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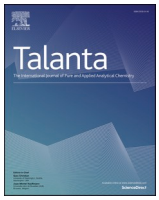

Comprehensive untargeted polar metabolite analysis using solvent switching liquid chromatography tandem mass spectrometry

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ABSTRACT

Metabolomics analyses enable the examination and identification of endogenous biochemical reaction products, revealing information on the metabolic pathways and processes active within a living cell or organism. Determination of metabolic shifts can provide important information on a treatment or disease. Unlike other omics fields that typically have analytes of the same chemical class with common building blocks, those that fall under the nomenclature of metabolites encompass a wide array of different compounds with very diverse physiochemical properties. Development of a comprehensive metabolomic pipeline therefore can be a troublesome and complicated process for the analyst. Often single liquid chromatography-mass spectrometry methods on unfractionated samples are carried out in order to be time-efficient, however this could potentially produce data with a low number of identifiable metabolites. In the present studies, we developed a comprehensive polar metabolomics pipeline for cell-based metabolomics. SH-SY5Y neuroblastoma cells were selected as the sample matrix for method development since they are one of the most widely used cell lines for human neurotoxicity studies. This was accomplished by investigating and optimising different mass spectrometry source and chromatographic conditions to enhance the signal of polar metabolites. Optimised hydrophilic interaction liquid chromatography (HILIC) based metabolomic methods at different pH values were examined in positive, negative, and polarity switching modes to determine which combination yielded the highest number of confidently identified metabolites. Additionally, the use of sequentially running two methods was also compared to determine the degree of overlap and whether there is merit in running two separate methods on one sample. It was determined that solvent switching between two optimised methods, acidic chromatographic conditions in positive mode and basic chromatographic conditions in negative mode, yielded the highest number of unique identifiable metabolites. This could be run in a single analytical batch due to the large pH range of the column. A quick switch method in-between each method allowed both conditioning the column and preparation of the MS source conditions for the sequential method.

1. Introduction

Metabolomic experiments can range from metabolome characterisation in which all endogenous and exogenous small molecule metabolites in a biological system are identified and quantified, to the determination of metabolic shifts due to a treatment or disease and can utilise samples from *in vitro* or *in vivo* studies [1–4]. While "omic" analysis aims to analyse all of the compounds of a class, it is not feasible to capture the entirety of an "ome" even with multiple analytical runs due to the complexity and variation in physicochemical properties within a classification of biomolecules [5]. This is perhaps even more apparent with metabolomics since, unlike similar fields like proteomics and lipidomics, there is a larger array of chemical classes with diverse physicochemical properties that fall under the umbrella term "metabolites". The metabolome contains chemically diverse compounds such as amino acids, carbohydrates, nucleotides, steroids, lipids and organic

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acids. Thus most untargeted metabolomic pipelines aim to detect as many metabolites as possible in order to classify phenotypes based on their metabolomic fingerprint.

Metabolomic methods generally employ nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) for analyte detection [6]. While both techniques have specific advantages and disadvantages, there are a higher number of MS-based studies published each year, most likely due to NMR being less sensitive and typically identifying fewer metabolites than MS-based methods [6]. NMR metabolomic data is often viewed primarily as complimentary to MS data [7,8]. MS-based approaches generally make use of separation techniques prior to MS analysis, most commonly gas chromatography (GC) [9,10] or liquid chromatography (LC) [11,12]. LC-MS metabolomics is usually preferred to GC-MS metabolomics as it allows larger metabolome coverage and requires less complicated sample treatment prior to analysis [13]. Tandem mass spectrometry (MS/MS) is used to obtain fragmentation data as it provides an additional layer of qualification in addition to the intact high-resolution mass to charge measurement [14]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is therefore the most commonly used platform for untargeted metabolomic analysis. Development of comprehensive LC-MS/MS methods however is complicated due to the large array of different chemical classes in the metabolome. This highlights the importance of optimising metabolomic methods to ensure the conditions allow maximum metabolite coverage, as well as fine-tuning parameters to favour the largest array of metabolites and not those of a particular class.

Development of a metabolomic pipeline typically starts at the extraction step. Sample extraction for metabolomic methods is usually performed on the day of the analysis to minimise degradation. Sample fractionation is commonly performed by the 'Matyash' or 'Bligh and Dyer' extraction methods or their many variations [15–18]. Some studies do not fractionate the sample to be more time efficient [19,20], potentially resulting in fewer identifiable metabolites and a less clean sample for the MS. Fractionation extractions divide samples into polar and non-polar fractions [15]. Polar metabolites comprise small molecules such as amino acids, organic acids and sugars, whereas nonpolar metabolites are typically referred to as the lipidome and are comprised of lipids and fatty acids [21]. These two fractions can be analysed in many ways, such as with variants of liquid chromatography, usually hydrophilic interaction liquid chromatography (HILIC) or reverse phase liquid chromatography (RPLC), under acidic, neutral or basic pH conditions and in negative or positive mode in the MS [22–27].

Many studies only examine the polar fraction of the metabolome using HILIC-MS/MS under acidic conditions in positive [28,29] and negative [30,31] ionisation mode, either individually or by polarity switching [32,33]. The disadvantages of polarity switching include decreases in sensitivity, inability to use different source conditions (as some conditions such as temperature take time to stabilise) for the different ionisation modes, and it requires the analysis to be conducted using chromatographic conditions less favourable for one of the ionisation modes [34]. However, since two ionisation modes are analysed in a single run it has the advantage of a shorter analysis time and less solvent consumption. The use of negative ionisation under acidic conditions has been previously shown to decrease the intensity of negative ions in MS [34]. Despite negative ionisation mode being hindered and potentially missing important metabolites, polarity switching is common practice in metabolomic experiments since it allows fast positive and negative mode analysis without the need to change solvents or develop another chromatographic method.

Mass spectrometry conditions are not normally optimised for metabolomic analyses, particularly ion source conditions [35–37]. Source conditions in which multiple ionisation modes are employed are usually performed at the default parameters of the instrument [35–37], and are identical for the different ionisation modes, with the exception of the spray voltage which is higher in positive mode (often the default setting on the instruments). This lack of source condition optimisation can reduce the number of identifiable compounds and decrease the signal intensity of other analytes, which may reduce the number of meaningful conclusions that can be draw from a dataset.

Source condition optimisation for untargeted metabolomic studies is not a trivial undertaking since, due to the diverse physicochemical properties of metabolites, beneficial conditions for one metabolite can be detrimental to another. Obtaining optimal source conditions for every metabolite is not feasible so the challenge is to identify conditions that benefit the largest number of metabolites, without being detrimental to many others, thus improving the analyte coverage. Additionally, optimal source conditions between positive and negative ionisation modes may also be different, meaning that different modes require individual optimisation and that polarity switching cannot be employed for a method with optimal source parameters (as some conditions such as temperature take time to change). These MS considerations alongside the choices made for chromatography highlight how many options there are for developing a metabolomic LC-MS/MS pipeline.

The diversity of the metabolome will always necessitate some compromise during metabolomic analyses, however some conditions such as buffer and source conditions are readily optimisable and will improve analyte coverage. The present study aimed to optimise and develop a comprehensive pipeline for the analysis of polar metabolites using a combination of HILIC-MS/MS methods, and then to apply this novel pipeline to the analysis of a human neuroblastoma cell line (SH-SY5Y) metabolome. SH-SY5Y cells are frequently used in neurology and toxicology *in vitro* metabolomic studies, researching conditions such as Parkinson's disease [38], Alzheimer's disease [39], algal toxin exposure [28] and polyfluoroalkyl substance exposure [40]. Optimising metabolomic methods to increase the number of metabolites identified can provide greater insight into the underlying conditions and will be applicable to other cell lines of interest. The final optimised method was then compared to different metabolomic methods that make use of single ionisation mode, and polarity switching in acidic, neutral, and basic pH mobile phase conditions.

2. Methods

2.1. Chemicals and reagents

LC-MS grade methanol (MeOH) and acetonitrile (ACN) was purchased from Chem Supply (NSW, Australia). LC-MS grade ammonium formate, formic acid, HPLC grade chloroform, and 99 % ammonium bicarbonate was purchased from Merck (Castle Hill NSW, Australia). The internal standard (ISTD) for the polar metabolites was reagent 1 of the EZ:faast™ amino acid derivatisation kit (Phenomenex®, NSW, Australia), containing 0.2 mM of homoarginine, D3-methionine and homophenylalanine. All water used was ultrapure water (18.2 MΩ cm) and was prepared in-house and filtered through a 0.2 μm filter. TrypLE™ Express Enzyme (x1 no phenol red), Glutamax™, fetal bovine serum (FBS), and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Thermo Fisher Scientific (Scoresby VIC Australia).

2.2. SH-SY5Y cell culture

SH-SY5Y cells (a human neuroblastoma cell line) were cultured in T75 flasks in DMEM supplemented with 5 % Glutamax and 10 % FBS. Cells were harvested by the following protocol: media was removed and 5 mL of TrypLE™ was added to each flask and left for 5 min in a 37 °C incubator. Cells suspended in the TrypLE™ were removed and put into a 15 mL tube and centrifuged at 300 g at room temperature for 5 min. The supernatant was removed and discarded, and 5 mL of phosphate-buffered saline (PBS) was added to the cells, the pellet was gently broken up and centrifuged under the same conditions in duplicate to ensure adequate washing. The remaining cell pellets were snap frozen with liquid nitrogen and stored in −80 °C until extraction.

2.3. Metabolite extraction

Throughout the extraction, all samples were kept on ice to ensure minimal sample degradation. To the frozen cell pellets, 250 μL of chloroform, 250 μL of methanol and 250 μL reagent 1 of the EZ:faast™ amino acid derivatisation kit was added. The samples were left in a rotary mixer in a cold room (4 °C) for 30 min. Samples were then centrifuged at 3000 g at 4 °C for 5 min to allow phase separation. 100 μL of the top layer containing the polar metabolites was removed and placed into a 1.5 mL tube. The tubes containing the polar metabolite samples were centrifuged at 15,000 g at 4 °C for 1 min 50 μL of the polar metabolite samples were placed into autosampler vials with inserts and 50 μL of ACN was added to each sample. 10 μL of each sample was taken and combined to create a pooled sample to use for quality control (QC) throughout each batch. Samples were analysed on the day of extraction.

2.4. Metabolite analysis via HILIC-MS/MS

LC-MS/MS was carried out on an Agilent Infinity II HPLC coupled to a Thermo Scientific Q Exactive Plus mass spectrometer. Instrument control was performed through Thermo Scientific Chromeleon version 7.3. The Q Exactive Plus was equipped with a heated electrospray ionisation (HESI) source with a HESI-II probe. Metabolite analysis was performed via two hydrophilic interaction liquid chromatography (HILIC) quadrupole orbitrap mass spectrometry methods (QOMS) run sequentially on a Waters® BEH Amide column (2.1 mm × 100 mm, 1.7 μm particle size) with column temperature set to 40 °C. Method 1 (M1) was performed in positive mode under acidic chromatographic conditions, and method 2 (M2) was analysed in negative mode with basic chromatographic conditions. M1 had a flow rate of 0.8 mL/min with mobile phase A consisting of ultrapure water with 10 mM ammonium formate +0.1 % formic acid, and B consisting of 90:10 ACN:Water with 10 mM ammonium formate +0.1 % formic acid. Metabolites were eluted with a stepped gradient of the following solvent B: 0.00 min 100 %, 3.00 min 100 %, 3.10 min 90 %, 6.00 min 90 %, 6.10 min 80 %, 9.00 min 80 %, 9.10 min 100 %, 12.00 min 100 %. M2 had a flow rate of 0.6 mL/min with mobile phase A consisting of ultrapure water with 10 mM ammonium bicarbonate and 90:10 ACN:Water with 10 mM ammonium bicarbonate. Metabolites were eluted with a gradient of the following solvent B: 0.00 min 100 %, 5.00 min 60 %, 7.00 min 60 %, 7.10 min 100 %, 10.00 min 100 %. HESI conditions are shown in Table 1 for both methods, and data was acquired via data-dependant acquisition (DDA) TopN. Data acquisition parameters were identical and are shown in S Table 12. The scan range was set to 66.7–1000 *m/z*, with the only difference between the methods being the polarity (positive or negative mode) and the collision energy. Both methods utilised stepped collision energy, the positive mode method had the stepped collision energy values set to 10, 20 and 30 collision energies, with the negative mode method having 20, 30 and 40 collision energies. The methods were run sequentially (referred to as M8 (Table 2.)) with positive being performed first followed by negative. Solvents for positive mode were put on lines A1 and B1 and negative mode solvents were put on lines A2 and B2. A switch method was developed to switch between the two methods and solvent systems, so that positive and negative methods could be acquired in the same batch. First the method swapped the lines being used i.e. swapped A1 and B1 to A2 and B2. The following B% and flow rate was used for the switch method 0.00 min 0 % 0.2 mL/min, 5.00 min 0 % 0.2 mL/min, 5.10 min 50 % 0.4 mL/min, 10.00 min 50 % 0.4 mL/min, 10.10 min 100 % 0.4 mL/min, 15.10 min 100 % 0.6 mL/min, 20.00 min 100 % 0.6 mL/min. This was followed by a blank and a QC using the negative mode method, with the swap method also changing the MS source conditions from the positive mode parameters to the negative mode parameters (Table 1). A pooled sample was created by combining 10 μL of all samples in a single autosampler vial, and this was used as the QC throughout the batch. Throughout the metabolite run a solvent matched blank and the QC pooled sample was injected every 15 injections to ensure no carryover or drop in sensitivity of the QOMS throughout the run. Injection volume was set to 5 μL, autosampler set to 4 °C and all samples were injected in triplicate. Two neutral pH and two polarity switching LC-MS/MS methods were used to compare against the individual acidic and basic methods and solvent switching method. All methods used in this study are listed in Table 2. See supplementary data for experimental details for additional methods used.

Table 1
HESI conditions of for the two QOMS methods.

Source condition	Metabolite (+)	Metabolite (−)
Sheath Gas	30	30
Aux Gas	10	10
Sweep Gas	1	1
Capillary Temp (°C)	400	350
Aux Gas Heater Temp (°C)	600	500
Spray Voltage (kV)	0.5	4
S-lens RF level	50	50

Table 2
Parameters for all methods used in this study. pH measurement was averaged over the course of a week. Error for pH denotes standard deviation (n = 5).

Abbreviation	Method details	Measured pH
M1	Positive ionisation mode in acidic pH conditions (10 mM Ammonium formate + 0.1 % formic acid)	3.47 ± 0.05
M2	Negative ionisation mode in basic pH conditions (10 mM Ammonium bicarbonate)	8.26 ± 0.04
M3	Positive ionisation mode in neutral pH conditions (10 mM Ammonium acetate)	6.75 ± 0.05
M4	Negative ionisation mode in neutral pH conditions (10 mM Ammonium acetate)	6.75 ± 0.05
M5	Polarity switching in acidic pH conditions (10 mM Ammonium formate + 0.1 % formic acid)	3.47 ± 0.05
M6	Polarity switching in basic pH conditions (10 mM Ammonium bicarbonate)	8.26 ± 0.04
M7	Solvent switching between methods M5 and M6	See M5 & M6
M8	Solvent switching between methods M1 and M2	See M1 & M2

2.5. Data analysis and processing

All data was exported as.raw files from Chromeleon™, with peak processing and metabolite identification performed using MS-DIAL [41]. The identification score cut off was set to 70 %, and "only report top hits" was selected to remove potential duplicates. Alignment reference file was set to a pooled sample, and remove features based on blank was selected. Both "keep reference matched metabolites" and "keep suggested (w/o MS2) metabolites" were unchecked. MS-DIAL settings that are not mentioned were kept to the default values. Once processed, the identified metabolite data was further filtered using the following criteria: non-metabolite features, non-gaussian peak shaped compounds, and compounds that only identified off intact mass in the fragmentation spectra were removed from the reference match list. The metabolite data was searched against a combined library of both metabolomic LC-MS/MS libraries provided by RIKEN on the MS-DIAL website and LC-MS/MS spectral libraries from the MassBank of North America (MBNA) [42]. This resulted in one combined positive library and one combined negative library. Spectral data for the internal standard homophenylalanine was added to both the negative and positive library, while the other ISTDs homoarginine and D3-methioine were already present in both negative and positive libraries. Data was normalised by a combination of normalisation to the ISTDs and locally weighted scatterplot smoothing (LOWESS) normalisation.

3. Results and discussion

3.1. Method optimisation

Developing methods for metabolomic analyses is complicated by the fact that parameters have to be optimised for compounds with highly diverse physicochemical properties. In addition, some metabolites are unstable in solution or are volatile [43]; thus, there is a need to analyse the extracts as quickly as possible, preferably on the same day. To achieve this, two separation methods were developed with an automated switch that allowed each sample to be analysed under conditions optimal for positive mode and then under conditions optimal for negative mode. The solvent switching method had positive mode solvents in two lines and negative mode solvents in the remaining two. A Waters® BEH Amide column was utilised as it has a wide pH range (2–11) allowing both acidic conditions and basic conditions to be run on the same column. Without this large pH range, the solvent switch would not be possible, and the column would have to be changed between positive and negative modes. Alternatively, two columns and two switch values could have been used to achieve a similar result. The large pH range of the Waters® BEH Amide column allowed the solvent switching method to be performed on one column reducing the cost, and had a simple instrument set up that did not require multiple switching valves.

3.2. Chromatographic conditions

For chromatographic and MS method optimisation, a combination of the total ion chromatogram (TIC) and representative metabolites for different metabolite classes were used (Table 3). 13 metabolites from 11 chemical classes (5 in positive mode and 6 in negative mode) were selected as representative metabolites (Table 3), these metabolites were selected as they are commonly detected in polar metabolomic methods and represent a large number of different chemical classes. These representative metabolites allowed examination of varying MS source parameters and their impact on different chemical classes commonly detected via polar metabolomic methods. Upon initial examination of what adduct form would be best to examine for the representative metabolites the ions detected were predominantly $[M+H]^+$ for positive mode (with the exception of choline which is natively charged), and $[M-H]^-$ for negative mode with only two instances of other adduct forms being identified ($[M+Na]^+$ for carnitine and $[M+Cl]^-$ for uridine). The protonated form of carnitine ($[M+H]^+$) was used for method development as initially no other form was detected upon manual inspection. Post-optimisation, a sodium adduct ($[M+Na]^+$) for carnitine was identified inconsistently through MS Dial. Manual investigation identified a chloride adduct for uridine ($[M+Cl]^-$) but was lower intensity and never prompted an identification through MS Dial despite being present in the libraries searched. As the deprotonated form was higher intensity and more readily identified it was selected for observation during optimisation.

Table 3
Representative compounds used for method development in both positive and negative mode.

Metabolite	Classification	Ion polarity	Adduct examined
Adenosine Monophosphate	Nucleotide	Negative	$[M-H]^-$
Carnitine	Quaternary amine	Positive	$[M+H]^+$
Choline	Quaternary amine	Positive	$[M]^+$
Glycolate	Organic acid	Negative	$[M-H]^-$
Guanine	Nucleobase	Positive	$[M+H]^+$
Leucine	Amino Acid	Positive	$[M+H]^+$
Isoleucine	Amino Acid	Positive	$[M+H]^+$
N,N-dimethylarginine	Amino Acid derivative	Positive	$[M+H]^+$
Pantothenic acid	Secondary Alcohol	Negative	$[M-H]^-$
Phosphocreatine	Phosphorylated compound	Negative	$[M-H]^-$
S-Adenosyl methionine	Nucleoside derivative	Positive	$[M+H]^+$
Uridine	Nucleoside	Negative	$[M-H]^-$
Valine	Amino acid	Negative	$[M-H]^-$

Both methods used ultrapure water as the aqueous component of the mobile phase, and 90 % ACN and 10 % water for the organic component. 10 % water was included in the organic solvent to allow the solubilisation of ammonia salts with only the additive differing between the two methods. For the acidic conditions, 0.1 % formic acid with and without 10 mM ammonium formate was compared to determine which would be better suited for metabolite analysis. Formic acid alone was unable to separate the isomers leucine and isoleucine, 10 mM ammonium formate with 0.1 % formic acid gave good responses for all representative metabolites and was able to separate isoleucine and leucine, and thus was selected for the acidic analysis. Leucine and isoleucine were chosen as representative metabolites as it is important to separate these isomers as they are common to all biological matrices and are of significant interest to several areas of research including dietary [44] and neuronal function [45].

For negative mode, 0.25 mM ammonium fluoride, 10 mM ammonium acetate and 10 mM ammonium bicarbonate, were examined with 10 mM ammonium bicarbonate giving the best results in negative mode as it could detect all representative ions at sufficient intensity, while the other conditions did not (See S Table 2). When optimising acidic conditions, a steep gradient (100 % B to 60 % B) was first employed, however, it was observed in the representative ions and the TIC that the majority of the analyte signal eluted at the beginning of the run. A stepped method allowed sufficient time for all compounds to be eluted and spread over a period of time to allow for an increase in the MS2 scans and thus potentially allowing for more compounds to be confidently identified. This method also allowed some isomer separation, namely the aforementioned isoleucine and leucine. The stepped gradient was unsuitable for the basic/negative method as compounds exhibited low intensity broad peak shapes with peak splitting (S Fig. 1). A steep gradient was employed instead which decreased the width of the broad peaks, allowing all compounds to elute before 7 min and a final run time of 10 min (S Fig. 2). While HILIC methods typically require long re-equilibration times, the high flow rates employed in the HILIC methods used in the present study (0.6–0.8 mL/min) allowed a relatively short re-equilibration time. For both methods (M1 and M2), 3 min was enough re-equilibration time to ensure repeatable injections with no discernible drift or change in peak shape. For each method, 7 repeat injections were performed on a sample during method development to ensure the methods were repeatable, determined via percentage relative standard deviation (%RSD) calculated for each representative metabolite. Both methods were found to be highly repeatable with all calculated %RSD values being less than 10 %. MS-DIAL analysis confirmed that all metabolites would elute before column re-equilibration. No carryover was observed in sequential blank injections. The pH of the mobile phase for both M1 and M2 was tested over the course of 7 days and was found to be stable with little variation over that period (Table 2).

3.3. Source conditions

Most metabolomic methods do not optimise source conditions and instead use default values [35–37]. In the present study however, some MS source parameters were optimised to increase the sensitivity. The main points of interest were observed from optimisation of the spray voltage, capillary temperature and aux gas heater temperature. Sheath gas flow rate, aux flow rate and S-lens RF level were left at default settings. When discussing the impact of MS source parameter optimisation, it is important to note that the improvements seen are in relation to protonated and deprotonated forms (excluding choline) of the representative metabolites. These are the two most common type of ions formed in their respective ionisation modes for metabolites and specifically for the representative metabolites selected for this study.

3.4. Aux gas heater temperature

The impact of aux gas heater temperature on peak height for the 13 representative analytes in positive and negative modes was compared between 100 °C and 600 °C (maximum) using 100 °C steps. Higher aux gas heater temperature was found to be optimal for both methods: 500 °C in negative mode (S Table 3) and 600 °C in positive mode (S Table 4). In negative mode, some of the representative ions had higher signal intensities at 500 °C and others had higher intensities at 600 °C, with one metabolite, adenosine monophosphate, having a lower preferred temperature of 200 °C. This metabolite signal didn't drop substantially, nor did the two that were optimal at 600 °C, thus 500 °C was selected for the aux gas heater temperature in negative mode. In positive mode the highest Aux gas heater temperature setting of 600 °C was selected. Four representative metabolites had their highest intensities with a temperature of 600 °C, the remaining three representative metabolites while not having their maximum intensities at 600 °C showed no substantial decrease from their maximum intensities (S Table 4). Suggested default aux gas heater temperature settings when using a flow rate of 0.5–1 mL/min (the flow rates closest to those used in optimised methods) are 300 °C–500 °C with no distinct values for positive and negative mode ionisation. The optimised value for positive mode (600 °C) was higher than the vendors suggested settings for this parameter. This was not the case for the optimised negative mode aux gas heater temperature value (500 °C) which fell within the upper limit of the values suggested by the instrument vendor.

3.5. Capillary temperature

In negative mode 350 °C was the capillary temperature considered to be optimal as intensities did not change greatly with this parameter (S Table 5). Positive mode ions had higher intensities either at a capillary temperature of 350 °C or 450 °C, therefore 400 °C was chosen as the optimal value (S Table 6). The instrument vendor suggests a capillary temperature of 380 °C–400 °C when using a flow rate of 0.5–1 mL/min. Similarly to aux gas heater temperature settings there are no distinct default values for positive and negative mode ionisation. The optimised value of 350 °C for negative mode was slightly lower than the lowest suggested default value whereas the optimised value for positive mode (400 °C) fell within instrument vendor's suggested range.

3.6. Spray voltage

Peak heights of the representative metabolites were very sensitive to the spray voltage. When voltages between 1 kV and 4 kV were examined in negative mode the ion intensity was maximal at higher voltages, with 4 kV being selected for the method (Fig. 1). The opposite was observed in positive mode, with all compounds preferring low voltages with 0.5 kV being selected for the optimal voltage (Fig. 2). This is similar to a targeted amino acid method [46] and has been previously demonstrated for small polar molecules [47]. While the optimised negative mode spray voltage of 4 kV is increased from the suggested default setting (2.8 kV), the optimal positive mode spray voltage of 0.5 kV was significantly lower than what is suggested by the instrument vendor (3 kV).

3.7. Collision energy

Fragmentation was also optimised, and various collision energies were compared. The best results were obtained from the use of stepped collision energy as it allowed multiple collision energies to be applied to analytes of interest, favouring a broader array of molecular classes. Stepped collision energy also produced a variety of fragments in the fragmentation spectra. For example, at higher collision energies the larger *m/z* identifying fragments, 264.09 *m/z* and 298.10 *m/z*, for S-Adenosyl methionine (S Fig. 3) dropped in intensity with 264.09 *m/z* not being among seen at 30 collision energy while smaller fragments (97.03 *m/z*, 203.06 *m/z* and 136.06 *m/z*) had lower intensities at lower collision energies. The stepped collision energy employed allowed both the small and large fragment groups to appear in the spectra at sufficient intensities. A similar pattern was seen in negative mode but required higher fragmentation energy than positive mode.

3.8. Sample preparation and internal standard selection

Better peak shape was obtained when the sample was diluted with ACN, most likely due to the sample being in a higher organic solvent concentration and a better match for the HILIC starting conditions. This resulted in a lower solvent volume being used for extraction so that the

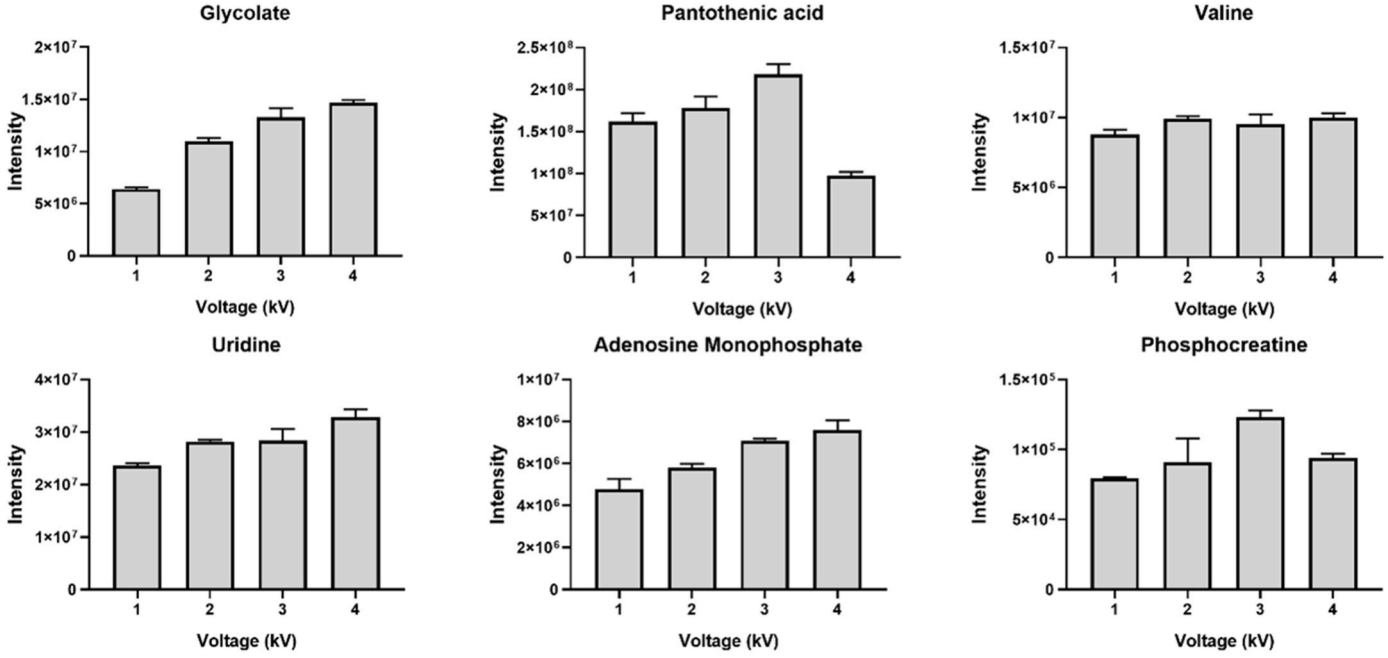

Fig. 1. Optimisation data for negative mode of the representative metabolites under different spray voltages. Error bars denote standard error of the mean (n = 2).

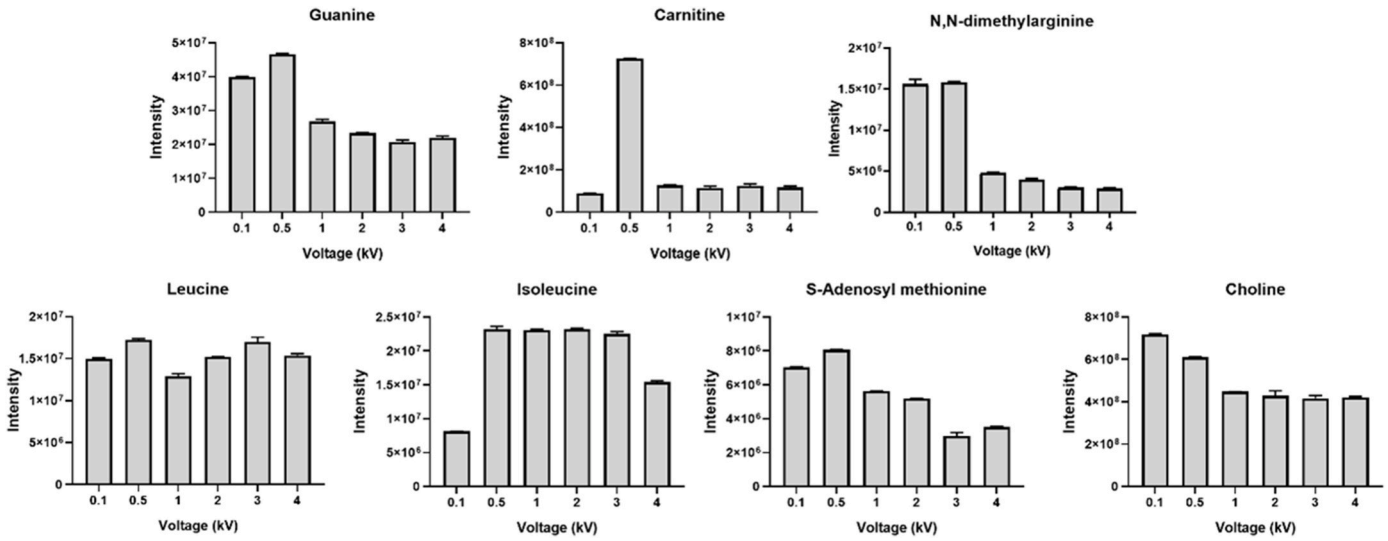

Fig. 2. Optimisation data for positive mode of the representative metabolites under different spray voltages. Error bars denote standard error of the mean (n = 2).

extract was more concentrated prior to the ACN dilution. The extraction procedure was based on that described by Sapcariu et al., [15]. A liquid-liquid extraction allowed separation of polar and non-polar metabolites and the removal of most lipids and proteins from the metabolite fraction resulting in a cleaner fraction. While ideally an ISTD representative of each class would be used, limited commercial availability and the high cost associated with purchasing several ISTDs would make this unfeasible. One ISTD was determined to be insufficient for the metabolite method so three ISTDs were chosen. Ideally these internal standards would elute at different times on the chromatographic run (beginning, middle and end) and would ionise in positive mode and negative mode. The non-protein amino acid ISTD mix included with the Phenomenex® EZ:faast™ amino acid derivatisation kit, specifically reagent 1, fulfilled these criteria. For both acidic and negative chromatographic conditions, homophenylalanine eluted at the beginning of the run, D3-methionine eluted shortly after and around the middle of the acquisition time, while homoarginine eluted at the end of both chromatographic runs. While D3-methionine is a deuterated amino acid and would not naturally occur in a biological sample, homoarginine and homophenylalanine are human metabolites. Recently homophenylalanine has been removed from the human metabolome database, regardless, neither it nor homoarginine were detected in the SH-SY5Y cells making them suitable candidates for this sample matrix. Both D3-methionine and homoarginine were already included in both the positive and negative combined libraries, and only homophenylalanine needed to be added.

The switch method was designed with two main factors in mind. The first was that there was subsequent time for the different MS source parameters (Table 1.), namely temperature, to transition. The length of the switch-over method (20 min) was shown to be sufficient for this to occur. The second factor was to allow equilibration of the column to the new conditions. The switch started in 100 % aqueous conditions of the new solvent to wash out the remaining buffer from the previous solvent and to minimise the chance of ammonium salts precipitating out on the column. The column flow rate was ramped over time up to the starting flow rate of the alternative method, and the percentage of the organic solvent was also increased over the duration of the method to match the starting conditions. This method was followed by a blank and QC to check that the switch would not affect the subsequent chromatography of the alternative method. While the switch method performed as intended, further optimisation could potentially be made to reduce the amount of time required. A limitation of this solvent switching is that due to all lines being used in the LC, a post-batch column wash with unbuffered solvents cannot be carried out and would have to occur after unbuffered solvents are loaded on the system.

3.9. Features and identification for solvent switching

MS-DIAL peak selection results are shown in Table 4. A total of 10308 peaks were identified (5685 in positive mode and 4623 in negative mode) using the solvent switching method. 3050 suggested identifications and matches were made when the identified peak's spectra were compared to the combined library (1955 in positive mode and 1095 in negative mode). 254 of the 3050 (177 in positive mode and 77 in negative mode) had matches to a metabolite in the spectral library. After additional filtering based on the prescribed criteria (See section 3.3) 104 metabolites were identified under acidic chromatographic conditions positive mode and 66 metabolites were identified under basic chromatographic conditions in negative mode, these identifications include 25 metabolites that were observed in both ionisation modes giving a total of 145 confidently identified metabolites. A list of confidently identified metabolites is shown in S Table 1.

3.10. Considerations for compound identification

Perhaps the most important step in a metabolomic workflow is compound identification/peak picking. Regardless of the program selected to handle the data, for confident identification to be established the output of the spectral library search needs to be thoroughly checked. When curating the metabolites identified in this study, further filtering of the metabolite identification results was put in place for more

Table 4
Results from MS dial and different stages of refining identifications for the solvent switching method.

Stage of identification	Basic (−) M2	Acidic (+) M1	Solvent switch M8
Features	4623	5685	10308
All suggested identifications	1095	1955	3050
Reference matched	77	177	254
Removal of intact mass only MS2 spectra and multiple adducts	69	137	206
Removal of non-human metabolites identifications	66	104	170
Unique identifications	41	79	
Shared identification	25		
Total confident identification			145

confident identification. Using MS-DIAL, peaks were identified, and data obtained was searched against a combined spectral library of the LC-MS/MS spectra provided by RIKEN on the MS-DIAL website and those provided by the MBNA. Two libraries were generated for this, one for each ionisation mode (i.e. one combined negative mode library and one combined positive mode library). Two search parameters were the most important regarding filtering the results obtained from the metabolite identifications based on matches to the combined spectral libraries. These were the "only report top hits" check and the "removal of features from blank". The "only report top hits" check allowed the program to filter duplicate matches and only match those that had the higher score. While there were a few duplicate identifications despite this being checked, it was mostly due to duplicate references with different naming conventions being present in the library. "Removing feature from the blank" allowed any identifications made in the blank to be removed from the list of identifications in the samples further filtering the identifications.

After processing in MS-DIAL, the compounds identified can be divided into two categories: 'suggested' compounds and 'reference matched' compounds. These categories are based on a scoring system in which the identified peak is matched to a compound in the spectral library, and takes into account factors such as accurate intact and fragment masses and the number of fragmentation matches to the spectral library candidate (with the option of retention time if the user wishes). This score is calculated and given a percentage value for how confident an identification is, and compared to a threshold that is set by the user. 'Suggested' compounds are those that did not meet the previously set threshold, 70 % in this case. Most compounds in this category have precursor m/z matches to a reference in the spectral library with no fragmentation. In MS-DIAL, for identifications to be classified as a reference match, fragmentation is required. Constitutional isomers would have the same high-resolution mass and, as the majority of metabolites do not have unique high-resolution masses [48], there is not enough information from intact mass alone to make a confident identification. Intact mass could potentially be used for identification if other information is known such as retention time. In addition, some suggested matches have fragmentation spectra but the fragment masses have a significant mass deviation to those of the suggested library match. Reference matched identifications match a library reference and exceed or equal the confidence threshold and require fragmentation matches. Further filtering of reference matched identifications was required for confident identification. Multiple entries in the library consist of only the precursor m/z in the fragmentation spectra, with some of these appearing in the reference matched identifications. Without additional fragments or retention time information identifications such as these cannot be validated, as the fragmentation spectrum gives no additional qualifying information and would be potentially shared amongst isobaric compounds. This is a common issue with almost all metabolite spectral libraries and compounds falling within this category were removed to increase confidence in the identifications made.

Another issue identified was that a small number of identifications had non-gaussian peak shapes. While the identifications may be correct, the occurrence of these peak shapes could be due to one of two factors. Firstly, the compounds are low in abundance or have low ionisation efficiencies and thus have a low signal-noise-ratio (S/N). The second factor is that the poor peak shape is due to the chromatographic conditions not being favourable. Comparison of these poorly shaped or low signal peaks may lead to misinterpretation of significant differences in metabolite abundance, and were removed from identification. The identification of non-gaussian peak shaped compounds may be a limitation of the peak picking algorithm for MS-DIAL, however there were not a substantial number of non-gaussian, or poor peak shaped compounds identified. The final criteria for filtering was the removal of non-metabolite features. The spectral libraries used contained compounds such as environmental contaminants and compounds previously detected in humans due to exogenous exposure. The latter is common to most metabolite libraries, however, the presence of some of these compounds cannot be justifiably explained in the cell samples used and were removed.

While isoleucine and leucine were reported to be identified using this method, it was only possible by manual inspection of the chromatograms and knowledge of the retention times. MS-DIAL generally identified and selected only one of the two isomers for automatic peak integration. The identification was correct for some samples and incorrect for others. Without prior knowledge of the retention times of these two isomers, their differentiation would not be possible. This is a limitation of untargeted methods, specifically for isomers that do not have unique fragmentation.

While a multitude of software options are available [41,49,50], one of the biggest limitations is the availability of relevant metabolite spectral libraries in the correct format for the program of choice (MS-DIAL in this case). This is mostly obvious in the number of identified features in negative mode. Negative mode had 66 identified features, which is approximately three fifths of the features identified in positive mode (104 identifications). When comparing combined spectral libraries, the positive library has 389,046 spectra (290,915 from RIKEN, 98,130 from MBNA and the ISTD homophenylalanine), whereas the negative mode combined library had 83,292 spectra (36,848 from RIKEN, 46,443 from MBNA and the ISTD homophenylalanine). While this is not factoring in repeated compounds or compounds without MS2 spectra/only precursor mass as the MS2 spectra compounds, this shows that the library for negative mode is approximately a fifth of the size of the positive mode library, explaining why the number of compounds identified in negative mode are less than those observed in positive mode. This is not uncommon in spectral libraries as there is more information pertaining to positive mode acquisitions available then there is compared to negative mode acquisitions.

3.11. Comparison of individual methods for different ionisation modes

It was important to compare the acidic and basic conditions in the present method to neutral conditions to ensure they were providing optimal metabolite coverage. Since analysts curate their data differently, we performed an in-house comparison using the same pipeline. In section 3.1 it was mentioned that neutral conditions were poorer for negative mode as they could not detect all representative ions and those detected were at lower intensities. Previously, comparison studies have suggested that neutral pH gives superior results to either basic or acidic conditions [51,52], however for most of these studies the chromatographic elution conditions (i.e gradient) were the same across all pH conditions examined. pH and additives may affect how the metabolite analytes interact with a given stationary phase (water in the case of HILIC). Additives and pH can affect the peak shape and retention of metabolites, thus for a true evaluation, the method should be carefully developed to allow a valid comparison. Two neutral (10 mM ammonium acetate) methods were developed for both positive (M3) and negative (M4) mode ion polarities. It was found that a sloped chromatographic elution was best for positive mode and stepped chromatographic elution was best for negative mode under neutral conditions highlighting the need to optimise each method individually. Similarly to what was observed for M1 and M2 the pH of the mobile phase used for M3 and M4 was found to be stable for 7 days (Table 2).

Methods M3 and M4 which utilise neutral conditions were compared to the previously developed methods M1 and M2 which utilise acidic and basic conditions respectively (Section 2.4). When comparing positive mode data, M1 had more features and identified more metabolites than M3 (Table 5). For negative mode, M4 had more total features than M2, however, for reference matched metabolite identifications this was not the case and M2 gave more confidently identified compounds than M4 (Table 5). Of the combined 110 confidently identified metabolites in positive mode 95 % could be detected using the acidic conditions (M1)

Table 5
Results from MS dial and refining of identification for different methods examined.

Stage of identification	Basic (−) M2	Neutral (−) M4	Acidic (+) M1	Neutral (+) M3
Features	4623	5660	5685	4028
All suggested identifications	1095	1204	1955	1377
Reference matched	77	56	177	88
Removal of intact mass only MS2 spectra and multiple adducts	69	45	137	62
Removal of non-human metabolites identifications	66	40	104	51

but only 41 % could be detected under neutral conditions (M3) and of the combined 73 confidently identified metabolites in positive mode 90 % could be detected using the basic conditions (M2) but only 55 % could be detected under neutral conditions (M4) (Fig. 3). Therefore, when comparing identified metabolites for each ionisation mode, neutral pH conditions in either ionisation mode were vastly inferior (Fig. 3).

This is not in line with previous comparison studies [51,52], which suggest that neutral pH was the superior condition however, this could have been due to lack of optimisation of the chromatographic methods, the sample matrix analysed, extraction techniques, or the data analysis pipelines utilised. When comparing all pH conditions of opposing ion polarity (See S Fig. 4 for Venn Diagrams comparing opposing ionisation methods for all pH conditions examined) it was observed that the average overlap for each condition when compared to the opposite ionisation mode was around 30 %, (with the exception of M1 which had 20 % average overlap due to hit having a higher number of identifications). This further highlights the importance of analysing each ion polarity and not a single ion polarity for metabolomic analysis.

3.12. Comparison of solvent switching to polarity switching

An accurate comparison of the method developed in the present study to published methods, based on the total number of metabolites identified, is difficult as different researchers curate metabolomic data differently. Thus, for an accurate assessment of the solvent switching method (M8), an in-house comparison was conducted comparing it to the more commonly used single methods with ion polarity switching (see Table 2 for details of all methods). When comparing M8 to the two polarity switching methods, one under acidic conditions (M5) the other under basic conditions (M6), M8 gave more confidently identified metabolites than both methods (Table 6). M8 had 145 uniquely identified

Table 6
Results from MS dial and refining of identification, comparing solvent switching (SS) and polarity switching (PS) methods examined.

Stage of identification	Acidic PS M5	Basic PS M6	PS SS M7	Single polarity SS M8
Features	8893	7888	16781	10308
All suggested identifications	2800	2073	4873	3050
Reference matched	162	117	279	254
Removal of intact mass only MS2 spectra and multiple adducts	141	106	207	206
Removal of non-human metabolites identifications	126	99	185	170
Shared identification	22	18	64	25
Total confident identification	104	81	121	145

metabolites, M5 had 104 and M6 had 81. M8 identified most of the metabolites in both polarity switching methods, covering 95 % of the metabolites identified in the M6 and 88 % of the metabolites identified in M5 (Fig. 4). Whereas just over half of the metabolites confidently identified using method M8 were covered in either polarity switching method (63 % in M5, 53 % in M6). Solvent switching was also superior when comparing total features to either polarity switching method (Table 6).

When comparing the two polarity switching methods (M5 and M6) to each other (S Fig. 5), there was a large overlap in the metabolites identified; 62 % of identified metabolites in M5 were identified in M6, and 79 % of metabolites in M6 were identified in M5. Thus, when comparing the total number of confidently identified metabolites, the acidic conditions with polarity switching (M5) was the superior metabolomic method of the two.

Polarity switching allows simultaneous analysis in both positive and negative mode but causes a decrease in sensitivity and a decrease in the number of points across a chromatographic peak for LC-MS methods [53]. These are not the only limitation, polarity switching DDA experiments will halve the number of MS2 scans per ion polarity when compared to single ion polarity DDA methods, as the total acquisition time remains the same but is now divided amongst two polarity modes. Another limitation of polarity switching methods is that source conditions cannot be optimised, as some conditions such as temperature requires time to change and would not have sufficient time during the polarity switching to reach the final settings for each ion polarity. It is most likely a combination of all of these factors that results in M8 (which makes use of 2 individual methods), being superior to either M5 or M6.

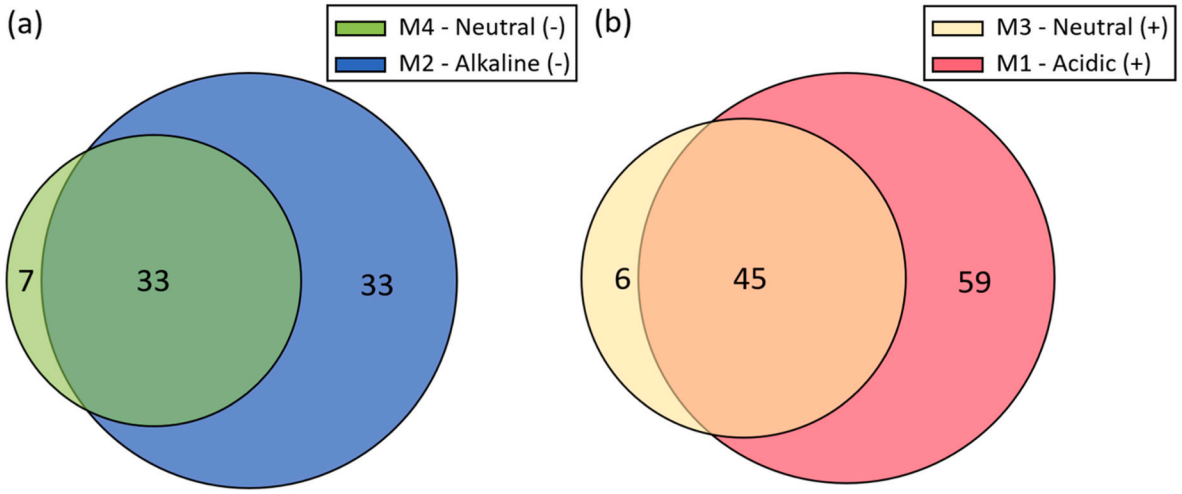

Fig. 3. Venn diagrams comparing confidently identified metabolites for each method of each ion polarity examined. A) Comparison of conditions for negative ion mode (M2 vs M4). B) Comparison of conditions for positive ion mode (M1 vs M3).

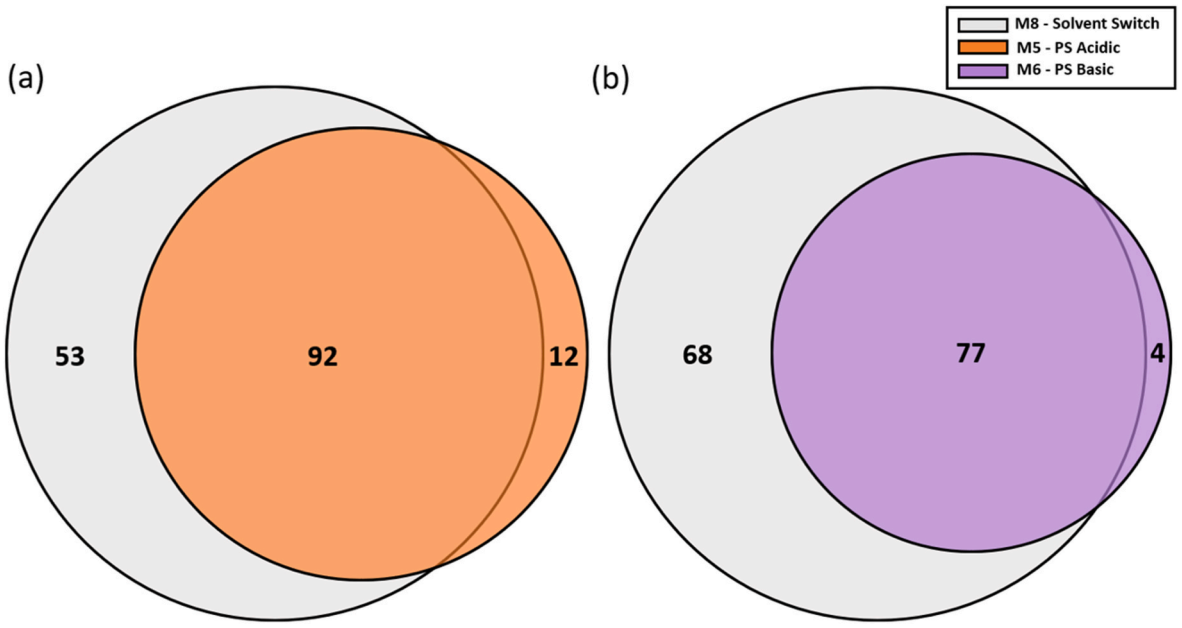

Fig. 4. Venn diagrams comparing confidently identified metabolites for the different polarity switching methods to the solvent switching method. 4a) Comparison of solvent switching (M8, grey) to acidic chromatographic conditions polarity switching method (M5, orange). 4b) Comparison of solvent switching (M8, grey) to basic chromatographic conditions polarity switching method (M6, purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Despite this, there is one area where the use of polarity switching is beneficial over solvent switching, that being time and cost of analysis, as solvent switching requires two separate chromatographic methods and thus has a higher solvent consumption rate.

Solvent switching can also be used in tandem with polarity switching methods, allowing two polarity switching methods to run sequentially with different chromatographic conditions. When this was performed (M7) and compared to M8, M8 had the higher number of identifiable metabolites (145 vs 121). This is most likely due to the large overlap in compounds detected between the individual polarity switching methods (M5 and M6) (S Fig. 5). M7 would also be subject to the previously mentioned shortcomings of polarity switching. When comparing the overlap between M8 and M7 there was a high number of overlap for each method (88 % of M7's confidently identified metabolites were identified using M8 and 72 % of M8's confidently identified metabolites were identified using M7) (Fig. 5), which is to be expected as they both utilise the same chromatographic elution for each method.

M7 had the most MS1 features (Table 6), most likely due to there being 4 data sets generated from this method (two positive mode and two negative mode). While M7 has the highest number of features, it is unclear how many of these are unique. When the identified features is used as a guide (S Fig. 5), it would appear there is a high level of overlap amongst the four M7 data sets. While identified data would suggest a large overlap, M7 could potentially be used for in-depth MS1 analysis if that is what the analyst prefers.

One limitation of using methods that have two ion polarities (either through polarity switching or solvent switching) with the current data analysis pipeline is that MS-DIAL does not allow simultaneous positive mode and negative mode data searching (i.e peak picking and library matching). From this, if M5, M6, or M8 is employed, two separate data searches need to be performed (1 positive mode and 1 negative mode), whereas methods that only use one ion polarity such as M1, M2, M3 and M4 only require one search. This issue is compounded when using polarity switching in conjunction with solvent switching (M7) as 4 separate data searches need to be conducted for full data analysis (2 positive mode and 2 negative mode). Ideally an analyst would prefer a method involving the least number of searches that yields the highest amount of unique data. As there is little overlap between positive mode and negative mode results (S Fig. 1), for the most comprehensive analysis, both ion polarities should be analysed and thus the minimum number of searches required is 2. Upon comparison of the methods that utilise both ion polarities (M5, M6, M7 and M8), M8 yielded the most confidently identified metabolites and only required two searches, once again demonstrating its suitability for comprehensive metabolite analysis.

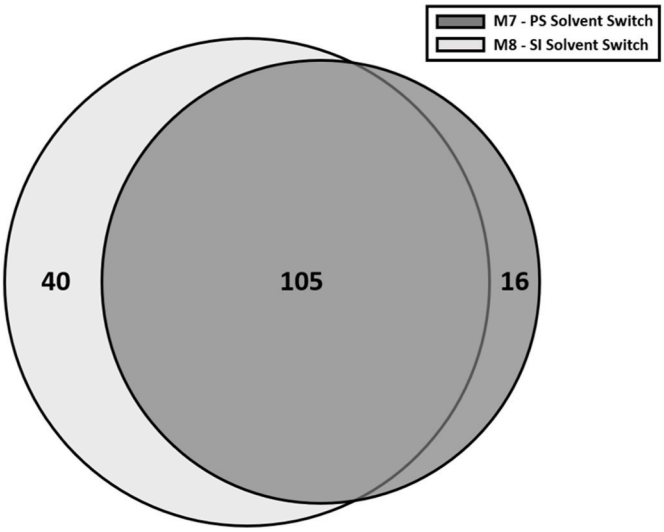

Fig. 5. Venn diagrams comparing confidently identified metabolites for the single ion (SI) polarity solvent switching method (M8) and the polarity switching solvent switching method (M7).

4. Conclusion

A novel metabolomic method was developed that allowed comprehensive analysis of SH-SY5Y cells under two pH conditions (acidic and basic) in two ionisation modes (positive and negative) in the same analytical batch using solvent switching. When compared to polarity

switching methods, the solvent switching method was superior in both features detected and compounds identified. If solvent switching is employed with two polarity switching methods, it allowed a higher MS1 feature count but was inferior on confidently identified metabolites suggesting it is ideal to run two single ion polarity mode methods for solvent switching then two polarity switching methods.

CRediT authorship contribution statement

Jake P. Violi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Connor R. Phillips:** Writing – review & editing, Investigation. **David S. Gertner:** Investigation. **Mika T. Westerhausen:** Writing – review & editing, Conceptualization. **Matthew P. Padula:** Writing – review & editing, Conceptualization. **David P. Bishop:** Writing – review & editing, Conceptualization. **Kenneth J. Rodgers:** Writing – review & editing, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.127610.

Data availability

Data will be made available on request.

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