Evaluation of Mobile Phase Characteristics on Three Zwitterionic Columns in HILIC mode for Liquid Chromatography-High Resolution Mass Spectrometry based Untargeted Metabolite profiling of Leishmania Parasites

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Abstract

It has been reported that HILIC column chemistry has a great effect on the number of detected metabolites in LC-HRMS-based untargeted metabolite profiling studies. However, no systematic investigation has been carried out with regard to the optimisation of mobile phase characteristics. In this study using 223 metabolite standards, we explored the retention mechanisms on three zwitterionic columns with varied mobile phase composition, demonstrated the interference from poor chromatographic peak shapes on the output of data extraction, and assessed the quality of chromatographic signals and the separation of isomers under each LC condition. As expected, on the ZIC-cHILIC column the acidic metabolites showed improved chromatographic performance at low pH which can be attributed to the opposite arrangement of the permanently charged groups on this column in comparison with the ZIC-HILIC column. Using extracts from the protozoan parasite *Leishmania*, we compared the numbers of repeatedly detected LC-HRMS features under different LC conditions with putative identification of metabolites not amongst the standards being based on accurate mass (±3 ppm). Besides column chemistry, the pH of the mobile phase plays a key role in not only determining the retention mechanisms of solutes but also the output of the LC-HRMS data processing. Fast evaporation of ammonium carbonate produced less ion suppression in ESI source and consequently improved the detectability of the metabolites in low abundance in comparison with other ammonium salts. Our results show that the combination of a ZIC-pHILIC column with an ammonium carbonate mobile phase, pH=9.2, at 20 mM in the aqueous phase or 10 mM in both aqueous and organic mobile phase components, provided the most suitable LC conditions for LC-HRMS-based untargeted metabolite profiling of *Leishmania* parasite extracts. The signal reliability of the mass spectrometer used in this study (Exactive Orbitrap) was also investigated.

Keywords: HILIC-HRMS (Hydrophilic Interaction Liquid Chromatography-High Resolution Mass Spectrometry); zwitterionic stationary phase; Metabolomics; untargeted metabolite profiling; *Leishmania*. 
1. Introduction

Untargeted metabolite profiling is a major challenge in metabolomics because of the wide variation in the physicochemical properties and abundance of the metabolites in a biological sample. Due to the rapid development of instrumentation in High Resolution Mass Spectrometry (HRMS) during the last two decades, HRMS-based analytical platforms are now able to achieve high sensitivity and mass resolution and this has greatly extended the coverage of metabolites in untargeted metabolite profiling [1, 2]. HRMS is usually interfaced with a separation technique providing retention/migration times as additional identity information for metabolites and reducing ion suppression during the ionisation process [3-6]. More importantly, the separation step offers the possibility of distinguishing isomeric metabolites; this is often unachievable with HRMS alone. In comparison with Gas Chromatography (GC) and Capillary Electrophoresis (CE), Liquid Chromatography (LC) demonstrates superiority in the requirements for sample preparation, metabolite coverage range and reproducibility [4, 5, 7]. Therefore it is the most commonly used separation technique in HRMS-based metabolomics studies.

Reversed Phase (RP) LC was the first LC separation technique hyphenated with HRMS and has been widely employed in metabolomics studies. However, its limitation is in the retention and separation of polar metabolites and these are major components in all biological samples. Hydrophilic Interaction Liquid Chromatography (HILIC) has been increasingly applied in recent HRMS-based metabolomics studies [8-11]. It not only enables superior retention of hydrophilic compounds but also improves their ionisation efficiency with ESI. The general retention mechanism in HILIC is believed to be the partitioning of the analytes between an organic-rich mobile phase and the aqueous layer formed at the surface of the polar stationary phase. A great number of HILIC columns are now commercially available and they show very different chromatographic performance [12, 13]. Therefore many column comparison/characterisation studies have been performed to investigate the retention mechanisms involved in HILIC [14-20]. They have often provided a theoretical understanding of chromatographic interactions in HILIC, but they are not a particularly helpful guide for column selection in metabolomics studies. A few column evaluation studies addressing the practical concerns in LC-HRMS based metabolomics studies have been reported and have provided information on the number of repeatedly detected LC-HRMS features, the
separation of isomers and the quality of the chromatographic peak shapes [21-23]. In these studies, however, the effects of mobile phase characteristics were not sufficiently investigated.

Current HILIC columns can be categorised into three groups based on the stationary phase chemistry: bare silica/neutral, charged and zwitterionic phases [11, 12]. Compared with the other two types of HILIC columns, the zwitterionic stationary phase is believed to have more potential for untargeted metabolite profiling studies. It has been demonstrated by many column characterisation studies that, depending on the chemical nature of the analyte and mobile phase composition, electrostatic interactions with permanently charged moieties and hydrogen bonding adsorption on the silica gel substrate could occur on the zwitterionic stationary phase [15, 18, 20, 24]. As there are also hydrophilic interactions, the solutes can elute with a mixed retention/separation mechanism in HILIC mode providing a unique selectivity in the separation of polar/ionisable metabolites. The first zwitterionic stationary phase in HILIC was fabricated by bonding sulfobetaine moieties on a silica-based substrate. The sulfonic group at the distal end of the zwitterionic moiety generates a negative surface charge which will produce an electrostatic attraction of positively charged metabolites at low pH values. Later the same moiety was bonded onto a polymer-based substrate to improve the pH tolerance and this polymer-based stationary phase has demonstrated improved chromatographic performance for negatively charged metabolites at high pH values [22, 25].

Both of the sulfobetaine zwitterionic stationary phases have been widely applied under HILIC conditions in untargeted metabolite profiling studies for various biological samples [26-29]. Recently a new zwitterionic stationary phase bonded with phosphorylcholine moieties has been introduced onto the market [30]. According to the manufacturer its opposite arrangement of the permanently charged groups to sulfobetaine will provide a different selectivity for charged solutes in HILIC mode. To date, there have been no reported applications of this new type of zwitterionic stationary phase in metabolomics, its application has only been reported in proteomic studies [31].

*Leishmania* is a group of protozoan parasites that can cause various disease manifestations depending on the infecting species. Our previous studies have demonstrated the potential of metabolomics for elucidating differences between *Leishmania* species with regard to their metabolomes [28, 32, 33]. In these studies, sample preparation for LC-HRMS was optimised...
for quenching of metabolism, cell washing and disruption, and metabolite extraction but there remained scope for further development of LC methods regarding stationary and mobile phases [34]. In the present study we have systematically evaluated the three zwitterionic stationary phases in HILIC mode under different mobile phase conditions with 223 metabolite standards. Cell extracts of Leishmania major promastigotes and extracellular medium after growth were used for assessment of each chromatographic condition with respect to the practical concerns in data processing for a metabolomics study. In addition the complementarity of RP LC to HILIC in the coverage of metabolites was investigated as was the reliability of the LC-HRMS signals for comparative analyses using a series of diluted biological samples.

2. Experimental

2.1 Chemicals and solvents

HPLC grade acetonitrile (ACN), chloroform and methanol were purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalAr grade formic acid (98%) was obtained from BDH-Merck, UK. Ammonium carbonate, ammonium formate, ammonium acetate, ammonium hydroxide solution (30-33%) and all standard compounds were purchased from Sigma-Aldrich, UK.

2.2 Preparation of metabolite standard solutions

Each metabolite standard was prepared at 1 mg/ml with HPLC grade methanol and water (1:1, v/v) as the stock solution and stored at -20°C. 100 μl was taken from each stock solution, about 56 metabolites were mixed and then the solution was made up to 10 ml with acetonitrile. Consequently, the final concentration for each metabolite standard was 10 μg/ml and 223 metabolite standards were distributed into four mixed metabolite standard solutions (detailed in supplementary material 1 (SM1)). In order to avoid identity confusion, isomers were distributed into different standard solutions and in-source fragments were also carefully verified.

2.3 Sample preparation of L. major cell extracts and spent media
**Leishmania major** promastigote were inoculated at $2.5 \times 10^5$ cells/ml in replicate 10 ml cultures at 26°C. Promastigotes were harvested after 6 days for metabolite extraction. The cell metabolite extraction was performed as described in our previous study [34] and the spent media metabolite extraction was performed as following: 75 μl of spent medium was added 300 μl of cold chloroform/methanol (20/60, v/v) followed by incubation for 1 h in a Thermomixer (14,000 rpm, 4°C). After centrifugation at 12,000 g for 10 min at 4°C, the supernatant was recovered and stored at -70°C until analysed. 200 μl of cell extract in chloroform/methanol/water (20/60/20, v/v/v) was obtained from each biological replicate. In order to ensure the equality of the samples tested under each LC condition, the obtained cell extracts from all biological replicates were thoroughly mixed together, redistributed into 25 aliquots and stored at -80°C until analysis. The same sample reconstruction was also carried out to the spent media extracts.

### 2.4 LC-HRMS conditions

Three zwitterionic HILIC columns (ZIC-HILIC and ZIC-pHILIC, both 150×4.6 mm, 5 μm; ZIC-cHILIC, 150×4.6 mm, 3 μm) and a reversed phase column (ACE C18-AR, 150×4.6 mm, 5 μm) were obtained from HiChrom (Reading UK). A Dionex Ultimate 3000 HPLC system (Camberley, UK) combined with an Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen, Germany) was employed as the LC-HRMS platform in this study. The MS parameter settings were described in our previous study [27] and the details of the mobile phase compositions and the gradient programs are described in Table 1. The flow rate of 0.3 ml/min was used under all LC conditions. In order to equilibrate the column for each LC condition, two solvent blanks (pure ACN) were run in advance and followed by three consecutive injections of the cell extract sample and then the spent medium sample and the four mixed metabolite standard solutions were run subsequently. The samples were kept at 4 °C during the experiment and a new set were used for each LC condition.

### 2.5 Data processing

The LC-HRMS raw data of the mixed metabolite standard solutions were processed using ToxID 2.1 (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) with ±3 ppm mass accuracy tolerance with both ESI positive and negative modes. The generated extracted ion chromatograms of metabolite standards under different LC conditions were visually evaluated...
with respect to peak shapes and are summarised with their retention times in SM 1. MZMine 2.10 was used for processing the LC-HRMS raw data of the parasite samples. The procedure and the settings were the same as described in our previous study [27] and the putative identification was also based on our in-house database used before. All representative chromatograms were produced by using Xcalibur 2.2 (Thermo Fisher Scientific Inc., Hemel Hempstead, UK).

2.6 Statistical analysis of LC conditions

The retention times of the metabolite standards generated by ToxID 2.1 were visually evaluated and corrected and then imported to SIMCA-P 13 (Umetrics, Sweden) for Principal Component Analysis (PCA) of 20 LC conditions. Prior to PCA, the data were mean-centered and unit variance (UV) scaled. Other statistical analysis were performed using the statistical functions in Excel (Microsoft Office 2010) associated with a few macros coded by Visual Basic for Application (VBA, Microsoft Office 2010).

2.7 Preparation of diluted parasite samples

To prepare a 3 times diluted sample, 100 μl of the original extract solution was diluted with 200 μl chloroform/methanol/water (20/60/20, v/v/v). A similar process was used to achieve 9, 27 and 81 times dilutions.

3. Results and discussion

3.1 Evaluation of chromatographic factors influential to retention mechanisms

We evaluated five chromatographic factors in our study: stationary phase chemistry; pH of mobile phase; nature of mobile phase additives (counter ions present or not); mobile phase additives added only in the aqueous mobile phase component or in both organic and aqueous components; and mobile phase buffer concentration. The ratio of aqueous and organic solvents in the mobile phase of the eluting program was not evaluated because this is the most influential chromatographic factor in HILIC mode and its effect is widely known. As described in Table 1, a gradient program of 80% ACN linearly decreasing to 20% in 30 min was applied for all the tested HILIC conditions. In order to evaluate the retention behaviour of 232 metabolite standards under different LC conditions, Principal Component Analysis (PCA) was
carried out based on their retention times. In the initial score plot, C18-FA was assigned far away from others as an outlier (data not shown), as expected because of the very different reversed phase retention mechanism. After excluding this set of conditions, the score plot generated by all HILIC conditions is shown in Figure 1. It is clear that the stationary phase chemistry and the pH of the mobile phase dominate the pattern. These two factors were represented by the colours and the shapes and almost reflected by the first and the second components, respectively, in Figure 1. The next most influential factor was the mobile phase additive used. At low pH (squares) the conditions with addition of formic acid (FA) in the mobile phases were separated from the ones with ammonium formate (AF) on both ZIC-HILIC and ZIC-cHILIC columns. The same situation could be observed with the HILIC conditions at high pH (stars). Regarding the question of whether to add buffer into only the aqueous phase or into both solvents, it seemed that the differential effect was greater at medium and high pH (a conclusion based on the increased distance between each pair of two relevant conditions).

Zwitterionic metabolites like amino acids and their derivatives were almost insensitive in terms of specific retention behaviour to these HILIC factors. For example, as shown in Figure 2, the retention times and the peak shapes of L-glutamine remained fairly constant under varying conditions except for a small decrease in retention time at high pH on the ZIC-pHILIC column. In contrast, however, variation in column and mobile phase conditions caused readily observable effects with basic and acidic metabolites (tertiary/quaternary amines and organic acids) such as choline and maleic acid (Figure 2). Under the same FA condition (0.1% formic acid in both aqueous and organic solvents: low pH without counter ions) the retention time of choline (a completely positively charged metabolite) was significantly less on the ZIC-cHILIC column compared to the ZIC-HILIC column, whereas that for maleic acid (a partially negatively charged metabolite) was significantly increased. This could be due to the electrostatic attraction and repulsion between the charged solutes and the charged surface of the zwitterionic stationary phases. It is believed that on the ZIC-cHILIC column the positively charged surface (phosphorylcholine) should attract negatively charged solutes but repulse positively charged solutes, with the opposite occurring on the ZIC-HILIC column. It should be also noticed that on the ZIC-HILIC column the peak shape of maleic acid was broad and split which might produce wrong/confusing outcome in the peak extraction algorithms used in
processing metabolomics data. This is most likely due to repulsion by the negatively charged groups on the surface of the stationary phase causing loss of peak integrity. On the ZIC-pHILIC column, which has a sulphonic acid group towards the surface of the phase, at high pH, with ammonium carbonate modifier, all three analytes have good peak shapes and the better peak shapes being at higher ionic strength with 10 mM buffer in reservoirs A and B. The retention time of choline decreases at higher ionic strength due to increased competition from the ammonium ion. In contrast, the retention time of the maleic acid increases at higher ionic strength due to less electrostatic repulsion due to masking of the negative charge by increased levels of the ammonium ion. Often chromatographers only incorporate modifier into the aqueous phase reservoir (A), partly because the modifiers used in LC-MS are not that soluble in 100% acetonitrile. The outcome of this approach was studied for the ZIC-cHILIC column and it was found that incorporation of ammonium acetate into A alone led to poorer peak shapes due to lower ionic strength overall whereas having the modifier in both reservoirs resulted in better peak shapes due to the same factors as discussed for the ZIC-pHILIC column. The optimal peak shapes on the ZIC-cHILIC column were with ammonium formate in reservoirs A and B. This might be due to a higher level of ionisation of the two buffer ions at high levels of acetonitrile since formic acid will be less ion suppressed in an environment with low dielectric constant than acetic acid as it has a lower pKa value and thus be better able to donate a proton to ammonia and keep in its protonated form. The ZIC-pHILIC column, in which the stationary phase is based on an organic polymer rather than silica gel, was the only one of the three zwitterionic HILIC columns suitable for testing at high pH (9.2). It was found that, consistent with the data of Figure 1, the retention behaviour of the metabolite standards were quite distinct on this column under these conditions even for the zwitterionic metabolites such as L-glutamine (Figure 2). However, the unknown surface chemistry of the polymer substrate obstructed the understanding of this stationary phase to some extent. Apart from hydrophilic partitioning, ion exchange interaction plays an important role. For instance, the decrease in the retention time of choline with increasing the buffer concentration from 5 mM to 10 mM is typical of ion exchange interaction. Compared with partially ionised status at low and medium pH, the two carboxylic groups in maleic acid are completely deprotonated at pH 9.2 which should improve the electrostatic attraction to the positively charged betaine group thus causing it to elute later at high pH in comparison to low and medium pH. Moreover, ammonium ions in the mobile phase will associate with the
negatively charged sulfonate groups facilitating the interaction of negatively charged solutes with the betaine group which is closer to the surface of the stationary phase support. This hypothesis is supported by the increase of the retention time of maleic acid with increasing the buffer concentration because the maleate ions are able to stay longer in the stationary phase if the charge repulsion by sulfonate groups is reduced by their association with ammonium ions. The peak shape of maleic acid was also improved when the buffer concentration was increased, which could be attributed to reduced charge repulsion. This phenomenon at high pH on the ZIC-pHILIC column could be observed with all completely negatively charged metabolites (almost all acidic metabolites) including diphosphate or triphosphate nucleotides (see SM 1).

3.2 Examples of poor chromatographic peaks and their interference to data processing

Elucidation of the retention mechanisms is not the aim of LC optimisation in LC-MS-based metabolomics studies. In untargeted metabolite profiling, optimisation of the number of metabolites detected is the highest priority in selecting a LC-MS method. However, this number can be potentially determined by the data processing. In our experience, the quality of the chromatographic peaks generated has a great effect on the output of the data processing. Poor peak shapes are more likely to interfere with peak extraction (peak picking) and alignment in the data processing and consequently confuse the identification and produce wrong comparative information - both potentially resulting in misleading biological interpretation. This issue might be overlooked by biologists because of a lack of chromatographic knowledge and understanding of the algorithms used in data processing. Only few specific demonstrations of this issue can be readily found in the literature [22, 23]. Therefore we addressed the issue in this study and we present some examples and explaining their potential for interference with data processing. By visually checking the chromatographic signals generated by 223 metabolite standards under different LC conditions, it was possible to divide the poor chromatographic peaks into six categories: absence of a distinct peak; multiple peaks; double peaks; split peaks; broad peaks; and tailing peaks. Some chromatograms of metabolites are shown in Figure 3 to demonstrate typical poor peak shapes in each of these categories and we also show a comparative chromatogram of the same metabolite under different LC conditions where a good peak shape was produced. The worst case is generation of what amounts to baseline noise, which can be
described as a bunch of zigzag/splitting/irregular peaks covering a wide time range. The example shown in Figure 3 is putrescine under pHILIC-AC-10 mM-both conditions. These irregular patterns are usually non-repeatable even between two consecutive injections. During the data processing of this type of peak many features with the same m/z will be generated by the peak extraction algorithm; some of them would be removed by noise and/or peak shape filter algorithms, depending on the software used, which would itself introduce an artefact. After alignment, the remaining multiple features would confuse the subsequent identification because they are generated by a single metabolite; moreover, wrong comparative outcomes would be produced because of their irregular and fluctuating profile. The same issue would emerge if the chromatographic signal looked like the peak for isocitrate under cHILIC-AF conditions in Figure 3. In this case, however, several peaks could be observed repeatedly at certain retention times across samples which might produce correct peak extraction and alignment but still confuse the identification. If the chromatographic signal splits or forms two closely eluting features as in the case of maltose, features which are due to the equilibrium between and the α- and β-anomers of this reducing sugar under the pHILIC-AC-5mM conditions used, there would be an uncertain output of peak extraction. It may treat as two or one feature depending on the settings of the algorithm; it certainly would be confused with another situation in which two isomers elute very closely. Theoretically it is expected that in the former situation the chromatographic signal should be treated as one feature, but two features in the latter situation. Practically, however, there is no such ideal setting in any peak extraction algorithm to avoid this confusion in LC-MS data processing for untargeted analysis. Some metabolites can generate two peaks apart from each other such as nicotinate under pHILIC-AC-10mM conditions as also shown in Figure 3. In this case the chromatographic signal would be certainly treated as two features which would confuse the identification. Sometimes the shape and size of the peaks can vary from sample to sample depending on the composition of the sample solvent. Compared to the poor peak shapes described above, broad and tailing peaks might be acceptable in some cases, especially if their peak shapes are repeatable, because a single feature could be generated with proper settings (e.g. maximum peak span) in the peak extraction algorithm. However, if the peak is extensively broad or tailing with zigzag profiling across the top, like the pyridoxamine under pHILIC-AC-10mM conditions example in Figure 3, there would be high possibility of excluding the feature or splitting the peak into multiple features in the peak extraction algorithm.
Sometimes a combination of several poor peak shapes can be observed in one chromatogram and a brief comment on the chromatographic signal of each metabolite standard under different LC conditions is described in SM 1. The ideal peak shape for data processing is a single and sharp peak, like the ones in the right column in Figure 3. A little tailing or broadness could be acceptable although it might introduce inaccuracy in peak area integration.

### 3.3 Evaluation of LC conditions using metabolite standards

The number of metabolite standards detected under each LC condition, regardless of peak shape, is presented as a yellow strip in Figure 4. The main reason for a metabolite standard to be undetected is because it is unable to ionise under the conditions applied. This is primarily determined by the pH value of the mobile phase and the pKa value(s) of the metabolite and not the other LC parameters. The other two possible reasons are either that the metabolite was not able to elute within the given run time or that it was not stable in the testing solution and so degraded. For example, oxaloacetate will rapidly degrade to pyruvate in the water/methanol solution. As can be seen in Figure 4, all LC conditions showed quite similar numbers for the detected metabolite standards. As demonstrated and explained above, however, they would not be treated correctly by the data processing algorithms if their peak shapes are poor. Therefore it is more meaningful to compare between the different LC conditions the number of metabolites showing good peak shapes rather than the total number of detected metabolites. The number of metabolites exhibiting good peaks shapes is given as a purple strip for each LC condition in Figure 4. It is clear that the numbers of compounds detected dropped down dramatically under some LC conditions but not much under others and the fall in detectable metabolites seemed to be pH related (Figure 4). In order to elucidate the effect the LC conditions are highlighted with stars for high pH; triangles for medium pH and squares for low pH mobile phases in Figure 4 and also further classified the metabolites based on their detection according to ESI polarity: green strip if the signal was observed in both modes; red only in negative mode and blue only in positive mode. It can be observed that the green strips cover more than half length of the purple strip for each LC condition which represents the majority of the metabolites. More metabolites are detected for the ZIC-HILIC and the ZIC-chILIC columns when increasing the pH from low to medium (squares to triangles) and more still for the ZIC-pHILIC column with increasing the pH from medium to high (triangles to stars) and the increase in the buffer concentration. It should be
also noted that with low pH mobile phases the numbers of the metabolite standards observed only in ESI positive mode (the blue strip) were similar or slightly higher than in negative mode (the red strip), whereas with medium and high pH mobile phases these numbers decreased in ESI positive mode while greatly increased in negative mode. This change is attributed to the deterioration of the chromatographic signals of basic metabolites and the improvement of the peak shapes of acidic metabolites (e.g. organic acids with multiple carboxylic groups and diphosphate or triphosphate nucleotides), respectively. With respect to the number of metabolite standards showing good peak shapes (the purple strip), the best LC conditions would be the ZIC-pHILIC column with high pH mobile phases and buffer concentrations (pH, 9.2; 10 mM or 20 mM in both solvents and 20 mM only in aqueous solvent).

The other necessary evaluation in LC condition optimisation for untargeted metabolite profiling is the separation of isomeric metabolites; chromatographic separation is the only reliable way to distinguish these compounds. There were totally 32 groups of isomeric metabolites tested in this study and based on their acidity/basicity they were categorised as acidic, basic, zwitterionic and neutral compounds (SM 2). In general, there were no chromatographic factors showing clear influence on the separations. As shown in Figure 5, more groups of isomeric metabolites were separated on the ZIC-pHILIC column especially with high pH mobile phases. It appeared that the ionisable isomers would be more likely to be separated in their completely ionised modes because ion-exchange could be involved to greater extent in their interaction between mobile and stationary phase in HILIC mode. For example, betaine is permanently positively charged; therefore it was separated from valine where the ionisation of the amine group is dependent on pH under almost all LC conditions. This is also reflected by the observation that more acidic isomers (blue column in Figure 5) were separated at medium or high pH than at low pH. For the neutral isomers (sugars) the separations were difficult to achieve because hydrophilic partitioning was the only chromatographic interaction for them under all LC conditions. The improvement in the separation of acidic isomers on the ZIC-chILIC column at medium pH could be attributed to the unique selectivity for the negatively charged ions resulting from the positively charged stationary phase surface formed by betaine groups and the smaller particle size (μm) might be also contributing. It should be noted that the separation of some isomer groups was limited by their poor peak shapes, such as broadening and tailing.
3.4 Evaluation of LC conditions using a biological extract

A cell extract and spent medium of the protozoan parasite *Leishmania major* were prepared as described in experimental section and tested under each LC condition with triplicate injections. The raw data were processed using MZMine 2.10 with the settings described in our previous study [27]. Of most interest was the number of LC-MS features generated under each LC condition. After removing the interference from the solvent blank the remaining LC-MS features were filtered again by the relative standard deviation (<25%) of the integrated peak areas obtained from three consecutive injections. By searching an in-house database the features were putatively identified based by accurate mass (±3 ppm). The numbers of total (Total: yellow strip) and the putatively identified (ID: purple strip) features under each LC condition obtained from the *L. major* cell extract and the spent medium are shown in Figures 6 and 7, respectively. According to their ESI detection polarity, the features were classified into positive (Pos: blue strip) and negative (Neg: red strip) and if two features were detected in both modes with mass difference of 2.0146 ± 0.001 m/z within an Rt window of ±0.2 min they were considered as a single feature (Both: green strip) and counted once in the calculation of total and identified features. These data are the number of repeatable features in the data processing without considering their real identities. Thus false-positive features caused by poor chromatographic signals as explained above, MS non-proton adducts (any MS signals not generated by M ± H), ESI in-source fragments, complex ions (e.g. dimers: 2M ± H) and salt cluster ions were all possibly included in counts. It is easily seen in both Figure 6 and 7 that the total numbers of repeatable features generated under C18-FA conditions from both cell extract and spent medium samples were much lower than for any of the HILIC conditions. This could be explained by the serious ion-suppression effect due to the many polar metabolites eluting early and together under this condition, consequently leading to poor repeatability in the MS response and so exclusion from the total by the RSD filter. For instance, after removing the interference from the blank 141 features eluting between 4.6 and 6.5 min remained for the cell extract sample under the C18-FA condition in ESI positive mode; whereas only 59 of them survived through the 25% RSD filter. Unstable ionisation efficiency caused by high portion of aqueous at the beginning of elution gradient under C18-FA condition could also obstruct the MS signal repeatability of the polar metabolites.
Among the HILIC conditions, it is obvious that the total number of metabolites detected (yellow) are higher under the conditions of the ZIC-pHILIC column but only with a high pH mobile phase. The same column with medium pH mobile phases, especially for cell extract samples, did not out-perform the other columns, which means high pH might be the key factor to achieve higher coverage of metabolites. A similar observation has been reported in a previous column comparison study by Bajad et al. [23] who observed that an aminopropyl column at pH 9.45 was the best LC condition for measuring soluble intracellular metabolites. Based on the explanation previous outlined, the rise of the number of metabolites detected only in ESI negative mode (red) results from the enhanced detection and/or the improved peak shape of acidic metabolites like nucleotides in cell extract samples at high pH. Metabolite numbers detected in both ESI modes (green) and putatively identified metabolites (purple) also are greater under these conditions. It has been reported that ammonium hydrogen carbonate is an excellent buffer for LC-MS analysis because of its good volatility [35]. Ammonium carbonate was used in this study and its evaporation in ESI source appeared to be faster than ammonium formate and ammonium acetate because much higher background ion signals (dimer of formate: 91.00368 m/z, dimer of acetate: 119.03498 m/z in ESI negative mode and dimer of ACN: 83.06037 m/z in ESI positive mode) were observed if ammonium formate or ammonium acetate were present but such background was not observed for ammonium carbonate and also there was less evidence of deposition of condensed salt in the ion source. Therefore it is likely that faster evaporation of ammonium carbonate produced less ion-suppression effect and so facilitated the detection of the metabolites present in low abundance. It was expected that MS responses of basic metabolites would decrease with increasing mobile phase pH. In fact, however, the number of metabolites detected only in ESI positive mode (blue) remained at a similar level or even slightly increased, especially with high ammonium carbonate concentrations (AC-10mM-both and AC-20mM). It might be due to the enhanced opportunity to generate ammonium adducts \((M + \text{NH}_4^+)\) under these conditions. Another finding was that the total number of detected features under the conditions where the buffer was present in both solvents was always slightly lower than when it was present only in aqueous solvent regardless of the other factors.
Considering all the evaluations above using standard metabolites and parasite samples, the best HILIC condition could be either pHILIC-AC-10 mM-both or pHILIC-AC-20 mM. However, it should be noted that the poor chromatographic signals of highly basic metabolites like polyamines could not be avoided under these conditions and these would produce false-positive features in the data processing. The complementary coverage of metabolites under C18-FA condition to these two HILIC conditions was also investigated. Approximately 80 and 167 features were exclusively detected under C18-FA conditions for the cell extract and the spent medium samples, respectively. However, based on their m/z only 17 and 36 of these could be putatively identified as metabolites suggesting that many of these features might be false-positives. However, some isomers showed improved resolution under the C18-FA conditions, for example leucine and isoleucine.

3.5 Investigation of reliability of LC-HRMS signals

In an untargeted metabolite profiling study, it is optimal to record as many small but real and to remove as much noise as possible. The application of signal-to-noise ratio sometimes is not suitable for LC-HRMS data because in many cases there is no noise at all if the extracted ion window is only ±3 ppm. Therefore the differentiation between small true signals and noise is generally determined by the settings of two parameters in the peak extraction algorithm: minimum peak height and noise level. In order to find the proper signal intensity for these two settings, the original samples of cell extract and spent medium of L. major species were diluted as described in experimental section and measured under the pHILIC-AC-10 mM-both condition. By tracing some real signals confirmed by standard metabolites we would find the intensity level at which they disappeared or were undistinguishable from the noise. The LC-HRMS signals of three nucleotides in ESI negative mode were extracted with a ±3 ppm window for the original and the diluted samples and are shown in Figure 8 with the labelled retention times (RT), peak areas (PA) and heights (NL). The reason for selecting these nucleotides was that there is no effect of ionisation efficiency on MS signals intensity because they have a similar ionisable group. As can be seen above, at the intensity of 1.50E4 all the LC-HRMS signals could be confirmed as real. Below 1.00E4, there was one signal (GTP: 1/3) which looked like a chromatographic peak and two signals (UDP: 1/27 and GTP: 1/9) which were barely recognised as chromatographic peaks. Below or close to 1.00E3, there were almost no signals (GTP: 1/27 and 1/81) or only noises (UDP: 1/81). The evaluation was carried out with
more confirmed signals in this way and finally the noise level and the minimum peak height were determined as 1.00E3 and 1.00E4, respectively. It should be noted that the MS response could vary between instruments or with different settings, therefore we recommend that a trial experiment such as this should always be carried out before running a whole set of samples.

In LC-HRMS-based metabolomics studies, the level of a specific metabolite in a biological sample is reflected by its chromatographic peak area or height. When comparing the levels of this metabolite across multiple samples there is a question raising that whether the calculated ratios are able to reflect a true fold difference, especially with weak LC-HRMS signals. According to the intensity (peak height), the generated LC-HRMS features from the original sample were classified as three levels: low (1.00~3.00E4), medium (3.00~9.00E4) and high (>9.00E4) and based on the known dilution factors the reliability of the ratios calculated by peak areas at different levels was investigated and the results are shown in Figure 9. At each level the majority of the total LC-HRMS features showed R² value greater than 0.9. However, the number of features falling into the tolerance range (70-130%) of correct ratios dramatically deceased with increased dilution. At high level (green strip) only about 20% of the features were in the expected ratio range with 81 times dilution. At low level (blue strip) about 70% of the features even could not truly reflect 3-fold difference. This means in general there was a reasonable linear dynamic trend with dilution but that the calculated ratios could not practically reflect a real fold difference with large dilution factors especially when the LC-HRMS signals are low, which could be due to the loss of peak shape as can be observed as UDP: 1/27 and GTP: 1/9 in Figure 8. Therefore in real studies the fold difference of a metabolite across samples might be highly unreliable if the ratio is large plus calculated from weak LC-HRMS signals. This is where targeted screens based on tandem mass spectrometry are superior.

4. Conclusion

In this study on three zwitterionic columns the effect of varied HILIC factors on the retention mechanism and the output of the data processing for metabolomics studies were evaluated using metabolite standards and biological cell extract samples. In general, as well as stationary phase chemistry the mobile phase pH plays an important role in determining the
retention behaviours of the metabolites. Due to the fact that ion-exchange is greatly involved with hydrophilic partition in the retention mechanism on these zwitterionic columns the chemical nature and the concentration of the buffer can also contribute. The ZIC-chILIC column was tested for its applicability in metabolomics for the first time and in comparison with the ZIC-HILIC column it demonstrated improved chromatographic performance at low and medium pH, especially for acidic metabolites. With consideration of the quality of chromatographic signals, the separation of isomers and the number of LC-HRMS features generated in the biological samples, the optimum HILIC condition was using the ZIC-pHILIC column at pH 9.2 with 20 mM ammonium carbonate only in the aqueous mobile phase components. This can be mainly attributed to the high pH and the fast evaporation of the ammonium carbonate during the ESI process. By applying this method, clear differences between the intracellular metabolomes of three Leishmania species have been discovered and also between the composition of their suspending media after growth (manuscript in submission). With the Exactive Orbitrap MS used in this study, the minimum peak height and the noise level in data processing should be set to 1.00E4 and 1.00E3 respectively and care should be taken with regard to the reliability of weak LC-HRMS signals and avoiding multiplying these up unrealistically to obtain erroneous and misleading metabolite levels in whole cells.

References


Table 1 Mobile phase conditions

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<thead>
<tr>
<th>Mobile phase</th>
<th>Column</th>
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<tbody>
<tr>
<td></td>
<td>ZIC-HILIC</td>
</tr>
<tr>
<td>0.1% (v/v) FA in H₂O*</td>
<td>√</td>
</tr>
<tr>
<td>0.1% (v/v) FA in ACN</td>
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<tr>
<td>20mM AF pH=3 in H₂O*</td>
<td>√</td>
</tr>
<tr>
<td>ACN</td>
<td></td>
</tr>
<tr>
<td>10mM AF pH=3 in H₂O/ACN (9:1)*</td>
<td>√</td>
</tr>
<tr>
<td>10mM AF pH=3 in H₂O/ACN (1:9)</td>
<td>√</td>
</tr>
<tr>
<td>20mM AA pH=6.8 in H₂O*</td>
<td>√</td>
</tr>
<tr>
<td>ACN</td>
<td></td>
</tr>
<tr>
<td>10mM AA pH=6.8 in H₂O/ACN (9:1)*</td>
<td>√</td>
</tr>
<tr>
<td>10mM AA pH=6.8 in H₂O/ACN (1:9)</td>
<td>√</td>
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<tr>
<td>0.05% (v/v) A in H₂O*</td>
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<tr>
<td>0.05% (v/v) A in ACN</td>
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<tr>
<td>5mM AC pH=9.2 in H₂O*</td>
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<td>ACN</td>
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</tr>
<tr>
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<tr>
<td>ACN</td>
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<td>20mM AC pH=9.2 in H₂O*</td>
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<tr>
<td>ACN</td>
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<td>5mM AC pH=9.2 in H₂O/ACN (9:1)*</td>
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<td>5mM AC pH=9.2 in H₂O/ACN (1:9)</td>
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<td>10mM AC pH=9.2 in H₂O/ACN (9:1)*</td>
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<td>10mM AC pH=9.2 in H₂O/ACN (1:9)</td>
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<tr>
<td>20mM AC pH=9.2 in H₂O/ACN (2:8)</td>
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FA: formic acid  AF: ammonium formate  AA: ammonium acetate
A: ammonium hydroxide  AC: ammonium carbonate

*: Gradient program: mobile phase A was increased from 20% to 80% in 30mins and held at 92% for 5mins in HILIC mode and A was decreased from 95% to 10% in 30mins and held at 10% for 5mins in RP LC mode.
&: Gradient program: mobile phase A was increased from 12.5% to 87.5% in 30mins and held at 100% for 5mins
#: Gradient program: mobile phase A was increased from 0% to 100% in 30mins and held at 100% for 10mins
√: The column was tested with this mobile phase.
-: The column was not tested with this mobile phase.
**Figure 1.** Principal Component Analysis (PCA) of 19 HILIC conditions with retention times of 223 metabolite standards (the first two components explain 66.9% of the total variance). Column classification by colours: cHILIC, green; HILIC, blue; pHILIC, red. pH classification by shapes: low, square; medium, triangle; high, stars. For more detailed HILIC conditions see Table 1.
**Figure 2.** Extracted ion chromatograms (XIC: ±5 ppm) of one zwitterionic (L-glutamine), one basic (choline) and one acidic (maleic acid) metabolite under different HILIC conditions.
Figure 3. Examples of different types of poor chromatographic peak shapes compared with good peaks for the same metabolite standard.
Figure 4. Bar chart of the numbers of the total detected metabolite standards (yellow); the metabolite standards with good peak shapes (purple); the metabolite standards with good peak shapes and detected in both ESI modes (green); the metabolite standards only detected in negative mode (red) and in positive mode (blue) under different LC conditions. pH classification by shapes: low, square; medium, triangle; and high, stars.
**Figure 5.** Column chart of the numbers of separated isomer groups under different LC conditions with classification of their acidity/basicity (light blue, mixed; purple, zwitterionic; green, neutral; red, basic; and deep blue, acidic).
Figure 6. The numbers of total detected (yellow), only detected in ESI positive mode (blue), only detected in negative mode (red), detected in both modes (green), and putatively identifiable (purple) LC-HRMS features in the *L. major* extract sample under different LC conditions.
Figure 7. The numbers of total detected (yellow), only detected in ESI positive mode (blue), only detected in negative mode (red), detected in both modes (green), and putatively identifiable (purple) LC-HRMS features in the *L. major* spent medium sample under different LC conditions.
**Figure 8.** Extracted ion chromatograms (XIC: ±5 ppm) of AMP (the left column), UDP (the middle column) and GTP (the right column) from the *L. major* extract samples with different dilution factors (labelled on the left for each row). RT, retention time; PA, peak area; NL, peak intensity (height).
Figure 9. The numbers of LC-HRMS signals at low (1~3E4: blue), medium (3~9E4: red) and high (>9E4: green) intensity level in the original L. major extract (A) and spent medium sample (B) and in the diluted samples (3x, 9x, 27x and 81x dilutions). The data illustrate the numbers of the signals at each level showing R$^2$>0.9 across 1, 3, 9, 27 and 81 dilutions and the numbers falling into the expected ratio range under each dilution.

L. major cell extract ESI negative mode

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<th>1~3E4</th>
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<tbody>
<tr>
<td>Total</td>
<td>222</td>
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<td>208</td>
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<tr>
<td>R2&gt;0.9</td>
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<td>156</td>
<td>187</td>
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<tr>
<td>3±0.9</td>
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L. major spent medium ESI positive mode

<table>
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<th>1~3E4</th>
<th>3~9E4</th>
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<tbody>
<tr>
<td>Total</td>
<td>346</td>
<td>299</td>
<td>292</td>
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<tr>
<td>R2&gt;0.9</td>
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<td>264</td>
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<tr>
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<td>81±24.3</td>
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