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A tandem liquid chromatography-mass spectrometry (LC-MS) method for profiling small molecules in complex samples.

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Abstract

Liquid chromatography-mass spectrometry (LC-MS) methods using either aqueous normal phase (ANP) or reversed phase (RP) columns are routinely used in small molecule or metabolomic analyses. These stationary phases enable chromatographic fractionation of polar and non-polar compounds, respectively. The application of a single chromatographic stationary phase to a complex biological extract results in a significant proportion of compounds which elute in the non-retained fraction, where they are poorly detected because of a combination of ion suppression and the co-elution of isomeric compounds. Thus coverage of both polar and non-polar components of the metabolome generally involves multiple analyses of the same sample, increasing the analysis time and complexity. In this study we describe a novel tandem in-line LC-MS method, in which compounds from one injection are sequentially separated in a single run on both ANP and RP LC-columns. This method is simple, robust, and enables the use of independent gradients customized for both RP and ANP columns. The MS signal is acquired in a single chromatogram which reduces instrument time and operator and data analysis errors. This method has been used to analyze a range of biological extracts, from plant and animal tissues, human serum and urine, microbial cell and culture supernatants. Optimized sample preparation protocols are described for this method as well as a library containing the retention times and accurate masses of 127 compounds.

Key words: reversed phase; aqueous normal phase; mass spectrometry; metabolomics; tandem liquid chromatography

1. Introduction

Major advances have been made in the development of new analytical approaches for metabolomics experiments. However, the complete coverage of the cellular metabolome remains a formidable challenge, reflecting both the chemical diversity and the large concentration range of compounds in biological extracts. Hyphenated mass spectrometry (MS) approaches, such as gas chromatography-MS (GC-MS), capillary electrophoresis-MS (CE-MS) and liquid chromatography-MS (LC-MS) are the most common analytical techniques used for detecting the diverse range of compounds (Buscher et al. 2009; van der Werf et al. 2007; Allwood and Goodacre 2010). LC-MS is particularly amenable in metabolomics work-flows as samples can be analyzed without the need for complex sample preparation and derivitisation procedures and a wide range of compounds with diverse chemical properties can be resolved. Improvements in the MS instrumentation coupled with LC have greatly increased sensitivity, mass accuracy and acquisition speeds enabling more robust measurements, higher throughput and the ability to identify compounds using publicly available mass spectral data bases such as MassBank and Metlin (Horai et al. 2010; Smith et al. 2006). However, LC separation of complex samples is still necessary for the resolution of isomers and the minimisation of ion suppression. Also, the chromatographic retention time provides additional information which assists in compound identification as the degree of interaction with the stationary phase also provides information on relative hydrophobicity of the molecule, depending on the stationary phase applied.

To measure a larger subset of the total pool of small molecules in a biological sample (metabolome), two LC phases are commonly used, reversed phase (RP) and hydrophilic interaction chromatography (HILIC) or aqueous normal phases (ANP), which retain compounds through hydrophobic interaction and hydrophilic partitioning, respectively (Liu et al. 2008; Wang et al. 2008). RP and HILIC/ANP provide orthogonal separation, as compounds that interact with one type of stationary phase, are weakly retained or not at all to the other. When used individually, a large proportion of compounds in biological extracts are eluted in a non-retained fraction with significant loss of information. This problem has been partly overcome by multiple analyses of the same sample by LC(RP)/MS and LC(HILIC)/MS (Kind et al. 2007; Yin et al. 2009). Off-

line two-dimensional (2D)-LC methods have also been described in which fractions collected from the first dimension are subsequently injected onto a second dimension (Liu et al. 2008; Kiffe et al. 2007). These methods greatly increase metabolite coverage, but at the expense of increased analysis time, sample handling and complex reconstruction of 2-D chromatograms. An alternative solution is to incorporate two chromatographic separations in a single analytical run. The use of in-line, two-dimensional liquid chromatography (2D-LC) techniques, where columns of complementary chemistries have been coupled, is well established in the proteomics field (Wu et al. 2012). For example, peptides are resolved with 2D-LC configurations that employ a strong cation exchange (SCX) column coupled with a RP column, which exploits the diversity in ionic strength and hydrophobicity of peptides to separate a greater number of peptides/proteins in the sample (Nagele et al. 2003). However, the development of comparable 2D methods for untargeted metabolomics analyses, have been hampered by the technical difficulties associated with combining solvent elution conditions that are compatible with both RP- and HILIC-based LC conditions as well as MS detectors (Wang et al. 2008; Jayamanne et al. 2010). As a result, current 2D LC-MS methods generally use LC columns with similar chemistries, have complex plumbing configurations and/or don't allow detection of compounds that are not retained by either of the matrices being used.

The coupling of orthogonal stationary phases for increasing metabolite coverage has attracted more interest recently. One approach has been to couple columns of orthogonal stationary phases directly in series so that the one column follows the other with either a single LC pump or two LC pumps (K. R. Chalcraft and McCarry 2013; Greco et al. 2013). These methods have been shown to successfully fractionate both polar and non-polar metabolites in series and have been applied to metabolomics research (K. Chalcraft et al. 2014; Rajab et al. 2013). The serial column approach, however, does not allow the application of completely independent gradients which are optimized for both columns. An alternative two column approach has been published with uses two sample injections, one directed to a RP column and another to a HILIC column providing separate gradients, however, this approach requires two sample injections and a complex 10-port valve configuration (Klavins et al. 2014).

Here we describe a tandem LC configuration that links an ANP column with a RP column to serially resolve hydrophilic and hydrophobic compounds respectively in a single on-line chromatographic run with independent gradients for both columns. The ANP column is used in preference to the HILIC column as it allows shorter re-equilibrations times, and is less sensitive to retention time shifts in response to changes in pH or salts (Callahan et al. 2009). This procedure utilizes a trapping step, in which hydrophobic compounds are retained to a RP column, followed by a mobile phase conversion from high aqueous concentration to high organic concentration to allow hydrophilic molecules to be fractionated by the ANP column. Un-retained compounds, as well as compounds that are sequentially eluted from ANP and RP can then be detected by the MS in a single run. The application of independent gradients to both columns provides a flexible method so that new developments in column chemistry can be applied, for example, a high temperature fast gradient with a core shell column could be used for the RP column while a slower HILIC gradient is applied to the other ANP or HILIC column. A comprehensive review on these developments in relation to metabolomics has been published (Kuehnbaum and Britz-McKibbin 2013). Here we demonstrate the utility of this method with untargeted metabolite analysis of plant and animal tissues as well as biofluids and microbial extracts, including optimized extraction protocols for a number of different samples types.

2. Materials and Methods

2.1 Reagents and standards

LC-MS grade formic acid (FA), ammonium formate , ammonium hydroxide, valine ($^{13}C_5H_{11}{}^{15}NO_2$), sorbitol ($^{13}C_6H_{14}O_6$), 1-naphthyl amine, 2-aminoanthracene, pantothenic acid (unlabeled) and standards such as amino acids and sugars were purchased from Sigma–Aldrich (Sydney, Australia). Lipid standards, myristic acid ($^{13}C_2C_{12}H_{28}O_2$), 17:0 lysophophatidylcholine (LPC) and cholesterol ($C_{27}H_{39}{}^2H_7$ O) purchased from Avanti Polar Lipids. HPLC/pesticide grade acetonitrile (ACN) and methanol (MeOH) were purchased from Burdick and Jackson (Ajax, Australia). Deionized water (18.2 MΩ) was used throughout experiments (Millipore).

2.2 Sample preparation

Extraction protocols for cytosolic metabolites from *Mus musculus* muscle tissue, *Caenorhabditis elegans*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Leishmania mexicana* and *Arabidopsis thaliana* leaf tissue as well as metabolites from *Homo sapiens* urine and plasma biofluids were optimized for this method. In general, the workflow was to quench metabolic activity using various rapid chilling methods, wash cells where applicable and then extract metabolites in various solvents. Total extracts, containing polar and non-polar compounds, were concentrated under vacuum when necessary and finally re-suspended in 50% MeOH supplemented with 0.1% FA (100 µL) with vigorous mixing prior to LC-MS analysis. Samples must be reconstituted in a solvent mixture that contains at least 50% water (v/v). While an organic solvent content of up to 50% may be required to solubilize lipids and other hydrophobic compounds, higher concentrations of organic solvent reduces trapping efficiency of hydrophobic compounds on the RP columns, which is an important consideration with the tandem LC configuration described. A mixed internal standard containing $^{13}C_6$-sorbitol, $^{15}N^{13}C_5$-valine, $^{13}C_2$-myristic acid and $^{2}H_7$-cholestorol representing hydrophilic and hydrophobic compounds were added to samples to a final concentration of 20 µM of each standard. Online Resource 1 describes optimised extraction protocols for this method with the following samples types: urine, plasma, *E. coli*, *S. cerevisiae*, *C. elegans*, *L. mexicana*, *M. musculus* tissue and *A. thaliana* leaf tissue.

2.3 Instrument Conditions

An Agilent 1200 series LC (Agilent Technologies, Santa Clara, US, USA) system equipped with two vacuum degassers, two binary pumps, a thermostat controlled autosampler and a thermostat controlled column compartment containing a 10-port 2-position valve (a minimum of 6-ports are required). The method described here used 8-ports, the two extra ports (9 and 10) were not used and are not shown in figures. All connections were made using 180 µm internal diameter stainless steel tubing with the shortest practical lengths used to minimise band broadening. Chromatography was performed using an ANP Cogent Diamond Hydride column (2.1 mm × 100 mm, 4 µm particle size, MicroSolv Technology, Australia) and a Zorbax Eclipse XDB-C18 column (2.1 mm × 50 mm, 1.8 µm, Agilent Technologies, Australia; RP-A) with a matching guard

column (2.1 × 15 mm, 1.8 µm; RP-G) for trapping compounds. Normal operating pressures were less than 250 bar with this configuration. The LC was coupled with an Agilent 6520 QqTOF MS detector (Agilent Technologies, Santa Clara, USA) via a dual electrospray ionisation (ESI) source. The MS was calibrated before acquisition of each sample set using the manufacturers recommended standard calibration procedures. The API-TOF reference ion mix (Agilent Technologies, Santa Clara, USA) was continuously infused through the second ESI needle for in-spectrum mass calibration throughout each analysis. The ESI conditions were: nebulizer pressure 45 psi, drying gas flow-rate 10 L/min with a temperature 325°C, capillary voltage 3500 V for negative mode and 4000 V for positive mode, fragmentor 150 V and skimmer 65 V. The MS was operated in the extended dynamic range mode and data collected in *m/z* range 50–1700 u.

2.4 Mobile phases

All mobile phases were prepared in clean, freshly rinsed glassware and used within 5 days. For positive ionization pump 1 contained: mobile phase A 0.1% formic acid water, mobile phase B 0.1% formic acid in acetonitrile; pump 2 contained: mobile phase A 0.1% formic acid, 50% MeOH in water, mobile phase B 0.1% formic acid in acetonitrile. For negative ionization 10 mM ammonium formate adjusted to pH 7 with ammonium hydroxide was used in place of formic acid for mobile phase A in both pumps. The ANP Mobile phases, A1 and A2 were prepared in Teflon bottles (2100-0032, Nalgene) to minimize sodium ion contamination which can reduce chromatographic performance on a diamond hydride column (Fischer et al. 2010).

2.5 Tandem LC configuration

RP stationary phases require mobile phase gradients which start with high aqueous content while the ANP/HILIC stationary phases require initial high organic content for metabolite binding. The following description details how we have linked these two chromatographic sequences together. We use an LC system with an 8-port 2-position valve as shown in Fig. 1 and timed mobile phase gradients events as detailed in Table 1. The configuration of a 6-port 2-position valve is shown in Online Resource 2. The method

has four major steps: a sample loading step, then two independent resolving steps on the ANP column and RP columns, followed by re-equilibration of columns to the initial conditions.

During the loading step (Fig. 1A) both pumps run isocratically for 5 mins (RP column pump at 2% acetonitrile; ANP column pump at 100% acetonitrile). The sample is injected into the mobile phase stream from Pump 1 where compounds with some degree of hydrophobicity are retained by the RP guard column (RP-G). The hydrophilic compounds not retained by the RP-G pass through to a T-piece where the mobile phase stream is mixed with the mobile phase stream from Pump 2 containing 100% ACN. This increases the ACN concentration of the mobile phase to 91.8% ACN (at 600 µl/min) allowing retention of compounds that have hydrophilic properties on the ANP stationary phase. The flow-rates used in the loading step are important as the ratio of aqueous to organic solvents in the mixed flow greatly affects retention of hydrophilic compounds on the ANP column. For example, if the flow-rate of pump 1 is increased in the loading step, the organic solvent composition on the ANP column will be reduced and therefore reduce retention of highly polar molecules.

Following the loading of hydrophobic and hydrophilic compounds onto the RP-G and ANP columns, respectively, the valve is switched at 5 min to isolate the T-piece and allow independent mobile phase gradients to be run on each column from Pump 1 (RP) and Pump 2 (ANP) (Fig. 1B). As indicated in Table 1 and Fig. 1C, the timed events allow hydrophilic compounds to be eluted from the ANP column first, facilitated by a mobile phase gradient from Pump 2 (5-15 min). At 15 minutes the mobile phase flow from Pump 1 is increased to the RP columns optimal flow rate of 300 µl/min and Pump 2 is decreased to 300 µl/min to maintain a total flow into the ESI of 600 µl/min. The hydrophobic compounds are eluted from the RP columns using gradient elution from Pump 1 (15-25 min) and including a 5 min isocratic hold at 100% solvent B (25-30 min). Finally, both columns are returned to the starting conditions (30-31 min) and re-equilibrated (31-35 min), giving a total run-time of 35 min.

A typical mass feature extracted chromatogram from a human urine extract illustrating the three key chromatographic regions, trapping (isocratic analysis), ANP analysis and RP analysis is shown in Fig. 2. The chromatographic regions representing polar metabolites from the ANP column and hydrophobic compounds from the RP column can be seen. This setup is flexible and alternative stationary phases, such as a HILIC can be used in place of the ANP column. The gradients and time of analysis can all be varied. The method described here has been found to be optimal with regards to selectivity and throughput. The reverse configuration was also tested where an ANP guard column was used to trap hydrophilic compounds. However the column capacity of the ANP guard column was significantly lower than that of the RP column resulting in non-retention of hydrophilic analytes and reduced capacity for sample loading. The described configuration with the RP-G as the trapping column is therefore recommended.

2.6 Comparison to single column LC configuration

The tandem LC-MS method was compared to single column methods. The same corresponding LC gradients and MS source conditions were applied. The starting conditions equivalent to sample loading steps in the tandem LC-MS method were maintained from 0-5 minutes as the sample was loaded. The ANP and RP columns were then eluted with the same mobile phase gradient 5-15 min for the ANP column and gradient 15-25 min for the RP column (Table 1) including the same wash and re-equilibration steps.

2.7 Data analysis

Chromatographic deconvolution was carried out using the MassHunter Qualitative Analysis software (Agilent Technologies, Santa Clara, US, version B.04.00). The 'Find by Molecular Feature' algorithm was used to generate molecular feature lists that determined the charged and neutral mass of compounds and identified related masses corresponding to Na^+, K^+ and NH_4^+ adducts; neutral loss of H_2O; as well as dimers and trimers (clusters of more than one unit of the same molecule). A peak intensity threshold of 1,500 counts was used and resulting peaks were searched against the KEGG and the Human Metabolome Database (HMDB) databases for putative identifications with a 10 ppm *m/z* tolerance and a 30 sec retention time window when known (Wishart et al. 2013). For targeted analysis, the 'Find by Formula' algorithm was used with a

database of compounds with known retention times. Where sample alignment was required, the data was exported from MassHunter Qualitative Analysis software into mzData format with no filters in the settings and processed in MZmine 2 version 2.9.1 (Pluskal et al. 2010) .

3 Results and Discussion

3.1 Method validation and profiling

3.1.1 Linearity

Eight standards were selected to check linearity of both columns in the tandem LC configuration: valine, alanine, glycine, sorbitol and creatinine for the ANP column and pantothenic acid, 1-naphthylamine and caffeine for the RP column. A seven point calibration curve was prepared in the concentration range of 0.1-150 µM. All standards resulted in a linear response with an R^2 of at least 0.98 over this range (Online resource 3). The linearity observed showed that the RP-G column is able to trap hydrophobic compounds over a wide concentration range. Overloading of the RP-G column results in the detection of hydrophobic compounds in the loading phase of the analysis between 0-5 mins; this was not observed in the concentration range tested (0.1 – 150 µM) which corresponds to the normal linear dynamic range of the instrument used. The detection limit is dependent on the ionization efficiency of the analyte and MS detector and therefore not relevant for this method validation Linearity of spiked standards for RP resolved analytes was also tested in a sample matrix. A linear response for all three analytes was observed in the same concentration range (0.1 – 150 µM) showing the trapping efficiency in this range was not effected by the presence or concentration of a sample matrix (Online resource 3).

3.1.2 Precision

Ten replicate injections of a urine extract with added internal standards were carried out in order to assess retention time and peak area reproducibility on both phases in a complex biological mixture. The internal standards of $^{13}C_6$ -sorbitol (RT 3.6 min), $^{13}C_5{}^{15}N$ -valine (RT 9.6 min) was used for testing on the ANP column

and 1-napthylamine (RT 20.4 min) and pantothenic acid (RT 16.0 min) were used for testing the RP column; the endogenous metabolite creatinine was also monitored. For the standards, the percent relative standard deviations (% RSDs) for peak areas ranged between of 0.92-2.88 % RSD and 0.03- 0.4 % RSD for retention time (Online resource 4). The total number of mass features from the 10-repicate injections was also examined. The mean number of features using a 1,500 count intensity threshold was 1,181 with a % RSD of 1.1%. The minimum and maximum number of features was 1,163 and 1,203, respectively. These results show that the method reproducibility is similar to single column methods.

The reproducibility in the detection of the number of metabolites in a range of different samples types using the optimized extraction protocols described was also determined (Table 2). A total of eight biological replicates were extracted and analysed in order to map variability of sampling and analysis. Additional post-peak finding filtering can be applied to reduce the % RSD. However, this type of filtering is a decision to be made by the analysts and depends on the experimental design. The number of features detected per sample using the 'find by molecular feature' algorithm depended on the intensity threshold set for peak finding, instrument sensitivity and sample loading. Thresholds were adjusted so that the majority of low abundant peaks had a signal-to-noise of ≥ 3. In most cases the variability is due to low abundance peaks which only just make the threshold. As expected, unique metabolic profiles were detected for each sample type. All sample types tested contained the internal standards. The retention times and peak shapes were not affected by the sample type analysed. For each sample type it was important that the trap column was not overloaded. Overloading resulted in poor trapping on the guard column and a non-linear response at higher concentrations. It is therefore necessary to optimise column loading using the described sample preparation protocols as a starting point. Technical replicates typically produced %RSD of less than 5 for the number of mass features detected. These data show that the method can be used for typical metabolomics workflows for a range of sample types providing the correct quality control measures, such as, inclusion of pooled biological quality control samples, internal and external standards are followed (Dunn et al. 2011).

To measure long term (inter-day) reproducibility 80 plasma samples and 14 separately extracted pooled biological QC samples were prepared using the protocol described in Online Resource 1. These samples were analysed over a 3-day period; PBQC samples were analysed every six samples. A data matrix containing all aligned peaks for the PCQC samples was analysed. Typical response degradation was evident over the three days for the four internal standards, $^{13}C_5{}^{15}N$ valine, $^{13}C_6$ sorbitol and $^{2}H_7$-cholesterol (Online Resource 5). The reduction in the mean area (%50) of all detected metabolites was also consistent with single column analysis (Online Resource 5).[33] The %RSD of all mass features was less than 25% over the 3-day period.

3.2 Single column versus tandem LC configuration

The tandem LC-MS method provided higher metabolite coverage in comparison to a single ANP or RP LC-MS only method. For example, tandem LC-MS analysis of extracts of human serum resulted in the detection of approximately 1,500 molecular features. In contrast, analysis of the same plasma sample by either ANP-LC-MS or RP-LC-MS separately resulted in the identification of approximately 660 and 960 molecular features, respectively showing the tandem LC method detects similar numbers of metabolites as the two individual methods combined.

The retention times of individual metabolites in the tandem and single column LC-MS analyses were plotted against each other to compare the chromatographic profiles of the columns run independently and in the tandem LC configuration (Fig. 3). This plot was made by matching metabolites with a mass tolerance of 10 ppm and a library score, which includes isotope ratios, of ≥70. In region 1, the separation of metabolites was higher on the single columns than by tandem LC-MS. The RP only LC-MS had higher chromatographic resolution across the chromatogram. This is likely due to the trapping phase of the tandem LC analysis. Regions 2 and 3 show elution times of metabolites that were resolved in either the ANP-LC-MS or RP-LC-MS, respectively, as well as in the tandem-LC-MS analysis. Region 5 and 6 correspond to metabolites that are not resolved during individual either RP-LC-MS or ANP-LC-MS analyses, respectively, but are well resolved on the tandem-LC-MS. Region 4 is the void volume and isocratic region for all LC configurations. Significantly, the chromatographic elution of the majority of metabolites that were resolved on the ANP (red dots) and RP

(blue dots) columns displayed similar retention times within the comparable gradient elution sequences in the tandem LC-MS. Overall, these analyses indicate that tandem LC-MS method gives comparable metabolite coverage to the combination of the two individual LC-MS methods.

Ion suppression for RP only, ANP only and the tandem-LC method was measured by infusing a $^{13}C_5{}^{15}N$ valine standard (10 µM) using a syringe and a T-piece connected between the eluent and the MS. A urine extract was used to test ion suppression regions. Three clear ion suppression regions were observed for the tandem-LC method (Online Resource 6). The first is related to the elution of the non-retained metabolites, the second relates to the valve switch which momentarily alters the flow to the MS and a third region corresponding to the elution of creatinine and glycine which are both present at high concentrations in the urine extract. In comparison to the single column RP method, the tandem-LC method shows a similar profile in the RP region. The ANP only method show a large drop in signal for the infused valine standard in comparison to the tandem-LC method showing a reduction in ion-suppression tandem-LC. Overall, gradual increases/decreases in intensity of the infused standard can be attributed to changes in solvent composition eluting from the column. As the organic content increases the desolvation efficiency increases and an associated increase in signal is observed e.g. RP only method. This effect is not as pronounced in the tandem-LC method as there is a more consistent flow entering the MS, during RP elution on the tandem-LC the solvent composition is from both pumps. For negative ionisation mode two ion-suppression regions were observed the first relating to the elution un-retained metabolites and second corresponding to the valve switch at 5 min. A region of ion-enhancement was observed at RT 7 min.

A comparison of the peak width at half height for the single column methods compared with the tandem-LC method was made (Online Resource 7). A small improvement in peak widths was observed with the RP only versus tandem-LC and a small increase was observed in the comparison of the ANP only and tandem-LC. The urine sample tested for this comparison contained 38% acetonitrile which results in some peak fronting on the RP only method for early eluting peaks. This effect is not observed in the tandem-LC method. Therefore

the peak widths are slightly narrower (0.05 compared with 0.059). This shows that the trapping step can actually improve column efficiency by removing sample and solvent matrix effects which can have deleterious effects on column performance and MS sensitivity. This was not observed on the ANP column, the peak widths were found to be wider, 0.13 compared with 0.10 peak width at half height, showing that the extra system volume prior to reaching the column has an effect on the peak width. This will reduce the ability to separate isomeric compounds such as isoleucine/leucine and reduce sensitivity. The extra system volume is not present prior to the RP column and therefor band broadening is not observed.

3.3 Limitations

The described method is more complex than a single column method and requires an additional LC pump However, it is comparable to other two dimensional LC methods and most modern LC/MS systems are now standardly equipped with two pumps. The setup times are also longer than a single column protocol, which may be an issue in facilities with many users and/or different applications. The increase in complexity also requires that quality control protocols are included which assess the system performance of both columns. The sample loading capacity (as discussed above) is also lower than single column methods, however, it was found that typically the MS detector signal was overloaded before the loading capacity of the column was exceeded. With regards to chromatographic peak shape the increase in the system dead volume as well as the holding time on the RP-G column did result in increased chromatographic peak widths for the ANP column of the tandem LC-MS method, however, peak areas were similar when compared to the single column methods. The reduction in chromatographic effeciency observed on the ANP column effects the resolution of isomers, for example,isoleucine and leucine. These two structural isomers are not resolved well using the tanderm LC method, however, they can be resolved as a single column method using conditions which target amino acids.

The gradient conditions reported here were selected to cover a broad range of metabolite chemistries rather than being optimised for specific target compounds. The mobile phase pH greatly effects the

retention characteristics of many polar metabolites therefore mobile phase composition is particularly important for the ANP column. Metabolites with either acidic or basic functional groups will be charged at certain pH ranges and mixtures of these charged species can result in poor chromatography, as has been reported for nucleotides, sugar phosphates and organic acids (Zhang et al. 2012). The mobile phase pH will also effect the stationary phase chemistry. These issues need to be considered when selecting either the low pH (formic acid) or high pH (ammonium carbonate) mobile phase. If a certain subset of the polar metabolome is required then the retention mechanism needs to be considered. For example, higher pH favours the peak shape of acidic compounds, such as succinic acid and citric acid which are detected in negative ionisation mode. Gradients which have been optimised for compound classes using either column can be reproduced in the tandem-LC configuration. A review of applied methods and a detailed study of the retention mechanism for the ANP column has been published (Kulsing et al. 2014; Pesek et al. 2013). Also, this method as described is suited to the analysis of polar to moderately polar metabolites and does not cover the lipidomics spectrum of molecules. The methodology would need to be adapted to include stronger solvents both for sample extraction and the reversed phase mobile phases.

3.4 Standard Library Curation

Pure standards for 127 typical metabolites were prepared (20 μM) and analysed using the described protocol. The elution times for different metabolite classes detected using tandem LC-MS are given in Online Resource 8. Polar metabolites such as monosaccharides (2-4 min), disaccharides (3-5 min) and trisaccharides (>5 min) were detected as their Na^+ adducts in positive mode. Organic acids (1.9-3.0 min) were detected in negative ion mode and amino acids (9-15 min in both polarities. Fatty acids, phospholipids and galactolipids eluted between 19-30 min, while sterols eluted between 21-32 min. It is important to note that the gradients and mobile phase composition can be modified to favor the separation of particular compound classes on each column. A number of methods using the diamond hydride column have been reported which include chromatographic conditions optimized for the separation of specific metabolite classes such organic

acids or amino acids (Callahan et al. 2009; Pesek et al. 2008; Pesek et al. 2009; Young et al. 2011). The tandem LC configuration is flexible and could incorporate these different gradients.

3.5 Other LC-MS configurations

While the LC configuration outlined here was found to be optimal for many biological samples, a number of alternative LC configurations and solvent elution conditions are possible. For example, it is possible to reverse the configuration of columns mobile phase gradients and load the sample on an ANP guard column and sequentially elute the sample from the ANP and RP analytical columns. This configuration can be used to load samples that have been extracted in high concentrations of organic solvent (>50% ACN or MeOH) without an intermediate drying step in which some volatile compounds can be lost. However, as mentioned above, the trapping efficiency of the ANP guard column is not as high or as reproducible as the RP guard column, limiting the amount of sample that can be loaded.

Using the MS in fast polarity switching enables the analyst to carry out an analysis which would be equivalent to four different methods from a single injection. This configuration allows the analysis of abundant relatively hydrophilic and hydrophobic compounds, but a significant loss in sensitivity was observed when polarity switching on a time of flight MS was applied as well as large increase in scan times.

For targeted routine analysis of relatively simple samples, it is possible to shorten the run time by eluting both the RP and ANP column simultaneously, enabling both hydrophilic and hydrophobic compounds to be detected from a single injection in the same time as a single column chromatography. Also, an alternative 6-port injector configuration can be used which is outlined in Online Resource 2. Finally, it is possible to include additional columns using this type of configuration (Online Resource 9), although each additional column requires another LC pump and switching valve.

4 Concluding remarks

The tandem LC-MS method described allows fractionation and sensitive detection of both hydrophobic and hydrophilic molecules on RP and ANP columns from a single sample injection using independent gradients on both columns. This method can produce accurate and precise metabolite measurements in a large range of biological matrices with a number of advantages over the use of single column LC-MS approaches, as well as previously described 2D methods. It also reduces data analysis times with the removal of separate LC configurations for polar versus non-polar metabolites and also reduces instrument setup time. Also, as data collection is the same as a single column analysis any in-house data processing algorithms, such as peak area normalization, can be applied. The tandem LC configuration is flexible and can utilise other chromatographic stationary phases such as graphitized carbon, HILIC, ion exchange and size exclusion, provided that the solvents are compatible between both columns and the MS. This flexibility allows new developments in hardware, such as the ultra-high pressure LC systems, as well as new chromatographic stationary phases to be utilized. In particular, the application of a UHPLC system using the described configuration could shorten run-times and further improve chromatographic selectivity thus increasing sample throughput. This method, coupled with the accurate mass and retention time library increases workflow efficiency in metabolomics without the compromises that normally accompany one- and two-dimensional chromatography.

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Table 1 Mobile phase gradients, flow rates and valve positions

Time (min)	**LC Pump 1 Solvent B (%)**	**LC Pump 1 Flow Rate (µL/min)**	**LC Pump 2 Solvent B (%)**	**LC Pump 2 Flow Rate (µL/min)**	**Valve Position**	**MS Detector**
0.0	2	50	100	550	1	Start
5.0	2	50	100	550	2	
14.9	2	50	40	550		
15.0	2	300	40	300		
25.0	100	300	40	300		
30.0	100	300	40	300		Stop
31.0	2	300	100	300		
35.0	2	300	100	300	1	

Table 2 The total number of mass features detected and the % RSD in the number of mass features from eight biological replicates in different samples types using the extraction protocols detailed in SI 1. %RSD average peak area was calculated from %RSD of each individual mass feature within the sample type. These data show both sample homogeneity, extraction reproducibility and the tandem LC method. No filtering was applied to these numbers

Sample source	Sample type	Mean number of features	% RSD number features	% RSD average peak area
E. coli	Wet cell extract	1228	9.8 %	25.12
H. sapiens	Plasma extract	2056	3.8 %	14.8%
H. sapiens	Urine extract	1212	8.0 %	15.4%
S. cerevisiae	Wet cell extract	1362	2.1 %	9.0%
C. elegans	Multicellular tissue extract	2472	12 %	18.4%
Mus. musculis	Multicellular tissue extract	4248	7.7 %	30.8%

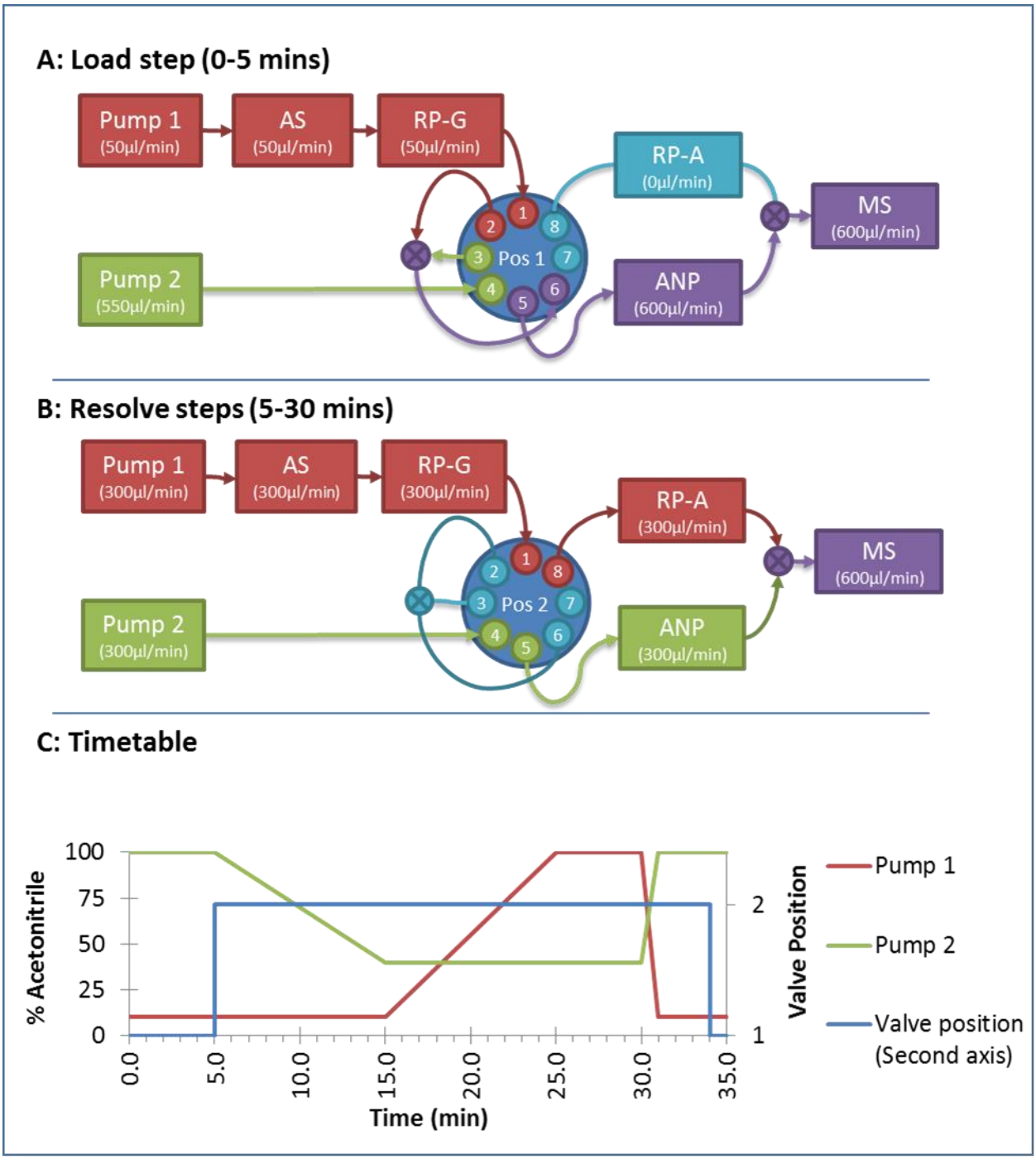

Fig. 1 The configuration of the tandem LC system and mobile phase flow during the loading and resolving phases. Red arrows indicate the flow path of solvent from Pump 1. Green arrows indicate the flow path of solvent from Pump 2. Purple arrows indicate the flow path after solvents from Pumps 1 and 2 are mixed. Blue lines indicate components that have no-flow. (A) Loading phase: the sample is injected onto the RP guard column using pump 1. The eluent (red arrows) is then diverted through an 8-port 2-position valve (Port 7 is blocked to prevent solvent back-flow). (B) Resolving phase: flow of samples when the system is eluting retained molecules with gradients from each pump. (C) Solvent gradients from Pump 1 (red) and Pump 2 (green) and the column valve position (blue) throughout an analysis

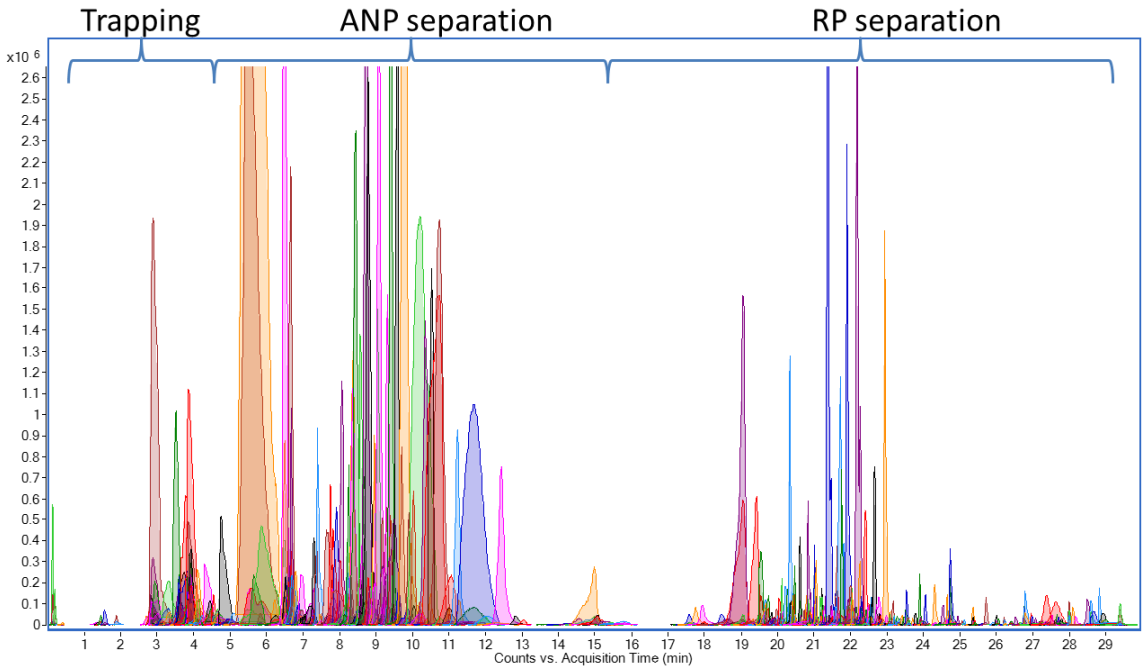

Fig. 2 A typical positive ion mass feature extracted chromatogram from a urine extract showing 1,570 peaks

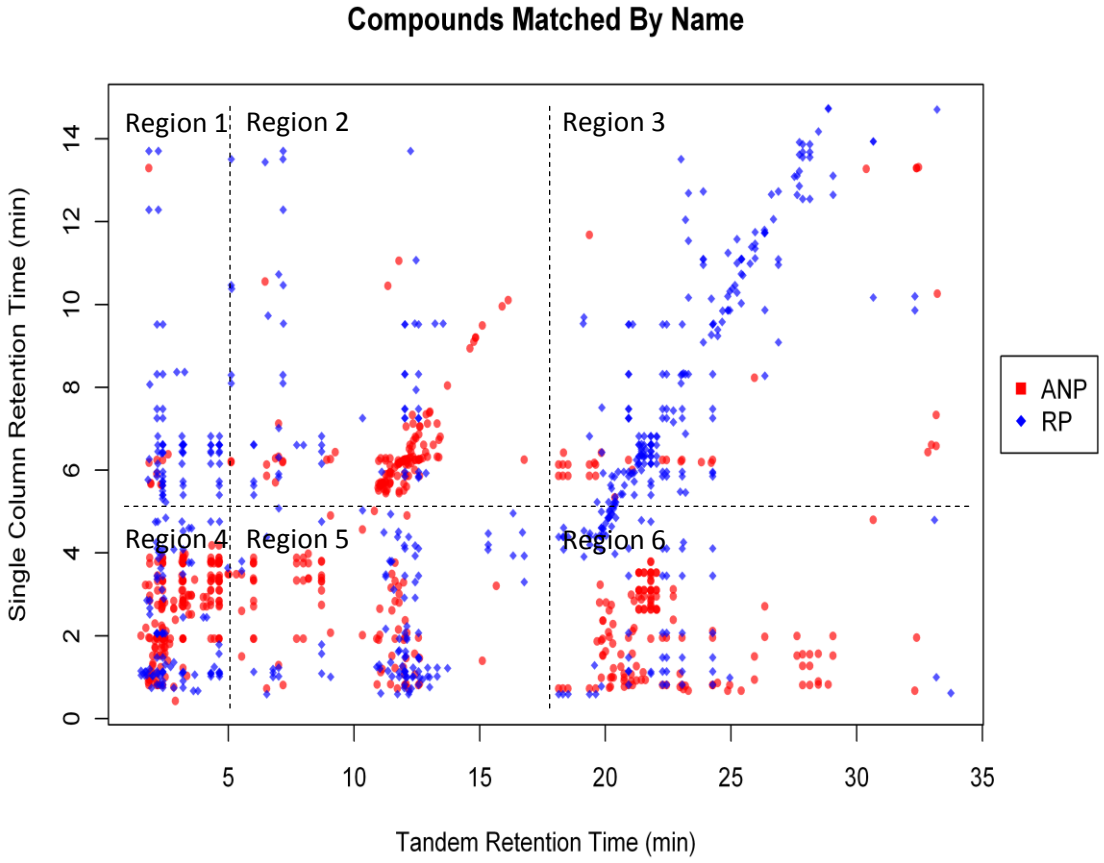

Fig 3 A plot of the same metabolites detected in the single column methods versus the tandem LC method from a human plasma sample. The y-axis corresponds to the retention time of the single column ANP (red) and RP (blue) (y-axis) methods plotted against the retention times on the tandem LC system (x-axis). R4 shows metabolites that were detected in the isocratic fractions of both the single and tandem LC runs. Region 1 (R1) shows metabolites that were detected in the unbound fraction of the tandem LC run. Regions 5 & 6 show metabolites that were detected in the void fractions of the ANP and RP single LC runs, respectively. Regions 2 and 3 show metabolites that were retained and eluted in solvent gradients in all three analyses. A significant fraction of metabolites were resolved on the tandem LC system, but not retained on either the ANP or RP columns (regions 5 and 6)