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Advances in high throughput LC/MS based metabolomics: A review

Robert S. Plumb [a, **], Lee A. Gethings [b], Paul D. Rainville [a], Giorgis Isaac [a], Robert Trengove [c], Adam M. King [b], Ian D. Wilson [d, *]

[a] Waters Corporation, Scientific Operations, Milford, MA, USA
[b] Scientific Operations, Waters Corporation, Stamford Ave, Wilmslow, SK9 4AX, UK
[c] Curtin University, Kent St, Bentley, WA, 6102, Australia
[d] Division of Systems Medicine, Department of Metabolism, Digestion and Reproduction, Imperial College, Burlington Danes Building, Du Cane Road, London, W12 0NN, UK

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ABSTRACT

Properly implemented, metabolic and lipidomic profiling can provide a deeper understanding of mammalian, plant and bacterial biology. These omics-tools have developed and matured over the last 40-years and are now being deployed to provide valuable information in epidemiological studies, drug toxicology and pharmacology, disease biology and progression and patient stratification. LC/MS has become the technology of choice for both metabolic and lipid profiling, due to its speed, sensitivity and structural elucidation capabilities. In the preceding two decades there have been many technological and methodological advances in LC/MS that have facilitated the evolution of the technology into a rugged, reliable, and easily deployed tool. These advances include, but are not limited to, improvements in chromatography (phases, columns, and delivery system), instruments for mass spectrometry, optimization of sample preparation, the introduction of ion mobility, data analysis tools, metabolite databases, harmonized protocols, and the more widespread use of quality control methods and reference standards/matrices. Here, recent developments and advances in high throughput liquid chromatography/high resolution mass spectrometry for metabolic phenotyping are described. These advances which may provide improved feature detection, increased laboratory efficiency and data quality, as well as "biomarker" identification, are discussed in relation to their potential application to the analysis of large clinical studies, or biobank collections.

1. Introduction

Untargeted metabolic profiling (metabolomics and metabonomics) [1,2] or metabolic phenotypying, whilst foreshadowed by earlier studies in the late 1940's and later in the 1970's (e.g., see Ref. [3]), only really emerged in its present form in the late 1980's (e.g., see Refs. [4,5]) with the introduction of advanced spectrometric techniques. Using this untargeted "holistic" approach provides information on the metabolite composition of cells, tissues and biofluids as well as highlighting changes in particular metabolite concentrations, which are indicative of normal physiological processes (e.g. ageing) or metabolic responses to disease, toxicological insults, pharmaceutical treatment etc. These "omics" studies rely on data provided by high information content analytical techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS). MS can either be used alone via the direct infusion (DIMS) of samples [6] for example or can be coupled to chromatographic/electrophoretic techniques such as liquid chromatography (LC), gas chromatography (GC), supercritical chromatography (SFC) or capillary electrophoresis (CE) [7]. When the data derived from these analytical platforms are used in combination with advanced statistical modelling it can result in the detection of "putative biomarkers", either as individual metabolites or combinations of several different molecules, which define a particular biochemical state. Thus, metabolic profiling provides complementary, downstream, information on a biological process to that provided by genomics, transcriptomics, and proteomics. In

* Corresponding author.
** Corresponding author.
E-mail addresses: rob_plumb@waters.com (R.S. Plumb), Lee_gethings@Waters.com (L.A. Gethings), Paul_Rainville@waters.com (P.D. Rainville), Giorgis_Isaac@Waters.com (G. Isaac), robertdtrengove@gmail.com (R. Trengove), Adam_King@waters.com (A.M. King), i.wilson@imperial.ac.uk (I.D. Wilson).

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2006 Griffin posed the question "does metabolomics get to go to the functional genomics ball?" highlighting the challenges faced in metabolic phenotyping (metabotyping) as a result of the chemical diversity, number and huge range of concentrations exhibited by metabolites in biological systems [8]; contrasting the hundreds of metabolites identified (as opposed to detected) in a typical metabolomic study to the 10,000's of peptides and 1000's of proteins identified in a proteomic experiment. Despite these challenges, since 2006 there have been in excess of 50,000 publications discussing metabolomics, with over 6000 directly related to the development and use of LC/MS-based metabolic phenotyping (based on a PubMed search using the following keywords; metabolomics, metabonomics and LC/MS Metabolomics (Dec 6th, 2022)).

Metabolic profiling via direct infusion or coupling separation techniques such as LC, GC or CE, and more recently SFC, to mass spectrometry has been extensively used in the fields of new-born screening and clinical diagnostics for decades [9,10]. The early pioneering work of Millington et al. established MS and LC/MS/MS as a fast, accurate and effective platform for the early diagnosis of inborn errors in metabolism [11]. Currently, every new-born in the USA is screened by a combination of Guthrie card and electrospray ionization (ESI) MS for conditions such as phenylketonuria (PKU), an inherited disease causing phenylaniline accumulation in the body and resulting in seizures and developmental delays [12]. Whereas the early clinical screening work employed a targeted MS/MS approach, the metabolomic/metabonomic approaches [1,2] which were emerging in the late 1980's employed an unbiased approach either using multiple reaction monitoring (MRM), full scan MS, accurate mass MS or ^{1}H NMR spectroscopy, with much of the early MS work performed in combination with capillary GC [13]. LC/MS in either targeted or untargeted "full-scan" mode quickly established itself as the technology of choice for mammalian based metabolic profiling. This rapid adoption was, in part, due to the increasingly widespread availability of suitable instrumentation and ease of both access and use, combined with good sensitivity, specificity and resolving power. Reversed-phase (RP) LC was also readily compatible with biological samples, and as a result, RPLC/MS has been employed in combination with accurate mass MS (QToF or Orbitrap technologies) or multiple channel MRM using tandem quadrupole MS to various degrees [14]. The key challenges that are faced when targeting either the metabolome or lipidome are analytical reproducibility, feature coverage, dynamic range, component annotation, data analysis and processing large sample sets/cohorts. These challenges have sparked many innovative chromatographic, MS and data analysis approaches aimed at either, increasing coverage (and identification), improving reproducibility, addressing data alignment issues and/or improving throughput.

1.1. High throughput metabolic phenotyping

The early use of LC/MS for metabolic profiling of urine and blood products used relatively moderate/low throughput methods with analysis times of 10–25 min/sample and moderate resolution chromatography systems (see Ref. [15] for an early review). LC/MS based metabonomics was first described by Haselden et al. [16] who employed reversed–phase HPLC-MS/MS for the analysis of rat urine samples, obtained after dosing a candidate drug, with accurate mass MS/MS allowing both the annotation of components of interest and drug related metabolites [17,18]. Many of these early approaches to LC/MS-based metabolic and lipidomic profiling employed columns packed with 3.5 or 5 μm particles and gradient elution over 10–25 min, resulting in 6–10 s wide chromatographic peaks at the base and yielding separation peak capacities of approximately 100. For relatively small-scale studies, this type of analysis was perfectly adequate, but the very long run times required for the analysis of several hundreds of samples (at rates of between 3 and 6 samples/h) provides a challenge, both in terms of resources and instrument stability. Indeed, scaling this sort of separation to high throughput (bearing in mind that analysis of the same sample in both positive and negative ESI would double analysis times) was clearly limited.

An early attempt to increase throughput, using sub 10 min analysis, was described by Pham-Tuan et al. [19] who investigated a number of column chemistries for the analysis of biofluids (urine/plasma) using HPLC with fluorescence and UV detection. They concluded that a short, 50 × 4.6 mm i.d. SpeedROD Chromolith monolithic column with gradient elution and an analysis time of ca. 5 min was suitable for deproteinized urine and plasma. However, for samples such as cell culture (containing cell culture media), a non-porous polymeric stationary phase (1.5 μm C18 Chromsper) packed into a 30 × 4.6 mm i.d. column was superior. Whilst this approach was not adopted widely the potential of LC/MS itself in metabolic phenotyping was appreciated and became rapidly established with studies demonstrating utility in plant biology, human disease, and response to treatments. Examples of this included Moco et al. who developed a metabolic database containing t_R, MS1 and MS2 information for the metabolome of the common tomato [20] allowing for the discrimination between peel and flesh tissue. Similarly, Chen et al. demonstrated the use of HPLC/MS-based metabolomics for the characterization of the nephrotoxicity induced by aristolochic acid (AA), a suspected kidney toxicant [21] whilst van der Greef et al. used RP HPLC/MS to examine the metabolic response to fasting in the mouse [22]. However, the use of HPLC with 3.5 or 5 μm stationary phases presented researchers with a conundrum of choosing between throughput or the number of detected features (which persists today). Greater chromatographic resolution results in increased feature detection, superior MS spectral quality and increased sensitivity. However, increased resolution required longer columns and hence longer analysis times which confounded the need the need to process the greater sample numbers found in e.g. epidemiological studies, etc. The need for higher resolution was addressed by the commercialization of analytical scale sub 2 μm ultra-high performance liquid chromatography (UHPLC) particle LC (first described by Jorgenson in the late 1990's [23] on capillary scale columns operating at up to 60,000 psi) in 2004 [24]. The optimal mobile phase velocity of these UHPLC columns was up to 3-fold that of the 3.5 and 5 μm columns and produced peak widths in the region of 2–3 s at the base, giving peak capacities of approximately 300 for a 10 min RPLC separation. This advance enabled a dramatic increase in resolution compared to HPLC and, with analysis times of 10–15 min this resulted in UHPLC providing not only greater separation power but, as a direct consequence, increased feature detection, superior MS spectral quality and increased sensitivity. The clear potential of UHPLC/MS for metabotyping was initially demonstrated by Wilson et al. [25,26] for the analysis of Zucker rat urine using UHPLC/ToF-MS. Since the introduction of UHPLC it has become the dominant platform for LC based metabolomics (as illustrated by recent applications such as [27–29]). However, the bulk of these applications still relied on the use of 10–15 min analysis times. The opportunity, to dramatically reduce analysis times ca. Ten-fold was demonstrated shortly after the initial application of the technique with a 1.5 min rapid gradient analysis of rat urine [26]. The problem however, with this early demonstration was that whilst analysis time was reduced, this reduction was accompanied by marked extra column band broadening which reduced the efficiency of the separation at the expense of feature count.

Subsequently Gray et al. [30] optimized the system towards

large scale metabolic phenotyping, firstly reducing the column diameter to 1 mm i.d., thereby greatly reducing solvent consumption (important in minimizing economic and environmental costs when analysing many thousands of samples) and secondly addressing the band broadening problem. In this work the fluid flow paths connecting the column to the MS ion source were optimized to reduce band broadening [30] and improve peak shape, with increased sensitivity an added benefit. Then, by reducing the length of the UHPLC column to 1 × 50 mm and operating at 4-times the optimal flow rate [31] an analytical cycle time of 3 min was obtained combined with a 75% reduction in solvent consumption and improved batch reproducibility. This was achieved whilst maintaining the group separation and "biomarker" detection (see Fig. 1), as demonstrated by analyzing a 700-sample batch of rat urine following the administration of acetaminophen. This **ra**pid **m**icrobore **m**etabolic **p**henotyping (RAMMP) approach therefore began to offer the possibility of large scale metabolic phenotyping for large sample sets at high throughput [31].

However, even though the "biomarkers" responsible for group separation were conserved between the conventional and "fast" methodologies, the reduced analysis time provided by this RAMMP methodology, as would be expected, came at the cost of reduced peak capacity and total feature detection. Whilst there are methods for increasing feature count, such as 2DLC, these usually inevitably result in an increase in analysis time. However, the inclusion of ion mobility (IM) in the analysis removes this as a factor, enabling an orthogonal 2nd dimension of separation to be achieved without a time penalty to the analysis. In addition using data from the IM step to measure the collision cross sections (CCS) of the analytes it provides a further means of metabolite characterization. As a result of these, and other, beneficial effects of adding IM to LC/MS, this approach is becoming an increasingly popular for metabolic phenotyping studies. Rainville et al. demonstrated that employing an IM enabled LC/MS approach for a 3-min UHPLC analysis of human urine, increased the number of detected features from 7300 to 10,400 [32]. It should be noted that IM will not redress ion suppression issues but does enhance feature detection by reducing spectral overlap [32]. The application of RAMMP-based RP-UHPLC/IM/MS to the IROA MS library standards (MSLS) and then control rat urine allowed for the identification of a number of urinary metabolites via a combination of t_R, CCS and MS data demonstrating the value of the addition of IM [33]. This work was rapidly followed by a comparison of the RP-RAMMP/IM/MS [34] approach with a conventional RP-UHPLC/MS method [35] for urinary metabotyping using urine obtained from rats on two different diets (control and methionine-choline deficient (MCD)) [34]. The resulting data, when analysed using multivariate statistics (MVA) showed very similar separations using principal components analysis (PCA) (Fig. 2). However, interestingly the use of IM meant that the components identified as being significant from the MVA, were different for conventional and RAMMP methods. Nevertheless, this example clearly demonstrated that, for relatively high throughput screening, RAMMP/IM/MS could clearly highlight differences between groups that mirrored those found by a more conventional profiling method. This would allow the rapid metabotyping of samples which could then be reanalysed in greater detail if it was deemed worthwhile, using in-depth conventional analysis.

Further examples of the use of RAMMP-based methods incorporating an IM separation include a hydrophilic interaction liquid chromatography (HILIC) UHPLC/IM/MS analysis of rat urine for polar metabolites with a cycle time of ca. 3.3 min/sample [36] following administration of tielinic acid. When applied to these rat urine samples this RAMMP HILIC/IM/MS method, detected ca. 6700 m/z-t_R features in the samples with IM enabled against ca. 3000 when IM was not used. Shortly afterwards a RAMMP-lipid profiling method was published [37] using sub 4 min microbore UHPLC/IM/MS that maintained the performance of a pre-existing 12 min conventional UHPLC/MS assay [38] whilst resulting in a 75% saving in mobile phase use and analysis time. The separation achieved using this RAMMP-lipidomic method, compared to the original 12 min assay, is shown in Fig. 3. As seen elsewhere the use of a second dimension of separation via IM improved the quality of the MS spectra and aided lipid identification. On application to human plasma extracts, over 5000 m/z-t_R features (+ve ESI) and 2000 (-ve ESI) were observed with ca. 3700 and 800 of these having CV's below 30% for the two ionization modes respectively. When applied to breast cancer patient plasma samples, the method was able to differentiate them from healthy controls based on differences in lipid phenotypes (with phosphatidylserines, triglycerides and diglycerides present at lower and phosphatidylserine at higher relative concentrations in patient samples) [38].

The ability to access the full potential of these RAMMP/MS methodologies is still however, not completely optimized. Thus peak dispersion, due to both within and post column band

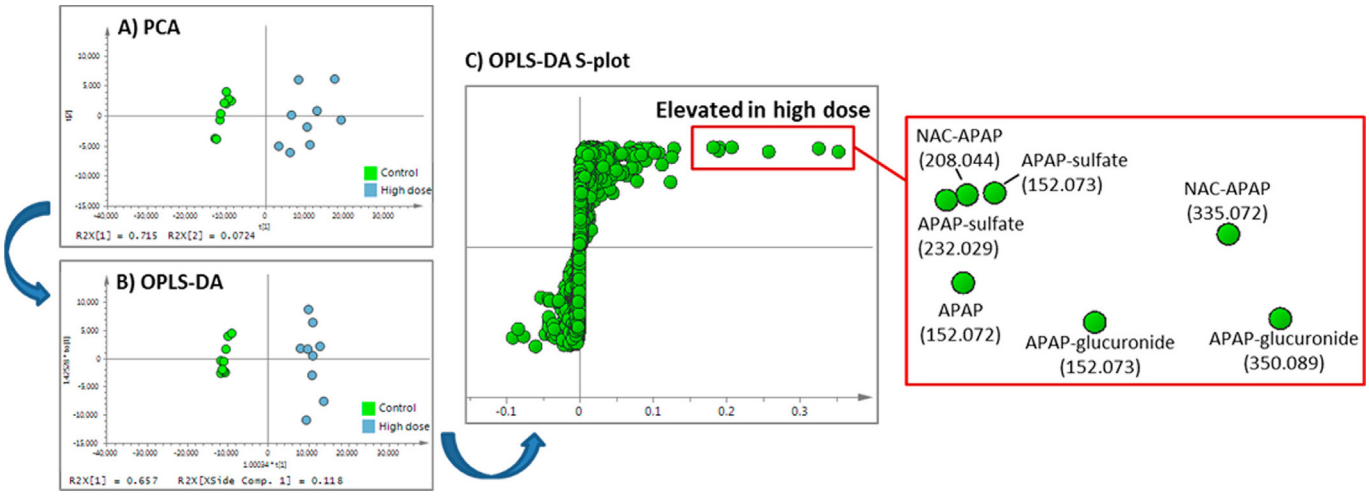

Fig. 1. PCA (A) and OPLS-DA (B) score plots and OPLS-DA loadings plots *S*-plot (C) comparing features (*m*/*z*) from control and high acetaminophen dose (800 mg/kg) rat urine samples at 0–24 h post dose obtained by UHPLC-MS (+ve ESI). Metabolite identification in high dose samples was by determination of the chemical structure using accurate mass measurements and from MS/MS data. **From Ref 31 with permission**.

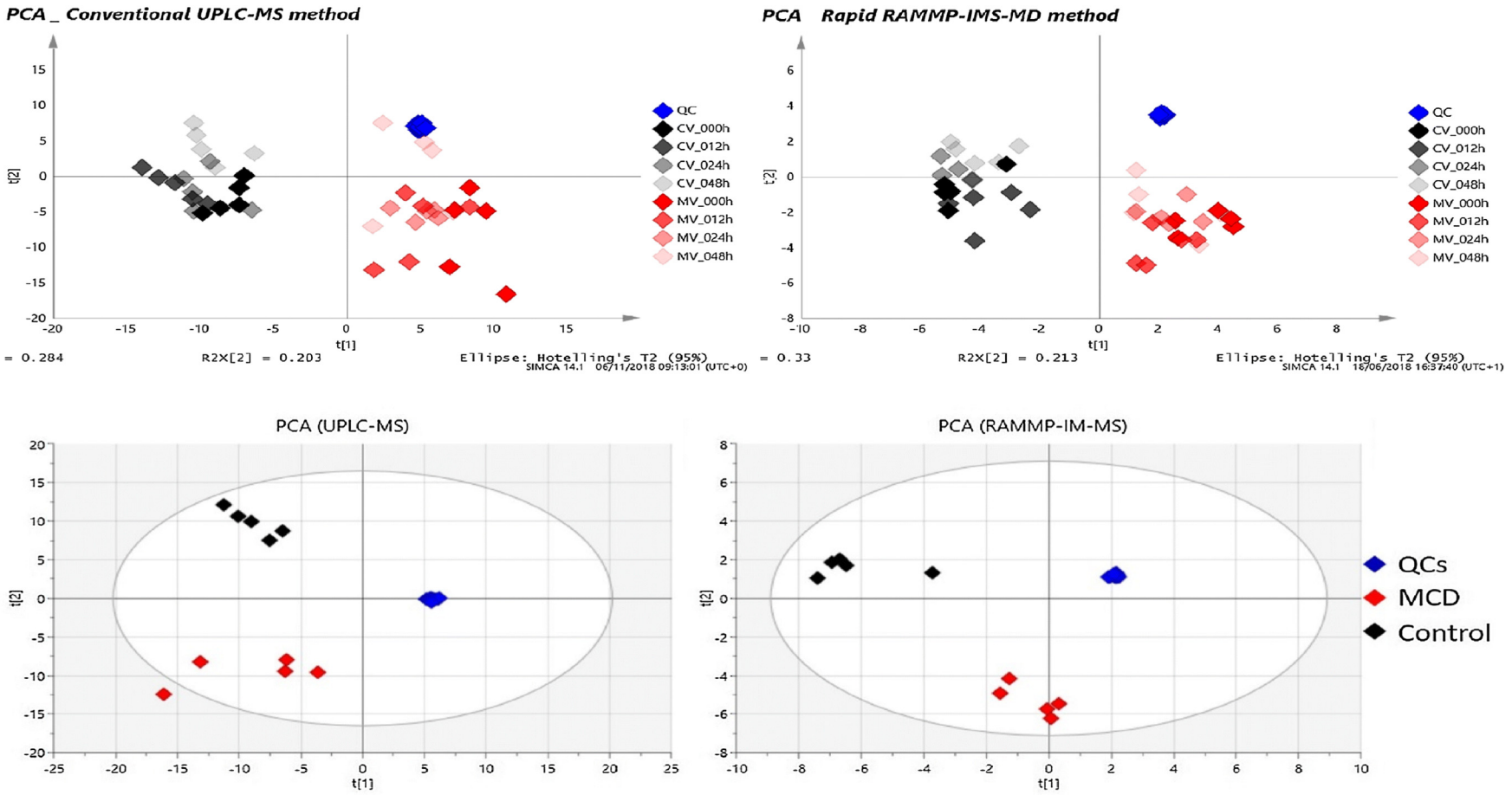

Fig. 2. PCA score plots showing the distribution of the urine samples according to their diet (control diet in black and MCD diet in red) at different time points after 8 days on the two diets. The PCA score plots show the distribution of the urine samples according to diet with control diet in black and MCD diet in red at different time points (i.e., 8 weeks + 0, +12, +24 and + 48 h, respectively)with the shade becoming lighter with time) and QCs (blue). Left upper: PCA score plots obtained when the samples were analysed by UHPLC–MS (4 principal components, N = 56 samples, R2X (cum) = 0.667 and Q2 (cum) = 0.561). Right upper: PCA score plot obtained when the samples were analysed using RAMMP/IMS/MS (2 principal components, N = 56 samples, R2X (cum) = 0.543 and Q2 (cum) = 0.495). The PCA plots in the two lower panels show the results for the −6 to 0 h samples analysed by either UHPLC–MS (Lower left) or RAMMP/IMS/MS (Lower right). **From Ref.** [34] **with permission.**

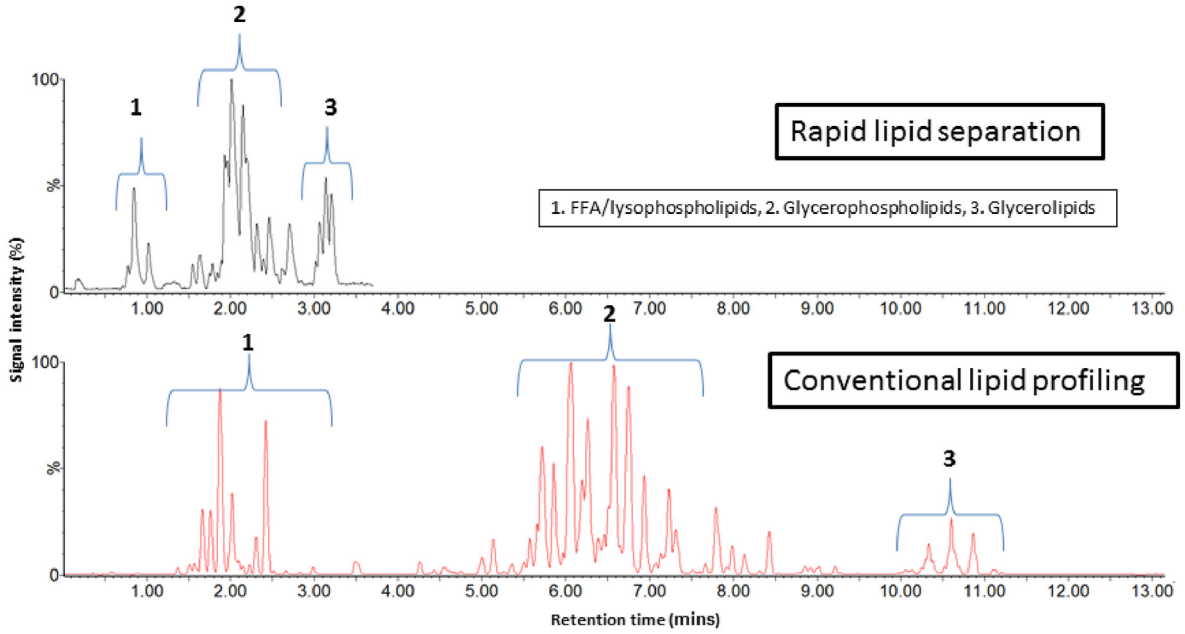

Fig. 3. Base peak ion (BPI) chromatograms of human plasma samples analysed using the rapid profiling method and a conventional lipid profiling method. Regions of lipid class elution are highlighted for both methods with free fatty acids (1) and lysophospholipids (2) eluting early and the larger more hydrophobic glycerolipids (3) eluting towards the end. **From Ref.** [37] **with permission.**

broadening, still results in broader peaks (often with peak tailing) than are desirable that reduce the potential peak capacity of the separation. On-column dispersion is often the result of frictional heating, whilst post column dispersion generally results from the tubing connecting the column, MS probe and liquid junctions/connections within the electrospray probe. These post column problems can be mitigated by e.g., using systems whereby the columns are located within the MS source as seen with chip-based approaches where this strategy has been shown to significantly reduce post column effects [39].

However, peak broadening arising from frictional heating within the column, caused by the solvent being forced through the tightly packed stationary phase under pressures of 10–15 kpsi, is unaffected by eliminating post column effects. The on-column band broadening induced by frictional heating is seen both in radial diffusion, resulting from the temperature differences between the core of the column and at its walls, and from longitudinal dispersion which takes place because of differences in the temperature at its inlet and outlet [40,41]. To remedy the effects of column frictional heating, the use of vacuum jacketed columns (VJC) combined with reducing the mass of the column end nuts was advocated to deal with the problem [42]. The effects of longitudinal heating were then attenuated via the application of heat to the inlet fitting resulting in an even temperature across the column. These modifications resulted in significant improvements, with column efficiency increasing by up to 30% [43,44]. Recently we have employed this novel VJC format, in a custom built housing located close to the MS source (to minimize the distance from the outlet of the column to the probe), to profile both endogenous and drug metabolites in human urine [45]. In this study we observed, with a 75 s VJC RP-UHPLC separation, illustrated in Fig. 4, that the average peak widths obtained were ca. 0.6 s for endogenous compounds. In comparison, conventional LC/MS under the same conditions gave average peak widths of 1.2 s. In addition, the use of the VJC system reduced the peak tailing factor from the 1.25 seen on conventional UHPLC to 1.13. In addition, peak capacity was increased from 65 on the conventional column to 120 with VJC, resulting in a 25% increase in feature detection). As well as enhancing resolution we also obtained a 10–120% increase in MS peak intensity, improving detection limits and raising the quality of the MS data. Reducing the gradient time to under a minute (37 s) gave peak widths of ca. 0.4 s with a potential peak capacity of over 80.

Subsequently the VJC system was applied to the analysis of lipids in plasma extracts [46] and again compared very favourably to conventional UHPLC/MS with, in addition to faster analysis, greater resolution and an increase in peak intensity of 2-fold. So, compared to the conventional approach, a 5 min VJC-based separation showed an increase in peak capacity of 66%, a reduction in peak tailing of up to 34%, and lipid detection increased by 30%. As seen with the metabolites detected in urine there was a significant improvement in the quality of the MS and MS/MS data which translated into a 22% increase in the lipids that were identified. Typical mass chromatograms for both UHPLC/MS and VJC UHPLC/MS where analysis was performed using the exactly the same conditions are shown in Fig. 5. From this figure, the improvement in peak shape and resolution resulting from the VJC UHPLC (particularly for the later eluting peaks (i.e., DG, TG, CE)) is clearly illustrated.

For the pooled QC samples analysed, when the VJC method was applied to mouse plasma samples, good reproducibility in terms of signal intensities, which ranged from 1.8 to 12%, and stable t_Rs were observed for the detected lipids [46]. Whilst we have not, as yet, combined VJC methods with IM we fully expect to obtain further improvements in metabolome coverage using such a combination. If the VJC system were implemented for a 3000-sample clinical or epidemiological study, and assuming that study and reference QC's were employed every 10 study samples, the overall batch size would be ca. 3600. Assuming a 45 s LC/MS analysis time, this would take ca. 27 h to process the batch. In comparison it would take ca. 25 days to perform the same analysis using a conventional 10 min analysis. In addition, the use of the VJC system would also save almost 20 L of solvent.

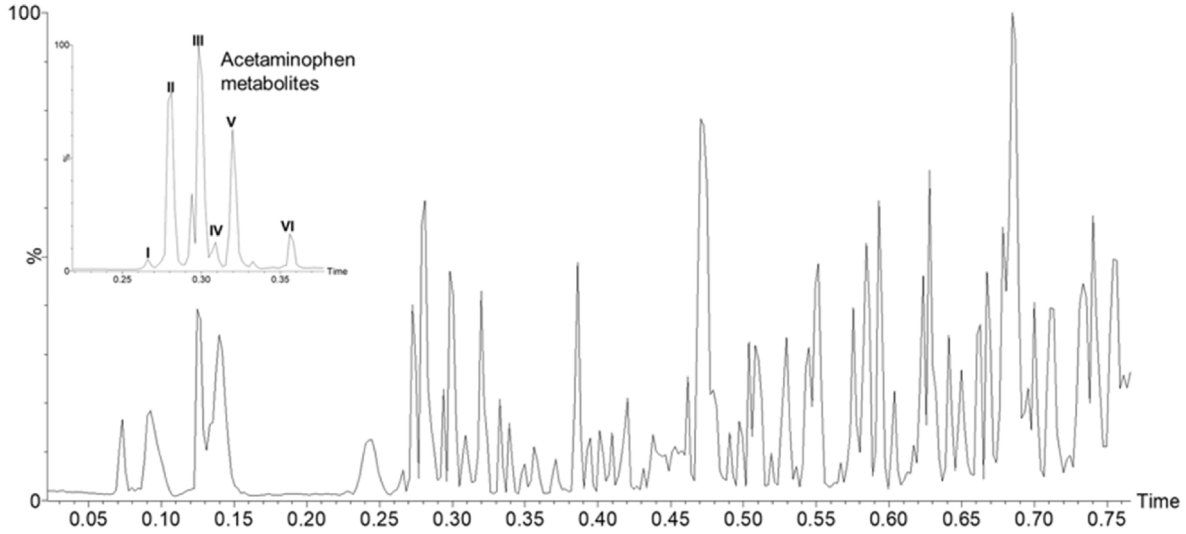

Fig. 4. Chromatographic traces obtained from the UPLC/MS (ESI^+) analysis of a urine sample obtained 1 h post-dose of 400 mg/kg APAP to a male human using the "0.5-min" VJC method performed on a HSS T3 2.1 mm × 30 mm 1.8 μm column. Inset, trace shows the extracted ion chromatogram of the acetaminophen metabolites where (I) APAP-OMe, (II) APAP glucuronide conjugate, (III) APAP sulphate conjugate, (IV) APAP cysteinyl conjugate, (V) acetaminophen, (VI) APAP *N*-acetylcysteinyl conjugate. **From Ref.** [45] **with permission.**

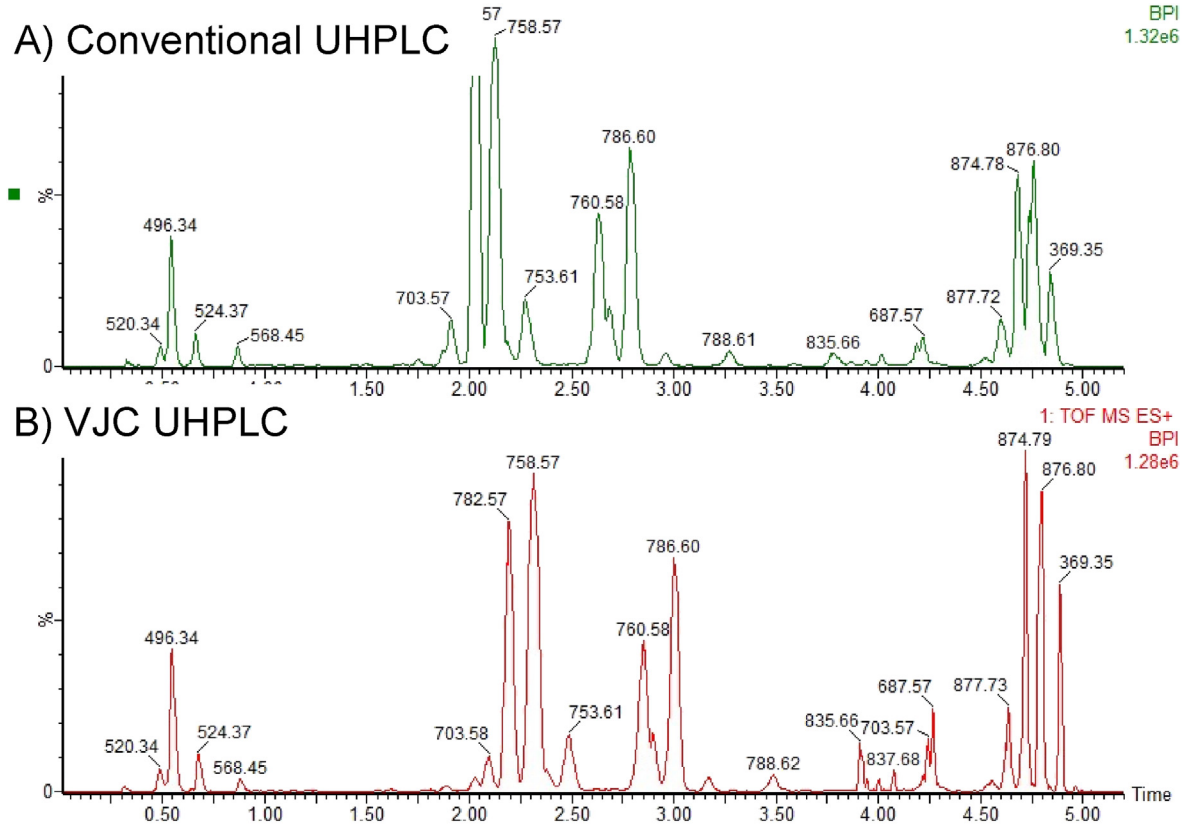

Fig. 5. Comparison of mass chromatograms obtained from the UHPLC/MS (+ve ESI) analysis of the NIST 1950 plasma (time scale in min). Upper trace (A): Data from the conventional method (ACQUITY UPLC CSH C18 1.8 μm 2.1 mm × 50 mm column). Lower trace (B): Data from the VJC method (ACQUITY UPLC CSH C18 1.8 μm 2.1 mm × 50 mm column). **From Ref.** [46] **with permission.**

1.2. Future directions in high throughput metabolomics (HTM)

The development of the VJC approach has enabled many of the potential benefits of UHPLC and rapid analysis for HTM to be realized by greatly reducing band broadening. However, the very narrow peaks and short analysis times now possible are not without their own problems. Very narrow (ca. Sub 1 s) peaks are challenging for MS detection whilst continuous sub 1 min analyses are not possible with many autosamplers as their wash cycles and preparation for the next injection often take ca 0.5–1 min. Both

problems should be solvable but will require investment by instrument manufacturers and users. Other approaches to HTM include the serial injection of samples onto a continuously flowing isocratic mobile phase with targeted MRM analysis [47], direct infusion of the liquid sample(s) into high-resolution MS or FTMS [48], direct infusion using targeted multiple reaction monitoring [49] and direct analysis of dried blood spots [50]. Although these methodologies are straightforward and ideal for small sample volumes they still only deliver analysis times in the 1–3 min range and may require the same solution in terms of instrumentation as VJC-based methods. So, whilst the removal of the separation process has benefits in eliminating the need for e.g., peak alignment etc., the absence of a separation can result in a significant increase in ion suppression, poor/no resolution of isomers (resulting in a significant reduction in feature detection, e.g., seen by Gray et al. [31]) and loss of biological information. All of these limitations of DIMS have meant that LC-MS has remained the dominant approach for comprehensive metabolic and lipid profiling. In an attempt to address the challenge of increasing throughput and ion suppression Baker et al. [51] combined rapid on-line solid phase extraction (SPE) with IM-MS for the analysis of endogenous and exogenous compounds in urine and plasma from large cohort studies. A similar approach was also employed by Taki and co-workers in the analysis of plasma samples from subjects with the serotonin syndrome [52] and by Rye and LaMarre for the characterization of pyrophosphate-dependent phosphofructokinase in *vitro* samples [53]. The use of this on-line approach reduced analysis times to as low as 10 s per sample, enabling the analysis of a biofluid sample in approximately 1 min (allowing for polar and non-polar stationary phases and +ve/-ve ion MS acquisition). The use of this on-line SPE approach boosted sensitivity by up to 3-fold compared to direct infusion, with good linearity ($r^2 > 0.99$) over 3 orders of magnitude (see Ref. [51]). The use of an IM separation prior to MS significantly increased confidence in analyte detection, as demonstrated by the resolution of thiabendazole from an isobaric endogenous compound in urine (see Ref. [51]). Although this approach shows promise it still required extensive sample pre-treatment for urine, including extraction, evaporation to dryness and reconstitution prior to analysis.

The batch processing times discussed above, with analysis by any of these methods including the VJC method, are not unattractive for sample batches in the 1000 to 5000 scale. However, for large biobank sample sets of 250,000 to 1 million, the analysis times would be on the scale of 2700 to 10,000 h. This may require alternative approaches to the challenge of HT-metabolic phenotyping which not only reduces analysis time but significantly simplifies analysis sample preparation. Approaches that may address some aspects of the issue of throughput, such as thermal desorption MS [54], acoustic ejection MS [55], desorption electrospray ionization MS (DESI) [56], and rapid evaporative ionization MS (REIMS) [57] offer speed and simplicity. They are also much simpler from an instrumental point of view eliminating the requirement for an autosampler by bringing the analyser to the sample rather than the other way round. However, these advantages do come at the expense of metabolome coverage. Nevertheless, for screening very large numbers of samples it may be that, as a first step in sample characterization, the potential throughput of a 1 sample/sec or better may make these very high throughput (VHT) approaches more suitable for biobanking, despite reduced metabolome coverage, with more in-depth analysis using LC/MS based methodologies of one sort or another to follow.

2. Conclusion

Over the last several decades developments in chromatographic LC/MS-based techniques have been impressive. These developments have greatly facilitated the use of MS, particularly LC/MS, for metabolic profiling (metabolomics/metabonomics). With each decade we have seen significant increases in the number of features detected, increased assay sensitivity, and improved MS methodologies that have aided the characterization and identification of potential actionable “biomarkers”. These advances have allowed metabolic profiling to be successfully employed in diverse areas such as disease biology, pharmaceutical drug development, patient stratification, nutrition and epidemiology studies. However, as the demand for this type of work has increased, the need for higher throughput assays delivering high quality data rapidly and efficiently has also increased. The need for further advances in HT-methods however, has still not entirely been addressed.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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