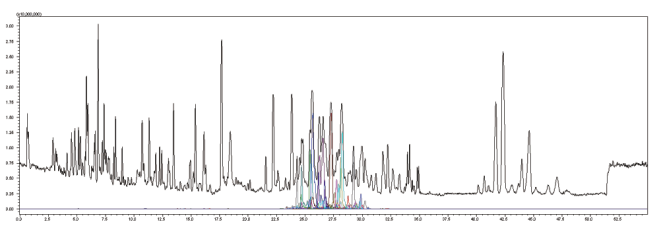

Fig. 1 LC conditions were optimized to separate different lipid classes balanced with retention of polar compounds such as amino acids and fatty acids. Optimized separation used curved binary gradient (-3) from 2% to 100% solvent B (gradient superimposed). Extracted ion chromatograms demonstrate effective separation of diacylglycerophospho-cholines.

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3. Results

Optimization of LC separation resulted in an accelerated binary gradient that succeeded in separating complex lipid species (Fig.1). Although not optimal for very polar compounds such as amino acids, analysis of these compounds was performed in another study using HILIC and PFP column stationary phase. Sample re-suspension buffer was investigated comparing 50% methanol to 95% and simply water. Ions measured *m/z* 150-1000 were chosen to represent sample composition. Solvent blanks were also included in the analysis to enable detection and exclusion of background ions from the analysis. Profiling Solution was used to align data using a novel ion binning alignment algorithm (Fig.2).

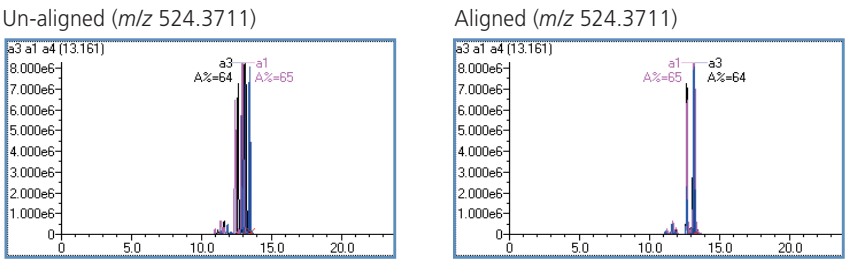

Fig. 2 Profiling Solution was used to align data using a novel spectral ion binning algorithm. 963 ions were detected in the chromatogram matrix, 43 of which were detected as background ions by inclusion of blank samples in the analysis (mass range *m/z* 150-1000; positive ion mode).

Sample preparation involved protein precipitation in methanol, centrifugation, supernatant dry-down and sample re-suspension. The choice of solvent used for sample re-suspension was investigated due to several different published methods in scientific literature.

Although re-suspension in methanol should guarantee no metabolites left un-dissolved, LC separation of polar compounds may be influenced and cause poor retention and peak shape.

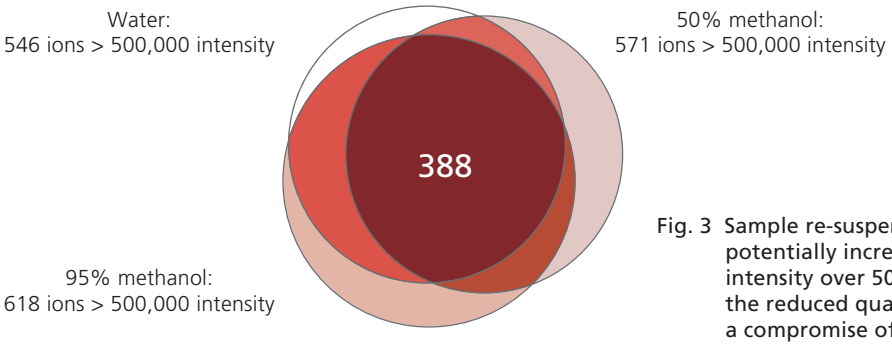

Fig. 3 Sample re-suspension solvent comparison: 95% methanol can potentially increase the number of metabolites detected with an intensity over 500,000 (*m/z* 150-1000 positive ion mode) however the reduced quality peak shape and column retention meant that a compromise of 50% methanol was used.

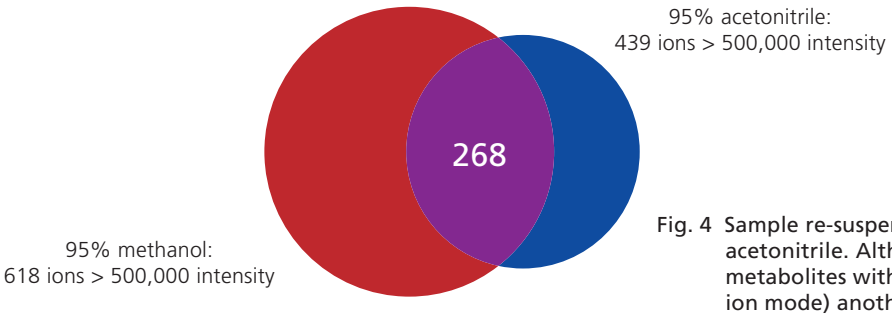

Fig. 4 Sample re-suspension solvent comparison: 95% methanol to 95% acetonitrile. Although one solvent may offer higher numbers of metabolites with an intensity over 500,000 (*m/z* 150-1000 positive ion mode) another solvent may enable better detection of different metabolites

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Sample preparation in water, an established method for metabolite analysis[1], can offer best separation of polar compounds however may not produce optimum signal intensity for less polar compounds such as lipids. In this study re-suspension in 95% methanol enabled detection of the highest number of metabolites over 500,000 intensity (Fig. 3), however to compromise between optimum solvent for peak intensity versus peak shape of polar compounds 50% methanol was chosen for further experiments. Re-suspension in different solvents can potentially realize many different compounds present in the metabolome[2] (Fig. 4), however the practicality of repeating all analyses multiple times with different preparation methods has to be balanced with other equally important factors such acquisition (positive / negative) and mass range (*m/z* 70-2000). With the potential of detecting several thousand metabolites using complementary techniques (GC-MS, LC-MS, HILIC, PFP, RP, ESI, APCI) untargeted approaches inevitably become semi-targeted as a generic method is adopted.

Sensitivity was maximized by performing data acquisition in mass ranges *m/z* 70-250, 150-1000, 1000-2000 (Fig.5). MS^n data directed acquisitions were included to enable proportionate scans through the mass ranges of interest.

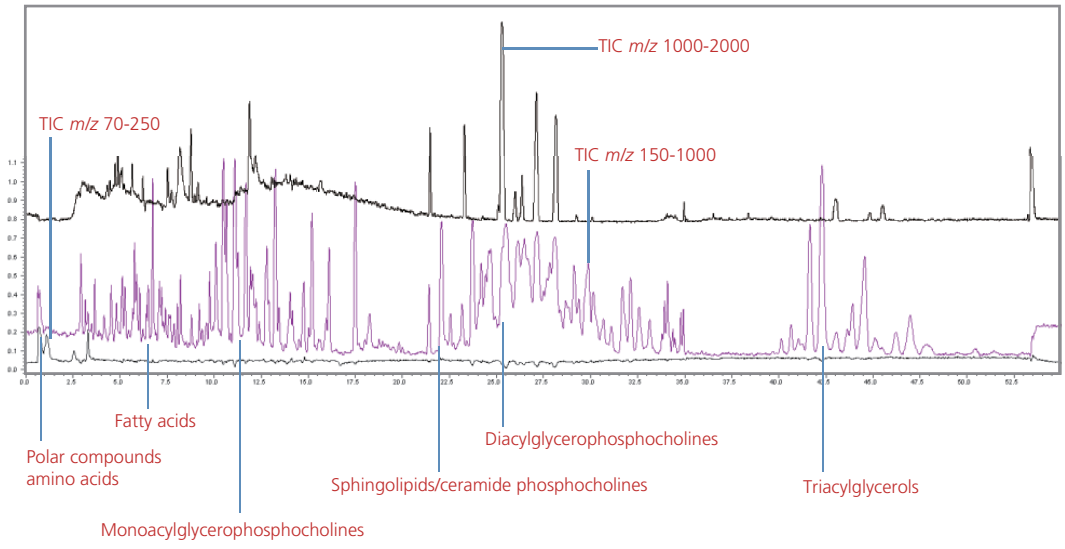

Fig. 5 Total ion chromatograms for different mass ranges demonstrated the majority of plasma metabolites were detected in the mass range *m/z* 150-1000 however significant numbers of lipid compounds were detected at higher masses.

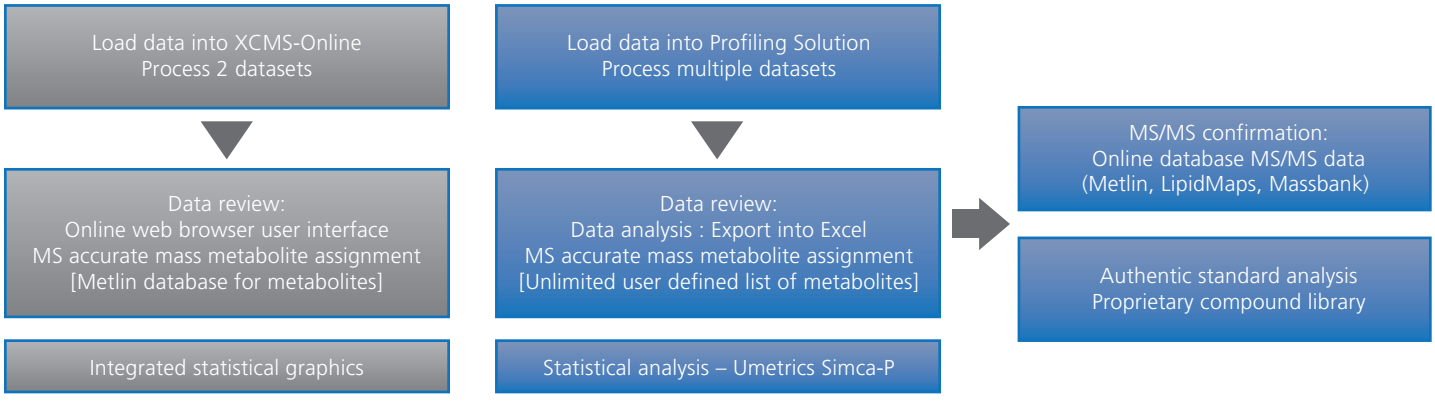

Fig. 6 Workflow describing the process of metabolite identification. This was performed using a number of complementary techniques because no single method proved universally definitive for reliably identifying an ion. XCMS-Online was easy to use with seamless integration of Metlin database and statistical functions. Although requiring more steps, the workflow highlighted blue was chosen due to the added ability to define several sample types for data alignment and unlimited user defined metabolite identification via an Excel workbook. On-line confirmation of MS assigned IDs using MS/MS data successfully confirmed a proportion of accurate mass MS identifications.

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Name	Formula	RT	m/z	MS/MS supporting ions	Accession
L-Carnitine	C7H15NO3	0.75	162.1125	103.0426	C15025
Acetylcarnitine	C9H17NO4	0.89	204.1231	145.0552	C02571
L-Phenylalanine	C9H11NO2	2.04	166.0863	120.0816, 131.054, 149.0829	C00079
Indoleacrylic acid	C11H9NO2	2.92	188.0706	118.0689, 146.0596	HMDB00734
Benzoylecgonine	C16H19NO4	3.66	290.1387	119.0512, 122.0983, 150.0877, 168.1015	EA282310
p-Acetamidophenol	C8H9NO2	3.80	152.0706	110.0515	C06804
Bilirubin	C33H36N4O6	5.96	585.2708	225.1020, 239.1180, 257.1241, 299.1388	C00486
Decanoyl-L-carnitine	C17H33NO4	5.98	316.2483	257.1673	HMDB00651
Piperine	C17H19NO3	6.41	286.1438	115.0536, 135.0425, 143.0512, 171.0450, 201.0543	MID43568
Umbelliferone	C9H6O3	7.30	163.0390	77.0438, 133.0280	C09315
3-HYDROXYCOUMARIN	C9H6O3	8.11	163.0390	133.0291	MID44078
PC(P-19:1(12Z)/0:0)	C27H54NO6P	9.77	520.3762	184.0738, 502.3292	LMGP01070010
PC(15:0/0:0)	C23H48NO7P	9.81	482.3241	184.0766, 405.2384, 464.3110	LMGP01050016
PC(20:4(5Z,8Z,11Z,14Z)/0:0)	C28H50NO7P	9.83	544.3398	184.0746, 526.3297	LMGP01050048
PC(P-19:1(12Z)/0:0)	C27H54NO6P	10.13	520.3762	184.0750, 502.3316	LMGP01070010
PC(20:4(5Z,8Z,11Z,14Z)/0:0)	C28H50NO7P	10.18	544.3398	184.0746, 526.3297	LMGP01050048
PC(16:0/0:0)[U]	C24H50NO7P	10.30	496.3398	184.0746, 258.1181, 313.2544, 419.2553, 478.3286	LMGP01050020
PC(16:0/0:0)	C24H50NO7P	10.72	496.3398	184.0746	LMGP01050018
PC(18:1(9Z)/0:0)	C26H52NO7P	10.85	522.3554	184.0747, 445.2662, 504.3455	LMGP01050032
PC(18:1(9Z)/0:0)	C26H52NO7P	11.28	522.3554	184.0738, 445.2708, 504.3448	LMGP01050032
PC(17:0/0:0)	C25H52NO7P	11.46	510.3554	184.0768, 433.2665, 492.3467	LMGP01050024
PC(O-16:0/0:0)	C24H52NO6P	11.59	482.3605	184.0799, 464.3494	LMGP01060010
PC(P-16:0/0:0)	C24H50NO6P	11.61	480.3449	184.0284, 240.0994, 326.3401	LMGP01070006
PC(O-18:0/0:0)	C26H56NO6P	11.73	510.3918	184.0768, 433.2665, 492.3467	LMGP01060014
PC(15:0/0:0)	C23H48NO7P	11.80	482.3241	184.0799, 464.3493	LMGP01050016
PC(P-18:1(9Z)/0:0)	C26H52NO6P	12.14	506.3605	181.0266, 240.1050	LMGP01070012
PC(18:0/0:0)	C26H54NO7P	12.26	524.3711	184.0750, 506.3592	LMGP01050026
PC(18:0/0:0)	C26H54NO7P	12.75	524.3711	184.0750, 506.3592	LMGP01050026
Bilirubin	C33H36N4O6	13.52	585.2708	225.1012, 271.1399, 284.1102, 299.1383	C00486
PC(P-18:0/0:0)	C26H54NO6P	13.71	508.3762	181.0279, 184.0754, 240.0989	LMGP01070009
PC(O-18:0/0:0)	C26H56NO6P	13.96	510.3918	184.0808	LMGP01060014
Oleoyl Ethyl Amide	C20H39NO	14.48	310.3105	163.147	MID44905
3-HYDROXYCOUMARIN	C9H6O3	15.52	163.0390	133.0289	MID44078
SM(d18:1/16:0)	C39H79N2O6P	23.33	703.5749	685.5667	LMSP03010003
PC(O-16:0/20:4(5Z,8Z,11Z,14Z))	C44H82NO7P	26.75	768.5902	585.4965	LMGP01020056
PC(16:0/18:1(9Z))	C42H82NO8P	26.90	760.5851	478.3266, 504.3397	LMGP01010005
PC(18:0/20:4(5Z,8Z,11Z,14Z))	C46H84NO8P	27.79	810.6008	506.3685, 626.5205	LMGP01010802

Table 1 Metabolites confirmed by MS/MS databases:
In many cases phospholipid metabolites shared common fragment ions making IDs uncertain with authentic standard analysis the only possible way to 100% confirm the ID of a metabolite.

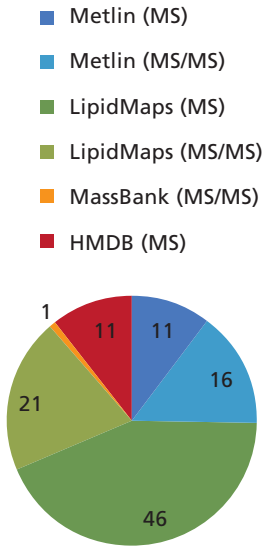

Fig. 7 The proportion of 106 most intense ions, IDs confirmed as metabolites by manual data analysis. 36% of identified metabolites could reliably be compared to on-line MS/MS data.

4. Conclusions

- Untargeted workflows become semi-targeted because sample preparation and the chosen analysis technique limit the scope and depth of the metabolome that can be analyzed.
- Metabolite identification is a complex process that can require the analysis of authentic standards due to limited database information. Limiting analysis to one database could significantly reduce the success of a metabolomics study.

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References

(1) *Anal. Chem.*, ***2009***, *81*, 1357–1364
(2) *Anal. Chem.*, ***2013***, *85*, 341–348

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