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EDITORIAL SUMMARY

A protocol for the analysis of polar and ionic metabolites using anion-exchange chromatography tandem mass spectrometry (AEC-MS/MS) with an untargeted or semi-targeted workflow for the identification of metabolites in biological samples.

PROPOSED TWEET

Metabolomics analysis of highly polar and ionic compounds by direct coupling of anion-exchange chromatography with mass spectrometry.

PROPOSED TEASER

Anion-exchange chromatography mass spectrometry metabolomics

KEY POINTS

- This protocol uses an integrated ion-chromatography-mass spectrometry system that incorporates in-line eluent generation and electrochemical ion suppression for the analysis of metabolites in cells, tissues and biofluids.

- Highly sensitive, selective, reproducible and robust, this protocol provides an alternative to methods such as HILIC-MS and ion-pairing-MS which can be more sensitive to analytical conditions and provide different metabolite coverage.

DATA AVAILABILITY

The authors declare that the main data discussed in this protocol are available in the supporting primary research papers (https://doi.org/10.1038/s42003-020-0957-6 and https://doi.org/10.1038/s41467-022-34095-x). The raw datasets have also been deposited in publicly available repositories for research purposes and any further data is available from the corresponding author upon reasonable request. Source data for Figure 1 is available in the Oxford University Research Archive with the identifiers

http://dx.doi.org/10.5287/bodleian:2abVOAvrg and http://dx.doi.org/10.5287/bodleian:eyq4Qj8AR (ref. 19). Source data for Figure 3 is available in Supplementary Data 6.

CODE AVAILABILITY

No code was generated during this study.

Metabolomics using anion-exchange chromatography mass spectrometry for the analysis of cells, tissues and biofluids

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ABSTRACT

The direct coupling of ion-exchange chromatography with mass spectrometry using electrochemical ion-suppression creates a hyphenated technique with selectivity and specificity for the analysis of highly polar and ionic compounds. The technique has enabled new applications in environmental chemistry, food chemistry, forensics, cell biology, and more recently metabolomics. Robust, reproducible and quantitative methods for the analysis of highly polar and ionic metabolites help meet a longstanding analytical need in metabolomics. Here, we provide the step-by-step instructions for both untargeted and semi-targeted metabolite analysis from cell, tissue or biofluid samples using anion-exchange chromatography high-resolution tandem mass spectrometry (AEC-MS/MS). The method requires minimal sample preparation and is robust, sensitive and selective. It provides comprehensive coverage of hundreds of metabolites found in primary and secondary metabolic pathways, including glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, purine and pyrimidine metabolism, amino acid degradation and redox metabolism. An inline electrolytic ion-suppressor is used to quantitatively neutralise OH^- ions in the eluent stream, post-chromatographic separation, enabling AEC to be directly coupled with MS. Counter-ions are also removed during this process creating a neutral pH, aqueous eluent with a simplified matrix optimal for negative ion mass spectrometry analysis. Sample preparation through to data analysis and interpretation is provided in the protocol, including a guide to which metabolites and metabolic pathways are suitable for analysis using AEC-MS/MS.

KEYWORDS

Anion-exchange chromatography; mass spectrometry; metabolomics; untargeted; semi-targeted; highly polar; anions; metabolic pathways.

[H1] INTRODUCTION

Metabolomics involves the comprehensive analysis of metabolites from biological systems to identify biomarkers, characterise biological responses and interpret metabolic functions. It is used to measure changes in the complex metabolic landscape of cells, tissues and biofluids that respond to endogenous and/or exogenous perturbations. However, the performance of analytical methods used in metabolomics remains a limiting factor; in particular for the comprehensive analysis of highly polar and ionic metabolites.

Polar and ionic metabolites are ubiquitous in primary metabolism, sustaining the function of living cells via processes such as energy transduction (e.g., involving glycolysis, pentose phosphate pathway, and tricarboxylic acid (TCA) cycle); formation of building blocks (e.g., purine and pyrimidine metabolism) and homeostatic functions (e.g., redox metabolism and anti-oxidation). Although many of these pathways are highly conserved across species, they respond dynamically to altered cellular conditions and are therefore often perturbed in disease states including cancer, diabetes, heart disease and infectious diseases[1-5]. The abundance of a number of metabolites can also change rapidly in response to environmental conditions, for example when energy requirements alter due to changing glucose levels or oxygen availability. There are a range of excellent protocols available for general metabolomics applications[6-9], including more specialised approaches[10-17], but methods which can sensitively, selectively and reproducibly analyse highly polar and ionic metabolites from complex biological samples remain limited. Therefore, new approaches for analysing the highly polar and ionic metabolome have an important role to play in extending our understanding of how biological systems function and respond at the small molecule level.

Analytical methods used in metabolomics often balance capabilities for metabolite coverage with those for identification and quantification. Current methods can be placed somewhere on a spectrum between fully untargeted (optimising hypothesis generation, but lacking fully comprehensive identification and quantification) and targeted (providing a highly selective quantitative response for a pre-defined number of identified metabolites[5]. Both approaches are important and are complementary. 'Semi-targeted' methods blend the analytical benefits of both targeted and untargeted approaches, incorporating untargeted detection methods with more rigorous metabolite identification and quantitation[18].

To expand the analytical methods available for the analysis of highly polar and ionic metabolites, we previously developed and applied an anion-exchange high-resolution tandem mass spectrometry (AEC-MS/MS) method suitable for untargeted, semi-targeted and targeted metabolomics applications focused on characterising highly polar and ionic metabolites extracted from mammalian cells[19,20]. We have since successfully applied this method to the analysis of a wider range of cell types, tissues and biofluids and have, along with others, demonstrated that AEC-MS/MS is a highly reproducible and robust technique that can provide advantages over other methods, particularly when analysing metabolites

that are ionised in solution[4,21-35]. Certain types of ionic metabolites have proven challenging to characterise using alternative liquid chromatography-mass spectrometry (LC-MS) methods, for example nucleic acids, phosphorylated sugars and their structural isomers. Given that many metabolic pathways involved in primary metabolism are driven by the interconversion of these, and similar acidic, negatively charged metabolites, AEC-MS/MS meets a latent need in metabolomics and its application has the potential to provide more detailed insights into cellular function under varied conditions. Here we describe in detail the analysis of highly polar and ionic metabolites using the AEC-MS/MS protocol with selected updates to our previously published method. The protocol provides an untargeted or semi-targeted workflow, with comprehensive metabolite data being collected and Level 1 assignment of metabolite identifications based on a panel of validated standards. The protocol described includes a complete workflow from sample preparation for cells, tissues and biofluids, sample analysis, data processing (with identification of metabolites), data analysis and interpretation of results using univariate and multivariate statistics and functional data analysis approaches.

[H2] Development of the protocol

Ion-exchange chromatography coupled to mass spectrometry (IC-MS) is well-suited to the analysis highly polar and ionic metabolites and its reproducibility and robustness enables efficient post-analysis metabolite identification. The mechanism of IC involves a direct interaction between the analyte and the stationary phase and has a well understood mechanism of retention and elution based on ionic interactions between functional groups on a resin-based stationary phase. Elution occurs by charge-exchange using high ion strength mobile phase ions; typically, a gradient of hydroxide ions (for anion-exchange) or protons (for cation-exchange). Compatibility with mass spectrometry is facilitated by an electrochemical ion-suppressor which interfaces the chromatography system and the electrospray ionisation (ESI) source of the mass spectrometer. The ion-suppressor is comprised of three flow-channels separated by two semi-permeable membranes with electrodes on the outer edges. For anion-exchange, the suppressor electrolytically generates H^+ ions from water in the outer channels. These H^+ ions cross the membrane into the central channel containing the eluent and combine with the mobile phase OH^- to form H_2O, while the mobile phase counter ions in the eluent (typically K^+) cross the semi-permeable membrane and go to waste, leaving resolved analyte ions in an aqueous solution to enter the ESI source of the mass spectrometer. As the counter ions are removed from the eluent, other positively charged ions are also removed. We provide a more detailed description of this suppression process for both cation and anion-exchange, in a recent review[36].

We initially developed an untargeted method using AEC-MS/MS to analyse ionic and highly polar metabolites in tumour cells investigating altered primary metabolism and related signalling pathways because we had found ion-pairing and hydrophilic interaction liquid

chromatography (HILIC) methods were not sufficiently robust and did not provide adequate metabolite coverage. This work revealed that AEC-MS/MS was able to achieve higher reproducibility, robustness, sensitivity, and coverage of metabolites associated with primary metabolic pathways. We optimised the OH^- gradient to maximise metabolite coverage paying particular attention to the separation of structural isomers that are common in primary metabolism, and which are in general challenging to resolve with other LC methods. For example, sugar phosphates (e.g., glucose-1-phosphate and glucose-6-phosphate) and various organic acids such as fumaric acid and maleic acid. We explored metabolite coverage using a wide range of metabolite standards[19] and have demonstrated quantitative responses for hundreds of metabolites of various types including sugar phosphates, nucleic acids, carboxylic acids and a range of co-factors and other charged metabolites. We used authentic standards spiked into biological samples, along with retention time (RT) and higher-energy collisional dissociation (HCD) tandem mass spectrometry, to identify and characterise these metabolites in biological extracts from cells, tissues, and bio-fluids. Formal targeted method validation was performed, demonstrating limits of detection, linearity, sensitivity and robustness for AEC-MS/MS (see '*Comparison with other methods*' section and Figure 1 for further details). Validation metrics for a selection of metabolites is provided in Supplementary Table 1. In general, whilst developing the protocol we observed: 1) the ability of AEC-MS/MS to characterise highly polar and ionic metabolites found in cells, tissues and biofluids. 2) High analytical reproducibility and robustness when characterising a wide range of chemical structures. 3) A predictable and manipulable chromatographic separation and retention mechanism.

The AEC-MS/MS method has some limitations in addition to benefits. For example, it is not well suited to resolving hexose and pentose sugars (monosaccharides and disaccharides), nor amino acids (with the exception of the acidic amino acids glutamate and aspartate). AEC-MS/MS was, however, notably successful at resolving nucleic acids derivatives (see Figure 1a-b) but we also observed that some de-phosphorylation of multiply phosphorylated metabolites can occur (e.g., adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), cytidine triphosphate (CTP) and similarly for other multi-phosphorylated metabolites) during the process of ionization, or potentially in the ion optics during ion transmission. For example, we observed some ATP was converted to ADP and ADP to adenosine monophosphate (AMP), other di and tri-phosphorylated metabolites are also susceptible. This is not surprising as it has previously been shown that dephosphorylation can occur during the process of electrospray ionisation[37]. De-phosphorylation is therefore not specific to AEC-MS/MS, and we have shown that the degree of dephosphorylation appears to be linear with concentration (as demonstrated in Figure 1c-d). Hence differences in abundances between these metabolites in different samples can still be measured and compared. We note that hydrolysis of phosphate groups can also occur in samples prior to AEC-MS/MS analysis. It has been shown by others that metal ions and lower pH environments can accelerate this process. Acidic metabolite extractions may therefore lead to increased phosphate loss and EDTA treated samples may

reduce any metal-ion catalysed hydrolysis[38]. The AEC chromatographic process in AEC-MS/MS is both metal-free and operates at basic pH, both of which should mitigate hydrolysis prior to electrospray. Since our first publications with the AEC-MS/MS method we have made minor modifications to the chromatographic and mass-spectral analytical conditions, and we publish the most up to date version of the protocol here.

[H2] Applications of the protocol

Given a rapid increase in metabolomics applications in recent years the target audience for this protocol has been growing. AEC-MS/MS has been applied in targeted, semi-targeted and untargeted studies in a wide range of research areas including recently cell metabolism[4,39-51]; physiology[21,52], oncology[22,23,53,54] and immunometabolism[24-26]. We, and other groups, have demonstrated the effectiveness of AEC-MS/MS in metabolomics applications exemplified across a range of biological sample types and extracts, including cells[24-27,49,50,53-55], tissues[28-31,51], plasma and serum[23,48,56], urine[56,57], microorganisms[32,33] and plants[34,35].

Given the coverage, robustness, and reproducibility AEC-MS/MS has shown in these studies, it provides excellent potential for new applications (including different sample types) being analysed in the future. For example, the analysis of saliva, microbiome-related faecal and caecal samples and cerebrospinal fluid (CSF) are yet to be explored in detail. There is also scope for targeting new metabolite classes using alternative stationary phases and ion-exchange modes (e.g., endogenous metal ion analysis and other cationic metabolites[58]). Isotope tracer and metabolic flux analysis studies benefit from the coverage of central carbon metabolism, making it a useful platform for such studies. Indeed, we have shown that AEC-MS/MS provides high fidelity coverage of metabolites and their isotopologues in dual ^{13}C tracer isotopic labelling experiments[31]. Another potential area for future development is alternative mass spectrometric configurations to enhance metabolomics experiments. For example, to date, most IC-MS methods have coupled the IC system to high-resolution Orbitrap MS with HCD-based tandem mass spectrometry to obtain fragmentation patterns, useful for metabolite identification. For some metabolite classes (e.g., phosphorylated metabolites), single-level HCD fragmentation may not provide structurally selective product-ion, and alternative modes may be worth exploring. For example, MS^n has the potential to provide greater structural specificity and, in the context of AEC-MS/MS based metabolite analysis, could provide greater structural fidelity for isomeric metabolites (such as sugar phosphates) in structural elucidation studies. Additionally, exploring alternative fragmentation methods may bring greater selectivity (e.g., in theory using Ultraviolet Photodissociation, Electron-transfer Dissociation, Infrared multiphoton dissociation etc). In addition, combining AEC-MS/MS with ion-mobility has not been implemented previously (to our knowledge) but has the potential to help address the major analytical challenge inherent in differentiating large numbers of structural isomers, such as closely eluting monosaccharides (glucose, fructose, galactose, mannose etc) and their phosphorylated products, for example. This is challenging as over half the highly polar

and ionic metabolites found in primary metabolic pathways have a chemical formula representative of multiple structural isomers[59].

[H2] Comparison with other methods

Currently, HILIC-MS, ion-pairing-MS and other mixed-mode chromatography-MS methods are used in metabolomics applications for the analysis of highly polar and ionic metabolites[60]. These methods are, however, often limited to a varying degree by their lack of robustness, metabolite coverage, reproducibility and/or quantitative capabilities, particularly when analysing extracts from complex biological matrices such as cells, tissue and biofluids.

HILIC-MS is probably the most commonly applied of these methods in metabolomics applications but can be subject to relatively high chromatographic RT and peak area, variability compared to other LC-MS methods[61]. In addition, sensitivity can also be relatively low for some metabolites,[62] and certain functional groups and classes of metabolite are generally not well characterised, including nucleic acids and some phosphorylated metabolites[63]. Mechanisms of analyte retention and elution in HILIC chromatography involve complex stationary phase-analyte interactions that are not fully understood and require careful column pre-conditioning[64] and choice of sample diluents. This is a limitation in untargeted metabolomics applications where sample throughput, analytical reproducibility and robustness are important considerations. The complex mechanisms of analyte retention associated with HILIC also often make it difficult to predict metabolite elution times and elution order making metabolite identifications more challenging[64]. Column conditioning requirements mean that metabolite extracts must be present in high organic content solvents (often acetonitrile) which can detrimentally affect the solubility/sensitivity of highly polar and ionic metabolites. Methods that mitigate the analytical challenges exhibited by HILIC-MS, particularly associated with the analysis of highly polar and ionic metabolites, therefore have the potential to improve metabolic profiling capabilities[61].

To provide a direct comparison between AEC-MS/MS and HILIC-MS/MS we previously benchmarked our AEC-MS/MS method against a commonly used HILIC-MS/MS method for metabolomic profiling[19]. Both methods were applied independently, analysing a selection of authentic metabolite standards (chosen to represent a range of chemical structures) and untargeted profiling of metabolites extracted from mammalian cell extracts. The same MS instrument was used with each chromatography approach. This comparison therefore specifically focussed on the differences in chromatography, controlling for MS type. In general, we found improved analytical performance with AEC-MS/MS compared to HILIC-MS/MS for the analysis of metabolite standards and cell extracts. Comparative results for typical analytical method validation parameters are shown in Figure 1 and further details

can be found in Walsby-Tickle *et al*[19]. Validation included chromatographic resolution (Figure 1a-b), quantitative loss of phosphate as illustrated by GTP and ATP (Figure 1c-d), testing linearity (Figure 1e-h), , untargeted reproducibility (Figure 1i-j), and the RT reproducibility (Figure 1k).

For untargeted metabolomics validation, the analysis of cell extract samples was performed using AEC-MS/MS and HILIC-MS (see PCA plots in Figure 1, panels i-j). HILIC-MS/MS fully resolved cells grown on high and low glucose media (reflecting changes in primary metabolism) but the isogenic LN18 glioblastoma cells with and without isocitrate dehydrogenase mutilations (mutIDH1 and wtIDH1) were not well resolved (grown at the same glucose media levels). In contrast the AEC-MS/MS method was able to completely resolve all four experimental groups (both glucose levels and IDH1 mutation status), illustrating strong performance for characterising variations in metabolism in both cases. The statistical analyses for both techniques followed the post-processing steps in this protocol with the same data normalisation, filtering, and zero-value imputation to enable direct comparison.

We identified three particularly notable outcomes from method validation and comparison with HILIC-MS: 1) AEC-MS/MS provided higher RT reproducibility and stability compared to HILIC-MS/MS, in turn making it more reliable and effective for metabolite identification when combined with other parameters (e.g., accurate mass measurements, isotope distributions and fragmentation patterns). 2) When full metabolite profiles from cell extracts were compared AEC-MS/MS provided noticeably higher peak areas response reproducibility compared to HILIC-MS/MS (using the same samples and mass analyser). 3) AEC-MS/MS was capable of fully or partially separating a wider range of 'hard-to-resolve' structural isomers relevant to primary metabolism, including sugar phosphates and organic acids. We are not aware of any published reports comparing AEC-MS/MS with ion-pairing methods to date.

[H2] Experimental design

Semi-targeted metabolic profiling using AEC-MS/MS (also referred to as simultaneous quantification and discovery (SQUAD) methods in a recent review[65]) aims to use untargeted detection of metabolites with comprehensive metabolite identification and quantification. For this AEC-MS/MS semi-targeted method, we use a database of authentic standards with RTs for identification and relative abundance changes for quantification. Optimal results from the protocol requires careful consideration of the entire workflow including experimental planning, sample collection, sample preparation, sample storage, the chromatographic and MS method, the setup and acquisition of data, data processing, data analysis and interpretation of results. An overview of the steps in the AEC-MS/MS protocol is provided as a schematic in Figure 2.

[H3] Planning experiments. The application of AEC-MS/MS has implications for experimental design as it informs the types of metabolites measured and the number of samples that are required in a study. Many hundreds of metabolites in cells, tissues and biofluids are sufficiently polar or ionic to be characterised by AEC-MS/MS and it is therefore realistic to conduct untargeted or semi-targeted studies. For in vitro studies we recommend a minimum of five biological tissue culture replicates (however, we find 8-10 is preferable) per experimental condition and approximately $5e^5$-$1e^6$ cells (roughly one near-confluent 6 cm diameter tissue culture dish). Additionally, we have found 12-well tissue culture plates (one well per replicate) enable reproducible analysis of metabolites, with coverage comparable to samples from individual 6 cm dishes (note some low-abundance metabolites are susceptible to dropping below limits of detection/quantification so care is needed when using 12 well plates). The advantage of 12-well plates it that they provide an efficient way to perform tissue culture and a straightforward way to expand the number of experimental groups used in an in vitro study[66,67].

[H3] Sample collection. Primary metabolism is primed to respond rapidly to changes in the cellular environment. For example, cell sensing mechanisms such as adenosine monophosphate-activated protein kinase and the mammalian target of rapamycin respond quickly to altered sugar or amino acid availability and initiate metabolic processes that lead to changes in ATP levels in cells within seconds. This can subsequently lead to rapid changes in the abundance of other metabolites that are measured by AEC-MS/MS[68]. From a metabolic perspective, it is therefore particularly important with AEC-MS to ensure that metabolites in cells, tissue and biofluids are extracted in such a way as to preserve their endogenous metabolic steady state abundances without perturbation. We recommend freezing cells and tissues in liquid nitrogen at the point of collection (or cooling the extraction solvent on dry ice) and ensure metabolite extraction, and further sample handling, occurs subsequently on ice, including cooled centrifugation.

[H3] Sample preparation and storage. The way samples are prepared, including which solvents are used to extract metabolites (and their pH) can affect the AEC-MS/MS metabolomics results. This is partly due to the versatility the method offers for sample preparation (different extraction solvents favour recovery of certain metabolites over others). We have found that in general both fully aqueous and fully organic sample extraction solvents and diluents are compatible with AEC-MS/MS, including Type 1 water, methanol, ethanol, butanol, and acetonitrile (including solvent mixtures). This provides a high degree of flexibility over which solvents can be used in a study and enables a wide range of metabolite extraction protocols to be compatible with AEC-MS/MS (note this is in contrast to HILIC-MS/MS, where high-percentage organic sample diluents are generally essential but can limit ionic metabolite recovery). Highly polar and ionic metabolites tend to favour extraction in highly aqueous and polar solvents[69]. We favour 80% (v/v) MeOH in Type 1 water which provides reproducible recovery of many highly polar and ionic metabolites.

However, 100% aqueous extractions may be favourable for some ionic metabolites. Another important consideration for AEC-MS/MS methods is the removal of protein content from biological extracts. We found this to be essential for efficient ion-suppression and to maximise ion-suppressor lifetime. Removal of most of the protein co-extracted with metabolites is a standard procedure in LC-MS-based metabolomics sample preparation; the most common method involves protein precipitation using an organic solvent or mixture of solvents. Our experiments comparing solvent-based protein precipitation and ultrafiltration of metabolite extracts from cells show that 3 kDa and 10 kDa molecular weight cut-off (MWCO) ultrafiltration is more efficient than solvent precipitation for protein removal (tested on cells, serum and plasma samples). We, and others, have found no detrimental effects using the MWCO protein removal method on recovery of ionic or highly polar metabolites[70]. However, some reproducible loss (and in some cases enhancement) of lipids, and other hydrophobic compounds, was observed when the samples (cell, tissue and biofluid extracts) were analysed using reversed-phase mass spectrometry (RPLC-MS). We recommend the use of MWCO ultrafiltration for AEC-MS/MS applications but caution against using the same filtrated samples with other LC-MS methods characterising more hydrophobic and lipophilic metabolites. See Box 1 for further discussion. Serum and plasma can both be used for AEC-MS/MS metabolic profiling but each produce quite different metabolite profiles. Serum preparation is relatively straightforward. For plasma, care should be taken with anti-clotting agents which are pre-prepared in blood collection tubes as they necessarily contain anticoagulants which can confound metabolite results and/or interfere in AEC-MS/MS analysis[9,80]. For example, citrate is sometimes used, but this is contraindicated for most metabolomics applications as it is also an endogenous metabolite present in blood. EDTA tubes are commonly used in LC-MS-based metabolomics, but EDTA is chromatographically retained by AEC-MS/MS leading to a very large chromatographic peak. It is therefore recommended to use Heparin-based anticoagulation tubes for plasma destined for AEC-MS/MS metabolomics analyses.

Box 1: Preparation of cell, tissue, and biofluid samples

Preparation of cell, tissue and biofluid metabolite extracts for AEC-MS/MS analysis

The process and efficiency of metabolite extraction is critical to the success of metabolic profiling experiments. The procedures for collecting and preparing metabolite extracts from tissue culture, physiological tissues and biofluids for AEC-MS/MS analysis are similar to those for other LC-MS based metabolic profiling experiments, detailed elsewhere[6-9]. For example, sample randomisation, appropriate controls, arresting metabolism rapidly and efficiently, keeping samples cold during extraction, sample storage at -80 °C and minimising freeze-thaw cycles are similarly important but some considerations tailored for AEC-MS/MS include extraction solvent choice, extraction buffer pH and protein removal steps. We provide a standard sample preparation protocol in the 'Procedure' section but some important sample-specific considerations for AEC-MS/MS profiling experiments are discussed below.

Amounts of sample and numbers of replicates

A guide to extraction volume requirements and recommended minimum numbers of biological replicates are given in Table B1 for each sample type but it should be noted these may vary depending on project objectives and biological variability between experimental groups.

Preparing tissue samples

In all metabolomics experiments it is essential to carefully preserve the endogenous metabolic profile when sampling. We prefer snap-freezing tissue samples in liquid nitrogen immediately after removal from their living biological state. Tissues that require prolonged manipulation post-mortem, prior to freezing, will likely start to reflect post-mortem changes in metabolism. Denser tissue samples (liver, heart, muscle, kidney, etc.) are usually best wrapped in labelled aluminium foil and stored at -80 ℃. Biofluids should be kept cool and processed as quickly as possible, then stored at -80 ℃.

Extraction solvent choice

A variety of extraction solvents are compatible with AEC-MS/MS including: H_2O, MeOH, ACN, EtOH. However, as the metabolites analysed by AEC-MS/MS are highly polar and ionic, more polar solvents usually provide increased extraction efficiency and solubility, therefore we recommend 80% (v/v) MeOH in Type 1 water or 100% MeOH. For plasma and serum extractions, we have tested 100% MeOH, 80% (v/v) MeOH in Type 1 water and 1-Butanol:MeOH (1:4,v/v). We have found samples extracted with 1-Butanol:Methanol (1:4, v/v) to have a higher extraction of lipids for analysis on other LC-MS platforms, but each of these extraction solvents provide near identical performance for detection of polar metabolites from plasma or serum.

Protein removal

The removal of protein from metabolite extracts is an essential sample preparation step for many LC-MS-based metabolomics methods. However, it is particularly important when using AEC-MS/MS due to the inline electrochemical ion suppressor which neutralises OH^- ions from the column eluent and removes counter-ions. We have found that protein in samples can become deposited inside the suppressor and builds up over time, eventually causing blockages leading to leaks and ultimately to catastrophic failure. In our experience this can occur in a month or two when samples are routinely analysed with residual protein levels around 200 µg/mL (which can still be present in plasma and serum samples after a solvent precipitation to remove protein - e.g. using > 70% (v/v) acetonitrile in Type 1 water). We have found that sample extraction combined with MWCO filtration (10 kDa or 3 kDa) typically reduces protein levels to < 20 µg/mL and applying this approach our ion-suppressors typically last for > 1 year of daily use.

Sample normalisation

Sample normalisation is essential for tissue samples, it is not essential for tissue culture samples (but strongly recommended), and not usually necessary for serum or plasma samples but is advisable for urine analysis. For tissues and tissue culture, total protein or total DNA concentration can be used for normalisation purposes – we typically recommend total DNA concentration normalisation for tissue culture as some protein precipitation is liable to occur during harvesting. For urine, specific gravity or osmolarity normalisation usually work best[71].

When samples are being prepared it is advisable to do so as reproducibly as possible but it is important to randomize the order in relation to the biological groups being studied. Avoid splitting sample preparation into batches or treating one group of samples differently from another. Ideally, samples should be analysed without a freeze-thaw cycle (i.e., directly after

sample preparation) but if this is not possible all samples should be stored at -80 °C until the day of analysis and multiple freeze-thaw cycles should be avoided. Time, oxygen, light, temperature, metal ions and pH can all influence the non-enzymatic degradation of metabolites. Highly polar and ionic metabolites can be particularly susceptible to these effects as many are easily interconverted, hydrolysed and oxidised (one of the reasons many are effective intermediates in primary metabolism). For example, nucleotides and other phosphorylated metabolites are easily hydrolysed, redox metabolites are easily oxidised, and coenzymes degrade rapidly at higher pH[38]. Quality control samples are an essential part of any metabolomics experiment to monitor analytical variability and analyte changes over time. For analysis of certain metabolite classes, adjusting the pH of the sample extraction solvent may help with recovery (e.g., redox metabolites such as $NADP^+$ and NADPH[35]).

[H3] Chromatographic method. Although it is feasible to perform AEC-MS/MS using a range of ion-exchange chromatography equipment, ion-suppression technologies and mass analysers, it is worth noting that one of the major benefits for metabolic profiling is the reproducibility and efficient eluent-suppression of an integrated ion-chromatography-mass spectrometry system that incorporates in-line eluent generation and electrochemical ion suppression. There are a wide range of stationary phases available for AEC, many of which have not been explored (as far as we are aware) in metabolomics applications at the time of publication. We have found anion-exchange (as opposed to cation-exchange), high-capacity stationary phases are the most effective for analysis of metabolites involved in primary metabolism, particularly those developed for characterization of a broad range of organic acids. Most metabolites found in glycolysis, the pentose phosphate pathway, the TCA cycle, pyrimidine and (to a lesser extent) purine metabolism are anionic at physiological pH and, in general, anion-exchange methods provide the most comprehensive metabolite coverage. Without recapitulating basic chromatographic principles, it is also worth noting some chromatographic variables that are particularly important in ion-exchange chromatography: column temperature alters resolution and organic modifiers in the water used for eluent generation can alter RTs (e.g., > 5% (v/v) organific modifier often reduces the RT and alters the elution order for organic acids). It should be noted that adding organic modifiers impacts not only the chromatographic process but may also affects electrospray ionisation conditions in the heated-ESI (HESI) source.

[H3] Mass spectrometry method. HESI has predominantly been used in AEC-MS/MS metabolomics applications to date (although inductively coupled plasma-IC-MS is of interest for metal ion analysis and atmospheric pressure chemical ionisation can also be used). The optimisation of ionisation source conditions is important for minimising in-source fragmentation and maximising ionisation efficiency[36,72]. Unlike some other types of chromatography applied in metabolomics, IC uses highly aqueous mobiles phases, therefore, desolvation (removal of solvent molecules, in this case water, bound to analyte molecules during ionisation) is worth considering. To enhance the desolvation process, the

eluent flow (post-column) can be combined with an organic solvent make up flow, prior to HESI. However, we have not found this to significantly increase sensitivity, and it can introduce greater variability in signal response. Our experience suggests that optimisation of carrier gas flow and source heating is preferable for sensitivity optimisation[19]. It is worth remembering that many metabolites are already in ionic form when they reach the ion-source in IC-MS. For these compounds, removal of a proton via the negative ion electrospray process is therefore not a requirement for mass spectrometric detection. In addition, the focus in this protocol is on high-resolution Orbitrap MS detection, triple quadrupoles can also be used for targeted (but not untargeted) AEC-MS/MS studies. Tandem mass spectrometry experiments are typically performed to obtain fragmentation spectra. Our recommended tandem mass spectrometry method uses an HCD 'Top 10' data-directed fragmentation acquisition (DDA) protocol to enable a range of metabolites to be automatically fragmented (see 'Methods').

Highly polar and ionic metabolites are found at a wide range of concentrations in cells and tissues and include some of the highest and lowest abundance metabolites. It is important to optimise the acquisition of MS^1 and MS^2 spectra accordingly, particularly for untargeted modes of operation. In our experience matching RT from authentic standards, along with accurate mass and isotope matching, provides reliable information for metabolite identification. Matching MS^2 fragmentation patterns for confirmation where possible, increases confidence in the assignment in many, but not all, cases. Balancing the time taken for MS^1 and MS^2 data acquisition modes is important. For example, Orbitrap detectors with scan rates of > 20 Hz tend to work well with 'Top 10' MS^2 methods for the analysis of cell and tissue extracts but care should be taken to ensure the number of MS^1 data points per chromatographic peak does not get too low for efficient and effective peak integration, which can occur for lower intensity peaks on any Orbitrap system. This is a particular challenge when using mass analysers with slower scan rates. It should also be noted that AEC chromatographic peak widths are typically wider than those seen for ultra-high-performance chromatography (UHPLC) methods for a variety of reasons including larger column particle sizes and the pressure limits of the PEEK connections (necessary for IC eluent compatibility and biological applications), usefully this allows for a slower MS^1/MS^2 duty cycle to acquire sufficient data across an eluting chromatographic peak. Mass spectral resolution, maximum injection time (IT), automatic gain control (AGC) target and other parameters which affect scan speed, may be useful to adjust for specific applications as well as mass analyser detector speeds. The mass spectrometry method in this protocol emphasises broad MS^2 scan coverage using the Q Exactive™ and Orbitrap Exploris™ instruments, aimed at untargeted profiling of metabolite extracts from cells, tissues and biofluids.

[H3] System optimisation and data collection. System optimisation, and confirmation of workflow and method performance, is an integral part of all analytical studies and system

suitability tests should always be performed prior to any new analytical sequence being acquired. Prior to performing new experiments, we recommend analytical system performance is checked via the results of the most recently run sequence. Our approach is to confirm that chromatographic peak width, peak area, RT and mass accuracy are all within the expected range for a selected metabolite or range of metabolites. We use citrate which is part of the authentic metabolite standard mixture (See Box 2) run at the beginning and end of all our sequences (note our metabolite standards are incorporated into a mixture designed for multiple LC-MS methods and not just AEC-MS/MS). Citrate was chosen as it is a common metabolite found in relatively high abundance in most biological matrices and sample types and is well characterised by AEC-MS. In addition to checking recent performance, we also perform a new mass calibration to ensure mass measurements accuracy is within 3 ppm (for Orbitrap mass analysers) prior to a new sequence being run. We always place at least 8 replicate Quality Control (QC) injections at the start of each sequence for the purposes of column conditioning and assessment of RT and abundance reproducibility. These QC samples are an equal volume mixture of each sample being run in the analytical sequence. When multiple batches are analysed, common QC samples can be used across batches, but these should represent all the samples being analysed. We have observed that AEC-MS/MS analysis of cell extracts and biofluids has considerably less intensity drift associated with it when compared to RPLC-MS or HILIC-MS. We have successfully run over 400 MWCO-filtered serum samples in a single sequence using AEC-MS/MS without the need for drift correction, illustrating its stability. When setting up analytical sequences we always randomise the order of experimental samples and place a process blank at the end of the sequence (after the QC samples and bracketing the metabolite standards). This enables confirmation that sample carry-over is minimal (< 1% for most metabolites) from QCs and standards. Box 2 illustrates our standard sample-type sequence for AEC-MS/MS metabolomics.

Box 2: Recommended sample sequence configuration

Sequence configuration

The configuration of the analytical sequence; i.e. where blanks, standards and QC samples are placed in relation to experimental samples, is important to ensure high quality results for untargeted (and semi-targeted) metabolomics studies. For the untargeted method described in this protocol, we use the general layout shown in the figure below which ensures experiential samples are interspersed with QC samples.

Quality Control (QC) samples

Pooled QC samples should be prepared for each experiment. These are used for column conditioning, assessment of reproducibility and, if needed, drift corrections. At least 8 replicate QC injections should be used at the start of the sequence to ensure the column is conditioned and to enable reliability assessments. After this, the QC sample should be interspersed after every 5-8 samples to enable comparisons across the whole breadth of the sequence. For shorter sequences, they can be interspersed more frequently.

Metabolite standards

Routine analysis of standard standards is essential for easy, broad system suitability assessment and accurate retention time matching. We recommend running a mixture containing a range of metabolite standards at the beginning and end of the sequence to evaluate chromatographic performance both before and after analysis and identify chromatographic problems quickly if they occur.

Process/Sample blank

Both process and sample blanks allow for system suitability measurements including carryover and assessment of conductivity detection limits across the analysis. A process blank enables assessment of contamination peaks associated with the sample preparation process.

[H3] Data processing, data normalisation and metabolite identification. The workflow for data processing is similar to that used for other types of LC-MS metabolomics. Briefly, data files are transferred from the acquisition PC to a networked server and uploaded to a preferred data processing platform. We use Progenesis QI for small molecules (Waters, Elstree, UK) to perform RT alignment, peak picking, and tabulation of data (into .csv format), other software platforms are available including Compound Discoverer™ and XCMS. A wide range of software is, in theory, suitable for processing AEC-MS/MS data, including both commercial vendor and open-source options. We describe the data processing method we originally developed which employs Progenesis QI for small molecules (Waters, Elstree, UK) for data extraction and processing followed by MetaboAnalyst (open source, online) for post-processing and data analysis. A Progenesis QI user guide is also available online here: https://www.nonlinear.com/progenesis/qi/v3.0/user-guide/. In addition to the Progenesis QI workflow, we also provide with this Protocol a similar workflow for data processing using Compound Discoverer software (Thermo Fisher Scientific) as an alternative. The step-by-step procedure for data processing using Compound Discoverer is provided in Supplementary Method 1. When analysing cell extracts (using the sample preparation and untargeted IC-MS method described) we typically measure 5,000-8,000 MS^1 distinct *m/z* and RT pairs and refer to these as 'compound-features'. For plasma and serum, the number of compound-features can be considerably higher. We use various approaches to try to reduce systematic and non-systematic variability and error in the datasets. Systematic variability associated with sample analysis can be identified via assessment of trends in QC samples, such as intensity drift over the analytical sequence. This can be corrected for using software such as MetaboDrift[73]. Other types of variability in metabolite intensities between samples can also occur (e.g. associated with biological and sample preparation variability). We correct these using sample normalisation (e.g. using total protein or DNA levels measured by spectrophotometry) and data normalisation (e.g. using a statistical normalisation of the data, usually sum, median or quantile data normalisation). In MetaboAnalyst[74] we use inter-quartile range filtering. Currently we do not filter samples using a RT cut-off as we observe some well-characterised compound-features can be obtained from the void-volume, but this is optional. Next, we perform principal component analysis (PCA) and non-clustered heatmapping of the dataset in its entirety (including QC samples) to look for sample outliers and class separation (using MetaboAnalyst). We tend to not remove outliers unless we know they occurred as part of the sample preparation process and are clearly non-biological in origin. Finally, we perform normalisation, transformation and scaling of the dataset to

optimise the data for further statistical analysis[75]. We assess the most effective data normalisation methods using PCA and un-clustered compound-feature heatmaps and scaling/transformation using Kernel Density plots (MetaboAnalyst). We often (but not always) find median normalisation to be the most effective data normalisation approach for untargeted AEC-MS/MS datasets collected on Orbitrap instruments.

[H3] Statistical analysis and functional interpretation of results. The AEC-MS/MS protocol is suitable for the analysis of a range of biological extracts and provides broad coverage of highly polar and ionic metabolites. Statistical analysis of AEC-MS/MS datasets can be performed on a wide range of software platforms suitable for metabolomics data analysis. These can be vendor specific or vendor neutral (e.g., MetaboAnalyst[75]; Compound Discoverer™[76]; XCMS Online[77]; MZmine3[78]) which provide a range of relevant statistical tools. Specific statistical tools can also be useful such as SIMCA® or various packages for R or MATLAB. In general, we tend to ask three analytical questions of our untargeted AEC-MS/MS datasets when performing data analysis:

1) Are there metabolite biomarkers or a metabolic phenotype associated with the experimental groups that are being investigated (e.g., using univariate statistical analysis)?

2) Can metabolite differences between experimental groups be modelled (e.g., using multivariate statistical analysis)?

3) Can altered biological functions be interpreted (e.g., using pathways and network analysis tools)?

Datasets can be analysed to answer all three questions either using the AEC-MS/MS method alone or alongside datasets collected with analytical platforms (e.g., RPLC-MS/MS, HILIC-MS/MS etc). The AEC-MS/MS method has been developed to provide broad coverage of highly polar and ionic anionic metabolites found across inter-connected primary metabolic pathways, making it feasible to ask questions about functional metabolic processes and pathways, in addition to biomarker discovery. As for all profiling assays, it is expected results would usually be used to generate hypotheses for further testing and validation using other methods.

[H2] Expertise needed to implement the protocol

The protocol and accompanying notes described here should make it possible for an experienced analytical scientist or trained researcher to apply the AEC-MS/MS workflow to analyse a broad range of biological sample types. The MS does not require modification to be coupled with ion-chromatography and used for AEC-MS/MS experiments. The same mass spectrometer can be interfaced with conventional LC and UHPLC systems when not being used for AEC-MS. For example, we have two separate chromatography systems interfaced

with our Q Exactive™ (an ICS-5000+ ion chromatography system and an Ultimate 3000 UHPLC (Thermo Fisher Scientific) system for RPLC or HILIC untargeted metabolomics applications). The expertise required for AEC-MS/MS does not go beyond what is needed for standard LC-MS metabolomics workflows. Researchers using conventional LC-MS-based metabolomics, for example, will be able to straightforwardly implement AEC-MS/MS using the protocol presented here with availability of the appropriate hardware.

[H2] Advantages and limitations of the method

AEC-MS/MS enables the chromatographic separation and high-resolution mass spectrometric analysis of highly polar and ionic metabolites extracted from complex biological matrices. It has several advantages over other LC-MS approaches. (1) The ability to analyse a broad range of compound classes including highly ionic compounds such as multiple carboxylated and phosphorylated metabolites which are generally poorly characterised using broad spectrum HILIC-MS/MS methods. (2) Analysis is highly reproducible, facilitating comparisons across multiple samples in an experiment. In particular, highly reproducible chromatographic RTs support accurate metabolite identification using authentic standard databases[19]. (3) Lower detection limits and higher sensitivity compared to common alternative methods[36]. (4) Pre-formation of ions and removal of counterions via electrochemical suppression reduces ion-suppression in the electrospray processes contributing to stability in complex sample analysis.

Although AEC-MS/MS provides extensive metabolite coverage of negatively charged metabolites in complex biological matrices, it performs less well at characterising zwitterionic metabolites. This is likely due to their removal by the current generation of ion suppressors as they are seen as a counter ion. Depending on the pKa value of the ionised groups, and overall balance of charges, some more acidic zwitterions can be measured (these include aspartate and glutamate for example) suggesting the overall charge is important. Care also needs to be taken with the macromolecule content of samples. We have correlated a reduction in suppressor lifetime with higher protein content biological samples (we believe proteinaceous material becomes deposited in the suppressor membranes over time). MWCO filtration (3 kDa or 10 kDa) of samples is effective at reducing these issues).

In summary, AEC-MS/MS is not suggested as a replacement for other LC-MS approaches that characterise highly polar and ionic metabolites. It has advantages and some disadvantages, which LC-MS method is optimal for a particular untargeted metabolomics application should be guided by the research questions. However, AEC-MS has the potential play an increasingly important role metabolomics. Table 1 provides a summary of some of the main analytical advantages and disadvantages of AEC-MS/MS for metabolomics applications.

[H1] MATERIALS

[H2] REAGENTS

- Water for sample extractions and IC mobile phase (≥ 18.2 MΩxcm; < 5 ppb Total Organic Carbon; Type 1 quality).
- Methanol for metabolite extractions: suitable for HPLC (P/N: 34860; Merck KGaA, Darmstadt, Germany or similar). **CAUTION**
- 1-Butanol: suitable for HPLC (P/N: 34867; Merck KGaA, Darmstadt, Germany or similar)
- Nitrogen for HCD: High purity N_2 gas supply (99% pure).
- Liquid Nitrogen
- Dulbecco's Phosphate Buffered Saline (PBS) (P/N D8537; Merck KGaA, Darmstadt, Germany or similar).
- Mixture of authentic metabolites. This is created in-house using individual metabolite standards bought in pure form and made up into aliquots at a concentration of 10 µg/mL using 80% (v/v) MeOH in Type 1 water. The specific selection of metabolites in the mix is not critical but it is useful to include those commonly encountered in experimental samples and to ensure they cover a range of chemical structures, RTs and *m/z* values. We provide a list of the composition of the mixture we use in Supplementary Data 1. The mixture of metabolite standards is used to check system suitability and for confirmation of RTs for key metabolites analysed in the same sequence (its composition is suitable for a range of LC-MS methods, not exclusively AEC-MS/MS I.e. not all compounds are detectable by AEC-MS/MS. Supplementary Data 2 contains the list of metabolites that can be measured by AEC-MS/MS). The standard mix should be run at the beginning and end of a sequence (see Box 2). Metabolites were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).
- Pierce™ Negative Ion Calibration Solution (P/N: 88324; Thermo Fisher Scientific).
- Pierce™ FlexMix Calibration Solution (P/N: A39239; Thermo Fisher Scientific)
- Dionex™ Combined Seven Anion Standard I (P/N: 056933; Thermo Scientific™).

[H2] EQUIPMENT

[H3] Metabolite extraction from cells and tissues

- Personal protective equipment (PPE) for lab work: gloves, lab coat, and goggles.
- Laminar Flow Hood
- Fume Hood
- CO_2 Incubator (New Brunswick™ Galaxy® 170 R; Eppendorf or similar)
- 1,000 µL, 100 µL and 10 µL pipette and pipette tips (e.g., Gilson or similar).
- Microscope with 4X magnification capability.
- 6 cm treated tissue culture dishes (P/N: 83.3901; Sarstedt or similar).
- Cell Scraper (P/N: 541070; Greiner Bio-One or similar).
- Low-lint or lint-free tissue (P/N: 7558; Kimberly-Clark Professional, Tadworth, Surrey, UK or similar)
- Microcentrifuge tubes (1.5 or 2 mL).
- Ultra-low temperature (-80 °C) freezer (P/N: U725G-86; New Brunswick or similar).
- Sparkfree fridge (Labcold, Basingstoke, UK or similar)
- Microbalance (e.g., XS105 DualRange, Mettler Toledo or similar).
- Weighing boats (plastic or aluminium foil).

- Forceps/spatula for sample manipulation/transfer.
- Small plastic homogenisation tube (maximum 10 mm diameter, minimum volume 4 mL). 5ml liquid scintillation vials are the most appropriate.
- Small blade homogeniser (minimum blade diameter 6 mm, minimum homogenisation speed 10,000 rpm). Polytron or Ultraturrax-type.
- Cooled centrifuge with capacity for the number of samples being prepared e.g., Thermo Megafuge 8R (P/N: 75007213; Thermo Fisher Scientific or similar).
- Vortex mixer suitable for microcentrifuge tubes (Grant-bio PV-1, Fisherbrand classic vortex mixer or similar).
- Ultrafiltration columns/centrifugal filters (0.5 mL volume, 10 KDa or 3 KDa MWCO; P/N: UFC501096 or UFC500396; Merck).
- NanoDrop™ One spectrophotometer for DNA quantitation (P/N: ND-ONE-W; Thermo Fisher Scientific or similar).
- Autosampler vials, e.g., Waters Total Recovery vials (P/N: 186000385C; Waters, Elstree). **CRITICAL**

[H3] AEC-MS/MS analysis

- Q Exactive™ Orbitrap tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).
- Orbitrap Exploris™ 240 Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany)
- ICS-5000+ ion-chromatography system with eluent generation and ion suppression (Thermo Scientific™, Sunnyvale, USA).
- ICS-6000 ion chromatography system with eluent generation and ion suppression (Thermo Scientific™, Sunnyvale, USA).
- ICW-3000™ water purification system (P/N: C85500; Merck KGaA, Darmstadt, Germany).
- Chromatographic column: Dionex™ IonPac 2 x 250 mm AS11-HC-4µm IC (P/N: 078035; Thermo Scientific™, Sunnyvale, USA).
- Suppressor for anion-exchange mode: Dionex™ AERS 500e 2 mm (P/N: 302662; Thermo Scientific™, Sunnyvale, USA).
- KOH eluent generator cartridge: Dionex™ KOH EGC 500 (P/N: 075778; Thermo Scientific™, Sunnyvale, USA).

[H3] Software

- Chromeleon™ Instrument control and acquisition software (Thermo Fisher Scientific).
- Dionex™ DCMSLink™ Software Plug-In (Thermo Fisher Scientific).
- Xcalibur™ MS data management software (Thermo Fisher Scientific). Progenesis QI (Waters, Elstree, UK).
- Spreadsheet software such as Microsoft Excel.
- MetaboAnalyst (www.metaboanalyst.ca).

[H3] Data processing

- Raw AEC-MS/MS data files for each sample need to be converted into a data table or data matrix providing peak areas/intensities for individually extracted compound--features. This is a common in processing metabolomics data and we treat the dataset in the same way as from other LC-MS metabolomics platforms. We use Progenesis QI for small molecules to perform data extraction and processing. There are a range of alternative platforms available that could also be used including Compound

Discoverer™, MetaboAnalyst, XCMS online etc. We illustrate, by way of example, the analysis of AEC-MS/MS data using our own processing workflow which incorporates Progenesis QI for compound-feature extraction, metabolite identification and MetaboAnalyst for further data processing and analysis.

[H2] REAGENT SETUP

- **Preparation of cells, tissues and biofluid samples**. See Box 1 for details and the Procedure section for step-by-step sample preparation.

- **Mobile phase for IC system**. There are no mobile phases to prepare as online eluent generation, using a water reservoir and KOH eluent generation cartridge, produce the OH^- gradient used for elution of analytes. Ensure the correct eluent generation cartridge is installed and functioning (cartridges typically last over a year with regular usage). Ensure new cartridges are conditioned on installation using the installation guide provided and several blanks and test samples are run after installation of a new cartridge to confirm analytical performance.

- **QC samples** should be prepared for each project by making an equal-volume mixture of all samples to be compared, e.g., pooling 5-10 µL from each sample into a single aliquot. Label the mixture 'QC' and transfer into a Total Recovery Vial. More than one QC vial may be needed for larger projects, in this case split the QC mixture into separate vials after first combing all aliquots from all samples, to ensure each vial contains an identical composition. Setup the samples in the autosampler sequence as illustrated in Box 2.

- **Metabolite standards**
  Prepare a mixture of authentic metabolite standards in 80% (v/v) MeOH in Type 1 water. Each metabolite should be at an accurately measured concentration of between 1-10 µg/mL. The choice of which metabolite standards to use is up to the analyst; a list of the composition of approximately 400 metabolites we use in our mixture is given in Supplementary Data 1. Prepare aliquots of 200 µL in microcentrifuge tubes and store at -80 °C to ensure samples of the same metabolite standard mix are available over a long period of time enabling longitudinal analysis. Thaw the aliquot and pipette to mix and transfer to a Total Recovery Vial prior to analysis. Only 2 × 5 µL injections are performed per analytical sequence, (see Box 2) and so a single aliquot of the standard metabolite mix can be kept at 4 °C in the autosampler and used across multiple projects and sequences for longitudinal system suitability checking.

- **Experimental samples**
  Experimental samples should be placed in Total Recovery Vials in the autosampler in a randomised order. Alternatively, the vials may be placed in the autosampler in any order and the order of the injections in the sequence randomised instead. See Box 2 for details.

- **Process or reagent blank**
  A 'sample blank' or 'process blank' should be prepared (it is up to the user to decide which is most appropriate for their study, a process blank is usually preferable). A sample blank simply contains the same fresh diluent solvent used to prepare

experimental samples (e.g., 80% (v/v) MeOH in Type 1 water). A process blank contains the same 80% (v/v) MeOH in Type 1 water solution but this has been taken through the preparation process as if it were an experimental sample (e.g., each of the sample preparation steps – extraction procedure, filtering, centrifugation, change of vial type etc). The blank sample is placed in the autosampler and run in the sequence as illustrated in Box 2. [71]

[H2] EQUIPMENT SETUP

We use a Thermo Fisher Scientific Q Exactive™ or Orbitrap Exploris™ 240 MS coupled to an ICS-5000+ or ICS-6000 ion -chromatography system with online eluent generation and ion suppression for AEC-MS/MS analysis. We use a gradient elution profile with online-generated KOH over a 37-minute analysis which incorporates a column flush and re-equilibration step at the end of the gradient. We have used this gradient to analyse sequences containing hundreds of samples.

Pipettes: Ensure all pipettes used are calibrated for accuracy. This can be checked by pipetting a known volume of water into a weighing boat (100 µL = 100 µg). Ensure +/- 3% over ten deliveries.

IC Column: For brand new AS11-HC-4µm columns, follow vendor guidelines for column start-up. We use the combined seven anion standard for ease. New columns can show initial drift in RTs until they are properly conditioned. This is in the order of half a minute for a compound eluting at 20 minutes for example. Lifetime of the column will vary depending on frequency of use and sample matrices. We typically see columns lasting >12 months with near continual use for the analysis of cells, tissues and biofluid extracts. With extended column use retention times start to get shorter and peak shapes can broaden, along with an increase in backpressure.

Mass spectrometer: Perform the calibration procedure as per vendor guidance using the appropriate calibration solution. For the Q Exactive™ MS, in addition to the Calmix calibration with Pierce™ Negative Ion Calibration Solution that is recommended, we also perform low mass range calibration routinely using the protocol developed by Liu *et al*[79]. It is recommended to calibrate the instrument in both polarities regularly. Ensure there are no warnings or errors on the instrument control software before starting any analyses.

IC system (AEC): Ensure the baseline conductivity reading is stable and < 1 µS to check that the ion-suppressor is fully suppressing the eluent before every sequence is started. To test the IC system fully in AEC mode (e.g., after a new column is fitted), run the system in at least triplicate as shown in the quality assurance report (QAR) provided with each column and compare the peak shapes, RTs (although note these may differ in absolute terms from the QAR due to system void volumes but should show a similar relative distribution), and peak areas to the original QAR or to a previous analysis of the same test mixture to ensure no significant deviations in RT, sensitivity or resolution. This should be performed when using

the method for the first time or after a hiatus e.g., if a new column or other component of the IC system is fitted or the system has been switched off for a period of time. Once you have established your method and are running experimental samples routinely, we recommend using data from your own metabolite standard mix run at the start and end of every sequence (see Box 2), to assess system suitability regularly. The metabolite standard mix data has the advantage of being acquired using the same AEC-MS/MS chromatographic and mass spectrometry method that experimental sample are run on. Any uncharacteristic deviation in RT, sensitivity, or peak resolution compared to previous values should trigger further investigation and rectification of the problem prior to experimental sample analysis. See Supplementary Table 2 for a small selection of metrics we use for system suitability using our standard mixes. Note, a gradual reduction in RT is observed as columns age (over several months of analysis time).

[H1] PROCEDURE

[H2] Metabolite extraction TIMING 2-4 hours

1. Perform metabolite extraction according to the appropriate procedure for each sample type below: Cells (A); Tissues (B) or Biofluids (C).

! CAUTION Take appropriate precautions when handling samples from organisms or primary cultures that may be disease-bearing. Use appropriate PPE and sample handling procedures, including a laminar flow or fume hood (as deemed suitable), gloves, lab coat and mask and minimise formation of aerosols during sample preparation. Note, this may require handling of samples in a Biosafety level 2 or 3 laboratory. Undertake a risk assessment and ensure appropriate screening of all samples.

CRITICAL STEP When collecting samples of biological origin ensure all ethical guidelines and sampling requirements from your institution, and those required by law, have been followed. Ensure informed consent has been obtained for samples from all human subjects and the requirements of the Human Tissue Act (and equivalent legislation) and best practice for the handling of rodents and other animals are followed where relevant.

(A) Tissue culture cell samples

CRITICAL This step is appropriate for adherent mammalian cells only.

CRITICAL STEP Steps (i-v) are time critical and should be carried out efficiently and swiftly to ensure cells do not alter their metabolism in response to the harvesting process.

(i) Grow cells and plate into 6 cm TC dishes for harvesting when 70-80% confluent (approximately $5e^5$-$2e^6$ cells). A minimum of 5 replicates of each experimental group are recommended.

(ii) Pour the remaining media from the dishes to waste and rapidly, but carefully, wash the adherent cells by adding approximately 5 mL of PBS buffer (at 4 °C), swirl gently and pour off to waste. Repeat for a second time. This removes excess

media and any dead cells. If your cells do not adhere strongly to the plate, consider pipetting off the media rather than pouring and pipetting the PBS onto the wall of the plate where there are no cells to disperse the force and reduce unwanted cell resuspension. Ensure all PBS is removed, aspirate the final amount if necessary.

(iii) Optional (but strongly recommended): add approximately 5 mL of liquid nitrogen to cover and snap freeze the cells and arrest metabolic processes. If no liquid nitrogen is available, it is strongly recommended to cool the extraction solvent on dry ice. If the cells are snap frozen, wet ice cooling is sufficient.

(iv) Add 200 µL of (ice cold) 80% (v/v) MeOH in Type 1 water to cover the cells in the dish. Note this extraction volume can be reduced to a lower limit of 180 µL, which in our experience enhances metabolite coverage, however, particular care is needed to ensure reproducible metabolite recovery if the extraction solvent volume is reduced. For larger dishes adjust the extraction solvent volume accordingly.

(v) Use a cell scraper to carefully lyse and remove all adhered cells into the extraction solvent suspension.

(vi) On ice: aspirate and pipette all the resulting lysate solution and cell debris into a 1.5 mL microcentrifuge tube (or equivalent for centrifugation). Check the dish using a microscope to confirm that > 90% of cells have been removed.

PAUSEPOINT If the samples will be processed on the same day, store on ice. If the samples will be stored at -80 ℃ prior to further processing, store on dry ice until transfer to the -80 ℃ freezer and do so as soon as possible. It is preferable to complete metabolite extraction (continuing to steps 2-9 below) in one continuous process to avoid multiple freeze-thaw cycles and potential batch effects.

(vii) Proceed to step 2.

(B) Animal tissue samples

CRITICAL This extraction method is optimised for small biopsy samples and will recover metabolites from tissues in the 10-80 mg range. A minimum sample size of 10 mg is recommended.

CRITICAL STEPS Preparing animal tissue samples is time and temperature critical. Ensure tissue samples are not allowed to thaw, and sample preparation is carried out swiftly, efficiently, and accurately. Reproducibility is critical.

(i) Divide snap-frozen tissues and carefully weigh on a balance, using aluminium foil as a weighing boat (plastic is unsuitable as tissue samples adhere). This step should be carried out ideally in a cold environment and rapidly to prevent thawing of the tissue sample. Differences in tissue weight between samples

should be minimised but are acceptable in the range 10-80 mg as they will be normalised to weight later in the protocol.

(ii) Transfer the individual samples to separate microcentrifuge tubes, pre-cooled and labelled in an ice bath.

(iii) Add ice-cold extraction solvent to the sample (80% (v/v) MeOH in Type 1 water). Table 2 provides suggested solvent extraction volumes per weight of tissue. Ideally, all samples will be within one size range and the same volume of extraction solvent used for all samples. If this is not possible different volumes can be used as the samples will be normalised by volume later.

(iv) Homogenise the sample using a blade homogeniser, taking care to ensure that the whole sample is equally distributed throughout the solvent. Tissues such as liver, brain, fat pads, spleen, etc. will require homogenisation for typically 3 × 10 seconds.

(v) Samples such as muscle, skin and kidney will require multiple rounds of homogenisation (typically 4+). Each round should last no longer than approx. 10 seconds to minimise heating of the sample.

(vi) Return the homogenised sample to a labelled microcentrifuge tube and store on ice.

PAUSE POINT Samples can be stored at this point in a -80 ℃ freezer if necessary but it is preferable to complete metabolite extraction in one continuous process to avoid multiple freeze-thaw cycles and potential batch effects.

(vii) Use a cooled centrifuge to pellet insoluble material (≥ 14,000 g for 15 minutes). The protein pellet can be retained for western blots, etc.

(viii) Recover the supernatant and transfer into a cooled small microcentrifuge tube.

(ix) Keep the sample ice cold and proceed to step 2.

(C) Serum, plasma and urine samples

CRITICAL If plasma is used, heparin-based anticoagulation tubes are recommended.

CRITICAL STEPS Prolonged exposure of blood to ambient temperature leads to changes in the metabolome profile over a few hours. It is essential that serum and plasma samples are kept cold and prepared within 1 hour. Consistency is highly important; serum and plasma samples should be stored at -80 ℃ once prepared if not analysed shortly after preparation.

(i) Thaw frozen plasma or serum samples at 4 ℃.

(ii) Centrifuge microcentrifuge tubes containing the plasma or serum in a pre-cooled centrifuge at 4 ℃ for 15 minutes at ≥ 14,000 g).

(iii) Take 90 µL of supernatant and add 210 µL of HPLC grade 1-Butanol: Methanol (1:4 v/v) (or another suitable extraction solvent such as 80% (v/v) MeOH in Type

1 water or 100% MeOH (see Box 1)). Maintain a matrix to solvent ratio of 3:7 (v/v).

(iv) Vortex the sample for 30 seconds and incubate at 4 ℃ in a fridge or other appropriate cold storage for at least 3 minutes.

(v) Vortex again and then centrifuge at 4 ℃ for 15 minutes at ≥ 14,000 g.

CRITICAL STEP The removal of high molecular weight material from metabolite extracts is an essential sample preparation step using MWCO filters (see Box 1 & 3 for further information).

(vi) Transfer the supernatant into pre-washed MWCO filters (see Box 3 for the washing procedure).

(vii) For MWCO filtration, centrifuge the samples at 4 ℃ for 30 minutes at 14,000 g.

(viii) Transfer the filtrate into a Total Recovery autosampler vial with a pre-slit PTFE cap. Samples are now ready for analysis (no normalisation step is required). Proceed to step 8.

Box 3: Prerinsing of MWCO filters

TIMING: 30 minutes

To remove excess glycerine contained in the filter membrane, a prerinsing step is recommended with Type 1 water. Once the filter is rinsed, it is recommended to use it immediately, do not allow the membrane to dry out.

Procedure

1. Place the filter into a provided microcentrifuge tube.
2. Add 500 µL of Type 1 water into the filter and close the cap.
3. Place the capped tube and filter into the centrifuge rotor with the microcentrifuge tube hinge aligned towards the center of the rotor. Ensure the centrifuge is appropriately balanced.
4. Run the centrifuge for 30 minutes at 14,000 g.
5. Remove the tube and filter from the centrifuge.
6. Remove the filter from the tube. Invert and tap the filter on a paper towel to remove any latent Type 1 water from the filter to avoid diluting the sample.
7. Place the rinsed filter into a new, labelled, microcentrifuge tube for use to remove protein from a sample.

PAUSE POINT Samples can be stored at this point at -80 ℃ if necessary but it is preferable to proceed with the sample preparation and analysis to avoid multiple freeze-thaw cycles and batch effects where possible.

[H2] Protein Removal and Sample Normalisation. TIMING 1-4 hours

CRITICAL We find that the DNA concentration in the pre-filtered sample, the filtrate and the sample retained by the filter, varies proportionally cell number and amount of tissue being analysed. Therefore, DNA concentration can be used for normalisation purposes[66]. This is illustrated below for the material retained in the MWCO filter.

CRITICAL STEP The removal of high molecular weight material from metabolite extracts is an essential sample preparation step using MWCO filters (see Box 1 & 3 for further information).

2. Keep samples on ice and centrifuge at ≥ 14,000 g for 30 minutes (a cooled centrifuge is strongly recommended).
3. Transfer the supernatant to a pre-washed MWCO filter in a labelled microcentrifuge tube.
4. Filter the samples by centrifuging at 14,000 g for 30 minutes. Using a pre-cooled centrifuge at 4 °C is strongly recommended.
5. Estimate the total DNA concentration of the filter residue for each sample using a 'NanoDrop™ One' spectrophotometer or similar by pipetting carefully from the dead volume in the bottom of the filter, this is most easily seen when looking through the clear side of the filter with the numbers (see Box 4 for the procedure).
6. Normalise each sample by adding an appropriate volume of 80% (v/v) MeOH in Type 1 water (or other extraction solvent used in step 1). The goal is to achieve an equal DNA concentration in each sample. Optionally, total protein can be used for normalisation instead of DNA concentration, however it is not recommended for cell lysates due to the possibility of partial protein precipitation during the harvesting process. **TROUBLESHOOTING**
7. Example of how to normalise samples: If you have 3 samples and you measure the total DNA content for each as 100 ng/µL, 75 ng/µL and 50 ng/µL respectively. You should normalise to the 50 ng/µL sample by diluting the others with the appropriate sample solvent to bring them to the same concentration (e.g., total sample volume is 500 µL, then mix 250 µL solvent with 250 µL sample for the 100 ng/µL sample, and for the 75 ng/µL sample, mix 333 µL sample and 167 µL solvent).
8. Transfer the filtered and normalised sample extract into a Total Recovery Vial with a pre-slit PTFE cap.

Box 4: DNA measurement using a NanoDrop™ One

TIMING: 0.5 - 2 hours

The order of sample measurement should be randomised to avoid bias in the measurements. To ensure reproducible measurements, check there are no bubbles in the droplet and endeavor to have a consistent pipetting angle to the pedestal as this can reduce variability.

Procedure

1. Select ds DNA measurement from the Nucleic Acids menu.
2. Clean the pedestal with Type 1 water and a lint-free tissue.

3. Blank the instrument by adding 2 µL of the appropriate extraction solvent onto the pedestal. Close the lid to initiate a measurement.
4. Once the measurement has completed, lift the pedestal and clean it with Type 1 water and a lint-free tissue.
5. Pipette 2 µL of a sample onto the pedestal and close the lid to initiate a measurement.
6. Once the measurement has completed, lift the pedestal and clean it with Type 1 water and a lint-free tissue.
7. Repeat steps 5-6 until 2 concordant results (± 10% or 6 ng/µL, whichever is smaller) are achieved for each sample.
8. These concordant results should then be averaged to use as the concentration for normalisation purposes.

PAUSE POINT Samples are now ready for analysis or can be stored at -80 °C until the day of analysis. On the day of analysis, they should be thawed on ice or in a cooled autosampler at 4 °C.

[H2] AEC-MS/MS sample analysis TIMING 39 mins per sample

CRITICAL STEP The quality of the results is dependent upon performing these steps correctly.

9. On the day of analysis allow samples to thaw to 4 °C in an appropriate fridge or other cold storage. Prepare a QC sample by taking an equal volume of each sample (often 5-10 µL depending on the number of samples and minimum required QC volume) and mixing in a single additional Total Recovery Vial labelled 'QC'. We recommend at least 20 µL should be calculated as excess QC volume after all injections are performed to account for sample vial dead volume. Ensure samples are well mixed by aspirating and dispensing multiple times or vortexing for 10 seconds prior to transfer to the QC vial (Note: QC samples can alternatively be made at the end of sample preparation and stored, along with samples, at -80 °C if preferred).
10. Setup the autosampler with QC samples, process blanks and metabolite standards. To ensure experimental samples are analysed in a randomised order throughout the sequence, they should be either placed within the autosampler sequence in a randomised order or alternatively the run order in the AEC-MS/MS sequence should be programmed to take samples in a randomised order from the autosampler. If you have more samples in your sequence than there are spaces in the autosampler, it is imperative that these additional samples are also part of the same randomisation process. **TROUBLESHOOTING**

11. Program the analytical sequence using the diagram in Box 2, injecting 5 µL of each sample using a *PushPartial_ls* loop injection. Other injection modes can be used, see Box 5 for further considerations. Additional autosampler parameters are given in Supplementary Data 2

Box 5: Method settings for ICS-5000+ coupled to Q Exactive™

Here we provide anion-exchange chromatography and MS parameters for the protocol. These were optimised on a Thermo Scientific™ ICS-5000+ ion chromatography system coupled to a Q Exactive™ Orbitrap tandem MS (similar settings may be applicable to other ICS and MS instruments but may need optimising). Equivalent parameters for the newer ICS-6000 ion chromatography system coupled with an Orbitrap Exploris™ 240 MS are given in Supplementary Data 3.

ICS-5000+ ion chromatography system Column:

IonPac AS11 HC-4µm (2x250 mm), Sample injection volume: 5 µL, Mobile phase flow rate (pump 1): 250 µL/min, Suppressor regeneration (pump 2): 500 µL/min, Eluent generation type: EGC 500 KOH Multi-step gradient: (see Table B2), Sample tray temperature: 4 °C, Column Temperature: 30 °C, Detector Compartment Temperature: 20 °C, Electrochemical suppressor: AERS 500e (2 mm), Suppressor current: 62 mA (see note below), Injection mode: *PushPartial_Is*, Additional autosampler parameters: see Supplementary Data 2.

Injection mode

The injection mode can affect quantitation and reproducibility as illustrated in Supplementary Figure 1. *PushPartial_ls* uses the least amount of sample and takes only the amount of sample specified for the injection with no overfilling of the loop, this is useful when sample volume is at a premium which is why this was selected as the injection mode, we have found analyses using this method to have sufficient reproducibility. Note, however, *PushPartial_ls* is not as reproducible as other modes. To ensure the best sensitivity from *PushPartial_ls* injections, the autosampler transfer line volume (TLV) should be carefully calibrated to guarantee the most amount of the sample is centered properly in the loop. The *PushPartial* injection mode with a 2-5µL cut volume may generally provide enhanced sensitivity and reproducibility (see Supplementary Figure 1) with minimal sample loss.

Suppressor Operation

We apply a constant suppressor current to suppress up to the highest part of the gradient. This results in over-suppression across the majority of the gradient which may cause a slight decrease of suppressor lifetimes; however, we maintain that the largest impact on suppressor lifetimes is the protein content of samples. To reduce this affect it is possible to apply a stepped suppressor current to change based on the eluent concentration. For example, 38mA could be applied for the first 15 minutes for up to 60mM KOH, then 62mA would be applied for the remainder of the method to suppress up to 100mM. To implement this, the method commands must be edited in the 'Commands' or 'Script Editor' tab in the method file, adding new suppressor commands to change the current at the selected new time points. Care must be taken to ensure the suppressor current required is calculated correctly so no under-suppression occurs as this can have detrimental impacts on the MS. The first time such a method is applied it is strongly advised that the eluent out from the conductivity detector is sent to waste and the baseline conductivity is monitored over 3-5 blank injections to check adequate eluent suppression occurs across the whole method.

m/z range

The m/z range 60-900 was chosen to balance low and high m/z coverage. For lower molecular weight metabolites (e.g. acetic acid) the range can be adjusted.

Q Exactive™ Orbitrap Tandem MS system

The Q Exactive™ method collects full scan MS data followed by automatic (Top 10) DDA HCD fragmentation (Full MS - $ddMS^2$). See Table B3 for full details.

12. *IC gradient profile*. Box 5 provides details of the anion-exchange KOH gradient profile. With eluent generation the KOH concentration over the sequence is generated from the

concentrated KOH reservoir, providing highly reproducible gradient elution conditions. Equivalent parameters for the ICS-6000 ion chromatography system are given in Supplementary Data 3.

13. *AEC-MS/MS settings*. Program your IC and MS methods using the settings provided in Box 5. Equivalent parameters for the Orbitrap Exploris™ 240 MS are given in Supplementary Data 3.

14. *Instrument calibration*. Ensure the Q Exactive™ MS has been calibrated in negative ion mode according to the standard parameters using the Pierce™ Negative Ion Calibration Solution prior to sample analysis (if using an Exploris™ series instrument use the Pierce™ FlexMix™ Calibration Solution, e.g., for the Exploris™ 240). It is strongly recommended to calibrate the MS in both ionisation modes regularly. **TROUBLESHOOTING**

15. Start the sequence and observe the analysis of the standards and first QC samples before leaving the system to complete the rest of the sequence. If problems arise, they are often at the start of a sequence. **TROUBLESHOOTING**

[H2] AEC-MS/MS data processing: Progenesis QI TIMING 2-4 hours

PAUSE POINT Data processing and analysis is not time-critical and can be performed at any point after the analysis of the samples.

CRITICAL The processing and analysis of untargeted or semi-targeted AEC-MS/MS data follows a similar workflow as for data from other LC-MS platforms, however, there are a few critical steps of particular relevance for AEC-MS/MS. Box 6 describes the relevant data processing steps for AEC-MS/MS specifically, and provides additional commentary.

Box 6: Progenesis QI data processing procedure and settings

Data upload, alignment and peak-picking

We use the default settings for auto-processing using Progenesis QI for small molecules, except for possible adducts which we set to $[M-H]^-$, $[M-2H]^{2-}$ and $[M-H-H_2O]^-$ only. AEC-MS/MS tends to produce predominantly these adducts with less adduct variations than for RPLC-MS or HILIC-MS for example. Using a larger number of adduct possibilities is therefore unnecessary and increases the potential for false-positive adduct assignments. It is recommended to use a 0.1-minute minimum peak width when applying peak picking in Progenesis QI. This prevents the algorithm from picking peaks which have only 1 or 2 scans and likely to be uninformative noise peaks. We have tested a variety of minimum peak widths, with 0.1 showing the best balance of noise reduction and comprehensive peak detection without the risk of narrower chromatographic peaks being discarded which represent genuine metabolites. The best timing metabolite identification during the workflow can vary depending on the purpose of the experiment. This step is often performed during data processing when comprehensive metabolite identification is required – e.g., for functional analysis, pathways analysis, multiomics etc. For biomarker discovery, or when only the significantly altered metabolites are of interest, identification can be performed after data analysis has been completed.

Here we illustrate comprehensive metabolite identification during the data processing step to maximise the number of identifications from the dataset.

Metabolite identification

Automated metabolite identification can be performed with Progenesis QI by comparing analytical measurements and theoretical values using a variety of databases such as Metlin, LipidBlast, Chemspider and others that can be downloaded, including the Human Metabolome Database (HMDB) or a bespoke in-house database (using Progenesis Metascope). We recommend creating your own database using authentic standards for the main metabolites found in primary metabolism and/or those of particular interest. More than one database can be searched for the same dataset. Our in-house database is provided in Supplementary Data 4. Note that the retention times will only be indicative. When a different system is used, even with the same method, different path lengths, void-volumes and other factors can all affect retention times. Our preferred approach is to perform *Level 1* identifications[81] using the measurements from our metabolite standard mixture and in-house database. We automate metabolite identification from our experimental samples by scoring the match between experimental retention time, accurate mass, isotope patterns and HCD fragmentation pattern with our in-house database containing these values derived from the analysis of individual metabolite standards (See our Database in Supplementary Data 4). Bespoke fragment databases can be made using the tandem mass spectra derived from identification of authentic standards using Progenesis QI for small molecules and subsequent exportation of fragment database created (under 'File'). All metabolite identification matches are subsequently manually validated in Progenesis. The high retention time reproducibility of AEC-MS/MS makes retention time a particularly useful identification parameter in AEC-MS. Those with high scores in Progenesis QI (>50) are usually correct assignments but should be checked. Those with lower scores may also be correct but judgment is required. Note a lower score can occur because a metabolite is missing a score for one of the criteria. Commonly this can be fragmentation matching (e.g. if the compound was not fragmented in the DDA Top 10) or isotope similarity - see the Troubleshooting section. Note that over time, as columns age, retention times drift towards shorter retention times.

Exporting and configuring your dataset

It is sometimes appropriate to create a subset of the full .csv file including only the identified features. This can be used for targeted statistical or pathways analysis in MetaboAnalyst.

[H3] Data upload, alignment, and peak picking

16. Create and name a new experiment in Progenesis QI for small molecules. Select the type of MS as *'High resolution mass spectrometer'*, Profile data and Negative ion mode. Select $[M\text{-}H]^-$, $[M\text{-}2H]^{2-}$ and $[M\text{-}H_2O\text{-}H]^-$ as possible adducts. See table 3 for a summary of the data processing settings. Select *'Thermo (.raw)'* as the data type and select all raw data files in the experiment for import including the QCs, blanks, and standards.

17. Select *'Start automatic processing'* and choose *'Use the most suitable run from candidates I select'* and select only the QCs (leaving out the first and second QC samples as conditioning QCs). Check *'Yes, automatically align my runs'*. Leave the experimental design until later and select *'Perform Peak Picking'* with default settings but use a 0.1-minute minimum peak-width and finish automatic processing.

18. In the *'Review Alignment'* page, check that all files are uploaded and whether the scoring for the alignment of each biological sample is > 95% and alignment quality is 'good' with only a small proportion labelled as 'OK'. **TROUBLESHOOTING**

19. On the *'Experimental Design Setup'* page, select the appropriate design for your experiment, define your experimental groups and assign the available data files to the groups. Not all samples have to be assigned to an experimental group.

20. Select the *'Peak Picking'* tab – this is usually for information only, but manual peak-picking can be performed if necessary. Likewise, in the *'Review Deconvolution'* tab, adducts, mass spectra and chromatograms can be reviewed for each compound-feature. **TROUBLESHOOTING**

21. **CAUTION** Data normalisation is automatically performed in Progenesis QI (by sum) although it is possible to de-select or choose alternative data normalisation approaches manually. Data normalisation by sum is not always the most appropriate normalisation approach for untargeted metabolomics data and we prefer to apply no-normalisation to the dataset at this stage and perform data normalisation using MetaboAnalyst prior to data analysis (see Box 7). The optimal timing of metabolite identification in the workflow can be vary depending on the purpose of the experiment. Identification is usually performed during data processing when comprehensive metabolite identification is required – e.g., for functional analysis, pathways analysis, multiomics etc. For biomarker discovery, or when only the significantly altered metabolites are of interest, identification can be performed after data analysis has been completed. Here we illustrate comprehensive metabolite identification during the data processing step which provides a maximal number of identifications from the dataset.

Box 7: Data processing and analysis with MetaboAnalyst

Zero-value imputation and data filtering

The default data filtering is *'Interquartile Range'* filtering which filters out a proportion of features based on their variance, specifically those that have the lowest different between the 1st and 3rd quartile. Due to their low variance, the assumption is that they are unlikely to contribute to discriminating between groups. Other data filtering methods are available such as filtering based on reproducibility in QC samples or filtering based on absolute abundance.

Data normalisation, transformation and scaling

For AEC-MS/MS data we often find median normalisation, log transformation and pareto scaling is effective. However, the effect of a variety of data processing methods should be evaluated for each new dataset acquired. Additionally, certain data normalisation algorithms may be more suitable for specific sample types. PCA plots and heatmaps (hierarchically clustered and un-clustered) can be used to visually compare and identify the most effective normalisation, transformation and scaling approaches and to identify outliers. In common with other LC-MS datasets, untargeted AEC-MS/MS usually provides a non-gaussian abundance distribution. Transformation of the data makes this

distribution more gaussian and therefore more suitable for some multivariate statistical analysis tools (including PCA).

Univariate analysis

Univariate statistical analysis can be used to look for significant changes in metabolite abundance between two or more experimental groups represented by multiple samples. False-discovery rate (FDR) correction of the p-value to account for multiple testing is necessary as for most types of metabolomics data. A volcano plot is useful for combining the results of fold-change (FC) and FDR-corrected t-test values for each compound-feature when two experimental groups are being compared. When more than two experimental groups are present, one-way analysis of variance (ANOVA) with post-hoc analysis can test for significant differences in compound-feature abundance between the experimental groups. Box plots can also be subsequently generated from either analysis to illustrate the changes in abundance between experimental groups for a specific compound-feature.

Multivariate analysis

Multivariate statistical analysis can help to create predictive models and show relationships between metabolites within experimental groups. There are two main types used in metabolomics: unsupervised (PCA, hierarchical clustering etc.) and supervised (PLS-DA and OPLS-DA, random forest etc.). These statistical tools can be used effectively with untargeted AEC-MS/MS data because it generates metabolite information related to highly interconnected metabolic pathways, providing potential for strongly correlated compound-feature changes when primary metabolism is altered, for example. Supervised multivariate analysis approaches work best with larger numbers of samples in experimental groups. Care should always be taken when applying supervised modelling techniques to ensure the model is not over fitted and it's important to appropriately validate the model[82].

Functional analysis

Many metabolites involved in primary metabolism are anions at physiological pH and therefore amenable to characterisation by AEC-MS/MS. This makes it feasible to perform *functional analysis* and explore how changes in metabolite abundance are linked to biological function. A variety of online tools exist for these types of analyses including *Cystoscape*, *MetaboAnalyst*, *MZmine3*, *XC-MS online* and others. We have evaluated the effectiveness of the mummichog algorithm for untargeted pathways analysis by testing its metabolite annotations against our internal library of standards. We have found it to be relatively good at providing accurate pathway annotations, with 70-80% accuracy in our experience. We therefore use it as a tool for functional analysis and interpretation of untargeted AEC-MS/MS datasets at a pathways level but not as an alternative for accurate individual metabolite identifications.

[H3] Metabolite identification

22. Select *'Progenesis MetaScope'* then edit the search parameters and browse for your own in-house or downloaded metabolite database. Select a precursor tolerance of 5 ppm and a retention time window of 1 minute (recommended). We use an additional fragment database (note: this uses a *.msp* format) with a fragment mass tolerance of 12 ppm. Search for identifications and once complete, proceed to review the identifications in *'Review Compounds'*. We have provided our 'in-house' RT database as Supplementary Data 4 to give an indication of its format. Indicative RTs for a variety of metabolites

characterised on our system are also provided but note you should determine and use your own RT as these will differ between systems for the same metabolite due to differences in peak tubing, dead volumes etc., although the elution order is likely to be similar.

CAUTION Retention time differences occur when a new column is fitted (or potentially when other components are changed as a result of routine maintenance). The column used in this protocol is very robust and we have used it for 6-12 months with near continuous injection of cell lysates for example. However, as a column ages, we see a gradual reduction in analyte retention. The magnitude will depend on column use but for indicative purposes, a change of 1-2 minutes can occur over 12 months of heavy use for a compound eluting at 15 minutes. It is therefore important to monitor RTs on a regular basis using standards to ensure accurate identifications using RT databases.

23. All metabolites identified by the software should be manually verified where possible. One of the benefits of AEC-MS is that it can chromatographically resolve many metabolites found in primary metabolism which often occur as isobaric structural isomers. However, careful attention to retention time is needed to correctly assign identifications as, for example, some sugar phosphates are resolved by seconds rather than minutes and so accurate RTs for authentic standards may be needed to confirm accurate assignments. To visualise the extracted ion chromatogram (EIC) for a certain *m/z* value, select *'Review selected compound'*. In this window, it is often more useful to show the EIC in *'All runs'* or *'Runs in QC'* rather than the default *'Experimental Aggregate'*. This can then be compared to an authentic standard (such as in the metabolite standard mix) for further confirmation. Note, if '*Experimental Aggregate'* is used, the peak shape shown can have little relation to the peak shape in each individual sample if the RTs shifted slightly between runs that was not correctly accounted for. To assess the fragmentation matching, select the feature in the *'Identify Compounds'* tab. If an MS^2 spectrum was collected this will appear in the window and if there is a database entry that matches this feature it will be included to make a mirror plot. Careful assessment is needed for metabolites with more than one possible identification. **TROUBLESHOOTING**
24. Metabolite identifications are confirmed in Progenesis by selecting the star-icon next to the identification, under the *'Possible identifications'* tab on the *'Review Compounds'* page.
25. To ensure there are no features with the same identification, sort the data table by *'Accepted Description'* and run through the list of identifications to find and rationalise any duplicates. For some pairs of closely eluting isomers, it is helpful to note in which order they elute. Despite RT differences, this trend should be preserved and is useful to ascertain the correct identifications.

[H3] Convert the .csv file for upload to MetaboAnalyst.

26. In the *'Review Compounds'* window select *'File'* and then *'Export Compound Measurements'*. Deselect *'Normalised Abundance'* and save the *.csv* file. An example of this output is given in Supplementary Data 5.
27. Open the *.csv* file and carefully copy the identified metabolite names (column labelled *'Accepted Description'*) onto their corresponding position in the *'Compound'* column (first column).
28. Delete columns in the *.csv* table leaving only the *'Compound'* (first column) and the columns with the raw abundances for each experimental sample and QCs. Create *'Sample'* and *'Label'* rows directly above the cells containing the raw data, delete all other title rows. In the new *'Sample'* row add each sample identification. In the *'Label'* row, add each sample's experimental group assignment. Save this file as a separate *.csv*. This modified *.csv* file is now ready for uploading into MetaboAnalyst for statistical analysis. An example is given in Supplementary Data 6.
29. For functional analysis, reopen the original output *.csv* file from Progenesis QI and modify the compound feature information column to contain the *m/z* and RT in the following format to ensure compatibility with MetaboAnalyst:

    `accurate m/z'__'RT in *minutes'*

    This is most easily achieved by using the formula "=B4&"__"&D4" in a new column and filling this down for all features. This can then be copied into the first column by pasting the values only. Note the use of a double underscore between 'accurate m/z' and 'RT in minutes'.
30. Repeat step 26 for this new *.csv* file and save it as a separate file. This modified *.csv* file is now ready for loading into MetaboAnalyst for functional analysis. An example of a correctly formatted file is given in Supplementary Data 7.

[H2] AEC-MS/MS data analysis: MetaboAnalyst TIMING 2 hours

PAUSE POINT Data analysis is not time-critical and can be performed at any time after the data acquisition and data processing steps. Analysis of untargeted AEC-MS/MS metabolomics datasets follows a similar workflow to those from other untargeted LC-MS studies. However, there are some important aspects specific to AEC-MS/MS which we highlight below. There is a wide range of software available for data processing, both proprietary and open source. We predominantly use MetaboAnalyst 6.0 currently in our workflows and illustrate the data analysis here using MetaboAnalyst 6.0 which is open-source software available online. Similar data processing can also be performed using alternative software packages that perform a similar function. See Box 7 for further information about the data processing and analysis.

[H3] Zero-value imputation and data filtering

31. Open MetaboAnalyst 6.0 (https://www.metaboanalyst.ca).
32. Click on *'Click here to start'*. Select the *'Statistical Analysis [one factor]'* tab. Select *'Peak intensities'*; *'Samples in columns'* and click on *'Choose'* to upload your data. Load the modified *.csv* file output from Progenesis QI (see example in Supplementary Data 5). It is

worth noting that selecting the format of 'paired' or 'unpaired' will depend on your experimental design.

33. Proceed from the Data Integrity Check to the Data Filtering page. Press *'submit'* to use the default Interquartile Range (IQR) method then proceed to the next window.
34. Select the Data editor tab from the navigation pane and using the *'Edit Samples'* and *'Edit Groups'* tabs select only experimental samples and QC samples (remove blanks and standards from further analysis). Proceed to the Normalisation window.

[H3] Data normalisation, transformation and scaling.

35. In the *'Normalisation'* tab, select the appropriate normalisation, data transformation and data scaling parameters and click *'Normalise'*. Select the *'View results'* button to view the density kernel plots for both *'Feature view'* and *'Sample view'*. Careful assessment of normalisation, transformation and scaling parameters is required for each new dataset (see Box 7). Once the optimal data normalisation, transformation and scaling parameters have been identified, return to the *'Data Editor'* window and go to the *'Edit Groups'* tab. Exclude the QC experimental group from further data analysis and click 'Submit'. In the normalisation window, select the normalisation, transformation and scaling parameters to be used, click *'Normalise'* and then proceed. **TROUBLESHOOTING**

[H3] Data Analysis: univariate statistics

36. The statistical analysis can be carried out in two ways, depending on the number of sample groups:
    a. **Volcano Plot**: When two experimental groups are being compared, select the '*Volcano plot'* and select an appropriate FC and p-value threshold.
        i. Typically, we use a FC threshold between 1.2 and 2 to visualise metabolites of potential interest but this FC threshold should be considered carefully depending on experimental objectives. Some metabolite abundances are more tightly controlled than others in metabolism and a high FC threshold may lead to missing important metabolites or compound-features of interest. We often use a p-value threshold of 0.05 and always use false-discovery rate (FDR) corrected p-values. The data file containing features that pass these thresholds can then be output as a *.csv* file by selecting the *'Spreadsheet'* icon and clicking *'Download'*. To access the boxplot of any feature, click on the datapoint in the plot or select '*View*' in the data table.
    b. **ANOVA**: For more than two groups, select *'ANOVA'* (One Way Analysis of Variance).
        i. Choose an FDR-corrected p-value cut-off threshold (default 0.05) and click on *'Submit'*. The data file containing desired features can be outputted as a *.csv* file by selecting the *'Spreadsheet'* icon and clicking *'Download'*. To

access the boxplot of any feature, click on the datapoint in the plot or select '*View*' in the data table.

[H3] Data Analysis: multivariate statistics

There are a variety of multivariate statistical tools available in MetaboAnalyst, this procedure includes steps for those we most commonly use, but others may be of relevance for a particular study.

37. **PCA**: We use this mainly to look for sample outliers and visualise the impact of processing parameters. Select the *'PCA'* window to apply principal component analysis. The *'Overview'* tab by default shows pairwise comparisons of the top 5 components of the model. The *'Scree Plot'* shows the cumulative and individual variances explained by each component. The 2D scores and loadings plots can be viewed in their respective tabs.
38. **PLS-DA/OPLS-DA**: We use this to evaluate whether a metabolic phenotype can be modelled and if so which group of compound-features or metabolites drives the phenotypic differences. Select the *'PLS-DA'* or *'OrthoPLS-DA'* window to generate a model. It should be noted that OPLS-DA can only be used when there are exactly 2 experimental groups. The *'Overview'* tab by default shows pairwise comparisons of the top 5 components of the model. The 2D scores and loadings plots can be viewed in their respective tabs. The *'Imp Features'* tab displays the top 15 features ranked by Variable Importance in the Projection (VIP) score by default, which corresponds to the features that are driving the differences between the experimental groups in the model. In the '*Cross Validation'* tab, perform at least 5-fold cross validation and in the '*Permutation*' tab perform permutation testing of the model. Note, if your experiment has very few experimental/biological replicates, this testing will have less statistical power and may provide poor results. It is recommended to also externally validate new models that pass these tests where possible.[82]
39. **Top 25 Hierarchically Clustered Heatmap**: We use this to visualise the magnitude, consistency and similarity of changes in abundance between sample replicates and experimental groups. Select the *'Heatmap'* window. Under the *'Other view options'* section at the bottom of the page, select 'Use top 25' and *'T-test/ANOVA'* on the drop-down menu. Leave all other values as default and click *'Submit'*. The default settings show results show the top 25 compound-features across all samples in a heatmap format at the bottom of the page (the number of compounds shown can be adjusted).

[H3] Data Analysis: Functional analysis (or untargeted pathways analysis) using the mummichog algorithm.

40. To perform untargeted pathways analysis, select the '*Functional analysis [LC-MS]*' module in MetaboAnalyst; choose the *'A peak intensity table'* and load your dataset (note: the formatting requirements are slightly different from the statistical analysis table. See Supplementary Data 6 for an example). Select '*Negative ion mode'*; mass tolerance of *5 ppm*; retention in '*mins'*; data source '*generic'* and samples in '*columns'* and click *'Submit'*.
41. Perform the same data filtering and data normalisation, transformation and scaling as used for the statistical analysis.
42. On the *'Set Parameters'* page select *'Mummichog'* with version *'2.0'* for the algorithm with the default p-value cut-off. Select *'Edit adducts'* under the *'Advanced options'* section. Include only $[M-H]^-$, $[M-2H]^{2-}$ and $[M-H2O-H]^-$ adducts, as selected in Progenesis QI. Under *'Pathway Library'* select the appropriate species library for your experiment and then proceed.
43. The resulting *'Mummichog Pathway Activity Profile'* provides a visualisation of the metabolic pathways ranked according to statistical significance and impact. Any pathways towards the top right are both significantly altered and have high impact, suggesting metabolite abundances are most perturbed in these pathways.

[H1] TROUBLESHOOTING

Table 4 provides an overview of some of problems that can occur using AEC-MS/MS, how common they are, and proposed solutions.

[H1] ANTICIPATED RESULTS

The protocol can be applied for the analysis of anionic metabolites extracted from cells, tissues and biofluids, providing coverage of metabolites found in interconnected central metabolic pathways as well as some secondary metabolites. *Level 1* metabolite identification can be performed effectively using RT, fragmentation pattern, accurate mass and isotope pattern matching[81]. The removal of counter-ions by electrochemical ion-suppression, and the pre-formation of charged analyte ions prior to electrospray, reduces matrix effects and provides a high degree of reproducibility and robustness.

[H2] Anticipated results for identified metabolites

It is good practice to assess data quality and system performance on a continuous basis by analysing authentic metabolite standards at the end of all analytical sequences. Our mixtures of authentic standards contain over 400 metabolites for analysis by AEC-MS, RPLC-MS, and HILIC-MS methods, representing a broad range of chemical structures, *m/z* values and chromatographic RTs (the BPI chromatogram for AEC-MS is shown in Figure 3a). Elution order in AEC largely reflects the strength of analyte charge and polarity; e.g., in general sugars elute early, followed by organic acids, then mono-, di- and tri-phosphorylated metabolites in that order. The predictability of this can be useful in helping assign unknowns. We routinely assess the analysis of citrate in the standard mixture and investigate further if values are not within normal ranges. Supplementary Table 2 provides analytical

metrics for citrate and a selection of additional metabolites that come from analysis of our standard mixtures (Supplementary Data 1) which we use to assess system suitability. An example set of RT values specific to the AEC method presented in this protocol, for a selection of highly polar and ionic metabolites, can be found in Supplementary Data 4 alongside additional information (these metabolites include alcohols, carboxylic acids, phosphorylated and sulphur-containing compounds). We include RT values for the metabolites in Supplementary Data 4 for researchers setting up the method for the first time to have some indication of roughly where, and in what order, particular metabolites might elute. We have not included them to suggest in any way they are universal values, and we would not expect RT values to match between different instruments. It is also important to consider peak-width and peak-shape as AEC-MS/MS chromatographic peaks look different compared to RPLC-MS/MS and HILIC-MS/MS chromatographic peaks. AEC-MS/MS chromatographic peaks tend to be relatively broad (e.g., they can be > 1 minute in width), they can also have a rather jagged top with MS detection, which at first sight appears related to poor chromatography or ion-detection capabilities. However, our data (including from multi-instrument comparisons) shows this is not due to poor chromatography (as evidenced using inline conductivity detection). Additionally, the spikiness of the peaks becomes more pronounced when more data points are collected across a chromatographic peak (demonstrated in Supplementary Figure 2). We do not have a clear understanding of what causes this 'spikiness' at present but believe it is a phenomenon related specifically to ion-exchange chromatography coupled to mass spectrometry. We speculate it may be related in some way to the interaction of a large number of pre-formed ions during the electrospray processes. It is, however, reproducible and does not appear to interfere with peak area accuracy but care should be taken, e.g., when using automated peak integration algorithms, to verify peak detection and integration is accurate and reproducible.

[H2] Anticipated results for untargeted studies

Data quality and reproducibility from untargeted experiments can be assessed using QC samples interspersed between experimental samples and at the start of the analytical sequence (as illustrated in Box 2). The QC samples provide a measure of analytical reproducibility but can also indicate intensity drift and provide an overview of the proportion of well-characterised compound-features. Here we illustrate results from AEC-MS/MS analysis of INS-1 (832/13) rat insulinoma cells, a well-established model for pancreatic islet beta-cell function. The cells were cultured at different glucose concentrations as part of a study investigating the effect of hyperglycemia on β-cell function[15]. The data matrices for this experiment can be found in Supplementary Data 5-7. Sample to sample variability and class differences in intensity are visualised via an intensity heatmap (Figure 3b) as well as a PCA plot (Figure 3c). Both plots are helpful for assessing any sample or experimental group outliers. The heatmap shows a small amount of class-bias in the dataset which can be corrected using data normalisation (Figure 3b). Analytical reproducibility should be higher than experimental sample variability in general and this is demonstrated in the reproducibility of the QC samples which cluster closely in the PCA plot. There is no observable drift in intensity across QC samples in the heatmap. We seldom see drift in QC samples in AEC-MS/MS datasets (in contrast to some other LC-MS based metabolomics approaches) which may be due in part to the larger proportion of pre-formed ions and subsequent lower reliance on electrospray conditions for ion formation.

Finally, it is reasonable to perform functional analysis using AEC-MS/MS metabolomics datasets because the method provides relatively high coverage of metabolites found in the main pathways involved in primary metabolism (e.g., glycolysis, pentose phosphate pathway, extended sugar metabolism, TCA cycle, purine and pyrimidine metabolism, urea cycle and others). Application of pathway and network analysis tools are therefore useful for interpreting functional changes in these metabolic pathways using AEC-Ms datasets. Figure 3d illustrates the results of untargeted pathways analysis using the mummichog algorithm (via the Functional Analysis toolkit in MetaboAnalyst 6.0.). We analysed the processed, untargeted compound-feature dataset from AEC-MS/MS. Figure 3d illustrates some of the metabolites, grouped by pathway, for which AEC-MS/MS provides comprehensive metabolite coverage. Metabolites are also routinely characterised that are found in other pathways, including but not limited to, amino acid degradation, redox metabolism, and carbohydrate metabolism.

[H1] TIMING

Day 1, Step 1, Metabolite extraction: 2-4 h

Day 1, Steps 2-8, Protein removal and sample normalisation: 1-4 h (depending on number of samples)

Day 1, Steps 9-15, AEC-MS/MS analysis: 39 minutes per sample

Day 2, Steps 16-30, AEC-MS/MS data processing: 2-4 h

Day 2, Steps 31-43, AEC-MS/MS data analysis: 2 h

Box 3, Pre-rinsing of MWCO filters: 30 minutes

Box 4, DNA measurement using a NanoDrop™ One: 0.5-2 h

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Author contributions statements

J.S.O.M and J.W.T conceived and developed the original method. R.W. provided major updates. R.W, J.W.T, I.C.H, J.B.N, J.S and J.S.O.M performed laboratory experiments. R.W, J.W.T, I.C.H, M.M, K.L, E.P and D.H and J.S, developed methods and tested workflows. J.W.T, I.C.H, I.L, D.H, J.S, R.W, T.C.H and J.S.O.M worked on the data analysis pipeline. J.S.O.M supervised the project and co-wrote the manuscript with R.W. All authors edited the manuscript.

Competing interests

J.S.O.M has a research collaboration with Thermo Fisher Scientific and R.W's D.Phil. is supported by funding from Thermo Fisher Scientific. J.N contributed to this research whilst a postdoc in the McCullagh group. She currently works for Thermo Fisher Scientific.

Data availability

The authors declare that the main data discussed in this protocol are available in the supporting primary research papers (https://doi.org/10.1038/s42003-020-0957-6 and https://doi.org/10.1038/s41467-022-34095-x). The raw datasets have also been deposited in publicly available repositories for research purposes and any further data is available from the corresponding author upon reasonable request. Source data for Figure 1 is available in the Oxford University Research Archive

with the identifiers http://dx.doi.org/10.5287/bodleian:2abVOAvrg and http://dx.doi.org/10.5287/bodleian:eyq4Qj8AR (ref. 19). Source data for Figure 3 is available in Supplementary Data 6.

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SUPPLEMENTARY INFORMATION

Supplementary Data 1: Chemical compositions of our metabolite standard mixes.

Supplementary Data 2: Additional autosampler parameters for the AEC-MS/MS method.

Supplementary Data 3: AEC-MS/MS parameters for an ICS-6000 coupled to an Exploris™ 240.

Supplementary Data 4: AEC-MS/MS RT database for metabolite identification. We provide these data for indicative purposes only. Absolute RT values will be unique to each AEC-MS system and other laboratories should expect to have a different RT for the same metabolite on their system.

Supplementary Data 5: Example of the output from Progenesis QI after metabolite identification.

Supplementary Data 6: Example of the modified Progenesis QI output for use with Statistical Analysis within MetaboAnalyst.

Supplementary Data 7: Example of the modified Progenesis QI output for use with Functional Analysis within MetaboAnalyst.

Supplementary Figure 1: Comparison of Injection Modes for sensitive and reproducible analysis averaged across of a range of metabolite standards. Cross (blue) - peak area; circle (red) - % Coefficient of Variation (%CV) of peak area (n=3 per metabolite). Whiskers show 95% confidence intervals.

Supplementary Figure 2: EICs for inosine monophosphate (± 5 ppm) analysed with different mass resolutions by AEC-MS/MS to showcase the variation in scans across the peak and the subsequent peak shape. Circle (blue) - 17,500 resolution; square (red) - 35,000 resolution; triangle (green) - 70,000 resolution; diamond (purple) - 140,000 resolution. Each point is an individual MS^1 scan.

Supplementary Table 1: Validation metrics for a selection of metabolite standards analysed on the AEC-MS/MS, RPLC-MS and HILIC-MS methods including sensitivity, linearity, and RT stability.

Supplementary Table 2: Selection of metrics to assess system suitability for citrate, lactose, fructose-1,6-bisphosphate, and 2-hydroxyglutarate from analysis of our standard mixes.

Supplementary Method 1: AEC-MS/MS Data Analysis Workflow for Compound Discoverer 3.3

Supplementary Method 2: Workflow template for use with AEC-MS/MS data in Compound Discoverer 3.3

1946 # TABLES

Sample type	Typical amounts of sample	Minimum replicates per experimental group
Tissue culture	$5e^5$-$2e^6$ cells	5
Biopsy tissues	> 10mg	10
Biofluids (plasma, serum, and urine)	Approx. 1mL	10

1947 *Table B1.* Typical experimental sample requirements for preparation and subsequent analysis by AEC-MS/MS

Time (minutes)	Concentration OH^- (mM)	Curve
0.00	5.00	5
1.00	5.00	5
15.00	60.00	5
25.00	100.00	5
30.00	100.00	5
30.10	5.00	5
37.00	5.00	5

1948 *Table B2.* Gradient elution profile for AEC-MS/MS method

Parameter Setting	Full MS Settings	dd-MS^2/dd settings	Data Directed Settings
Polarity	-ve ion	-ve ion	-
Spectrum data type	Profile	Profile	-
Default charge state	1	1	-
Microscans	2	1	-
Resolution (at 200 m/z)	70,000	17,500	-
AGC target	1e6	1e5	-
Maximum IT (ms)	120	250	-
Scan range (*m/z*)	60-900	-	-
Loop counts	-	10	-
MSX counts	-	1	-
Isol. window (*m/z* units)	-	2.0	-
Isol. offset (*m/z* units)	-	0.0	-
(N)CE/stepped (N)CE	-	(N)CE: 35	-
Minimum AGC	-	-	5e3
Apex Trigger (s)	-	-	1-15
Charge exclusion	-	-	3-8, >8
Dynamic exclusion (s)	-	-	20

1949 *Table B3.* Q Exactive™ parameters for AEC-MS/MS method

Advantages	Disadvantages
Eluent generation provides highly reproducible chromatography.	**Chromatographic peak resolution** is generally lower in AEC than for UHPLC-based methods due to lower pressure limits and column design.
Counter ion removal by electrochemical suppression reduces sample matrix complexity and ion-suppression.	**Chromatographic peaks are broad** and can have spiked peaks when faster mass analyers are used.
Highly reproducible and robust chromatographic retention times.	**Limited to highly polar and ionic** (ionisable) metabolites that form a negative charge. Lower polarity metabolites are not retained.
Elevated sensitivity and linearity compared to	**Polarity switching** experiments are not compatible

HILIC-MS for many metabolites.	with AEC-MS/MS.
Retention and elution mechanism is simple and straightforward leading to predictable retention times from analyte structures.	**Longer run times** are generally required (compared to UHPLC methods) for the analysis of complex mixtures.
Highly charged anions are well characterised including multi-phosphorylated and carboxylated metabolites.	**Incompatible with most zwitterions** which are removed by electrochemical ion suppression, including most amino acids.
A wide range of sample diluents including 100% aqueous and organic solvents are compatible with AEC-MS/MS.	**Metabolites sensitive to high pH** may be affected by mobile phase gradient conditions.
100% aqueous mobile phases provide a greener and more environmentally friendly chromatographic footprint.	**Ion-exchange systems** are generally more expensive than UHPLC systems currently.

Table 1: Advantages and disadvantages of AEC-MS/MS compared to existing analytical methods for analysis of highly polar and ionic metabolites.

Sample size (mg)	80% MeOH (v/v) in Type 1 water buffer vol. (µL)	Homogenisation
Up to 10	150	3 × 10 seconds
11-20	250	3 × 10 seconds
21-40	500	3 × 10 seconds
41-80	1,000	4 × 10 seconds

Table 2. Suggested extraction protocols per tissue weight range. Including, solvent volume and homogenisation step length.

Setting name		Selected option
Type of machine		High resolution mass spectrometer
Data format		Profile data
Ionisation polarity		Negative
Adducts in this experiment		M-H; M-2H; M-H_2O-H
Runs to include		All
Peak picking limits	Sensitivity	Automatic (3)
	Chromatographic peak width	0.1 minutes
Retention time limits		None

Table 3. Summary of data processing settings for Progenesis QI.

Step	Problem	Explanation	Solution	Likelihood
6	Samples have low DNA concentration	This may be due to incomplete scraping of the culturing dish in the case of cultured cells.	If only 1-3 samples have a much lower DNA concentration, we recommend excluding these samples from any normalisation (e.g. normalise to the lowest DNA concentration excluding any anomalously low samples). If the whole batch of samples have very low DNA concentration (< 20 ng/µL) it is recommended to grow and harvest	Common depending on experience level

			new samples to ensure any further consumables are not wasted	
10	Alarm sounds on the autosampler	Leak sensor activated, as we keep plates at 4 °C this can lead to condensation in the plate tray	Check for blocked drain, wipe dry.	Common
14	Performing automated calibration fails	This can occur when the ion source is dirty or the electrospray is unstable.	Infuse the Calmix for several minutes before starting the calibration. If not successful, clean ion source if suspected of being dirty and try again.	Uncommon
15	Conductivity > 1 µS between analyses	Contamination of suppressor membrane leading to poor suppression. This can happen if eluent is run through the suppressor without applying a suppressor current	Proceed with suppressor regeneration and/or clean-up	Uncommon
15	Conductivity rises across the gradient	The suppressor may not be fully suppressing the eluent, or the CR-ATC may not be removing contaminants from the eluent effectively	Check the pH of the eluent post suppression, if it is neutral this suggests the CR-ATC is the issue. Either proceed with regeneration as per the manual or replace it. If the eluent has pH > 7, proceed with the suppressor regeneration or replace the suppressor.	Uncommon (maximum 1 time per year)
15	Suppressor leaks	The suppressor membrane may have ruptured or leaked due to high backpressure – this can be caused by build-up from macromolecules in samples over time.	Replace suppressor.	Uncommon (1-2 times per year)
15	Baseline pressure increases	This indicates a blockage. One likely place is the conductivity detector, especially if biological samples with unknown protein concentration have been run. Long-term high pressures can lead to suppressor failure.	Remove link between the suppressor and conductivity detector, watch the eluent pump pressure. If a decrease of > approx. 50-75 psi is seen, there may be a blockage in the CDet. Connect the suppressor outlet to the CDet outlet and run at 0.1 mL/min to backflush the cell and clear any blockage. Confirm the blockage has cleared by monitoring the pressure when reconnecting the CDet and running at normal eluent flow rates.	Uncommon
15	A mixture of standards has no signal	The autosampler transfer line and/ or the injection valve may have become blocked resulting in no sample being injected	Disconnect the autosampler transfer line from the injection valve, select *'Wash Sample Loop'* in the Sampler tab of Chromeleon/Direct Control. Observe whether any liquid is expelled from the end of the PEEK. If no liquid is observed, check the injection port in the autosampler for leakage. If leakage is observed, remove the injection port and sonicate in Type 1 water to dislodge the blockage, replace the injection port and rerun *'Wash Sample Loop'* to confirm that the blockage is removed. If there is no leak at the injection port, there may be an airlock, rerun the *'Wash Sample Loop'* command until water is observed at the end of the transfer line.	Uncommon
15	Low (or variable) sensitivity	This can be due to the position of the HESI probe or may indicate the suppressor is failing. It can also be due to a dirty ion-transfer tube	Check the pH of the eluent entering the HESI source, replace suppressor if faulty. Re-position the probe tip according to manufacturer	Uncommon

		(ITT), S-lens, and/or quadrupole leading to inefficient ion transmission	recommendations. Clean ITT and S-lens. Clean quadrupole if necessary	
18	Retention time not reproducible	This is unusual in AEC-MS/MS and may indicate gradient re-equilibration is not sufficient.	Increase the re-equilibration time in the AEC method.	Uncommon
20	Peaks wider, increased tailing, and/or earlier eluting than normal	This suggests the capacity of the column has been reduced.	Proceed with column regeneration overnight with a high concentration of KOH or a small amount of MSA. If this does not improve peak shape and/or retention, replace the column.	Uncommon (maximum 1 time per year).
20	Chromatographic peak has a spikey top	This is a feature of AEC-MS/MS chromatography when coupled to high resolution MS.	Reduce the number of datapoints points per peak (increase MS resolution) or apply peak smoothing prior to integration.	Common
20	Broad chromatographic peaks > 1 min in width	This is a feature of analytical scale AEC-MS/MS. Resin-based column chromatography does not provide UHPLC-scale peak widths.	None.	Common
23	Metabolite identification in Progenesis QI provides an empty isotope simulation score = 0	A low percentage of Progenesis QI compound-features have no isotope simulation score. This leads to a zero score for isotope simulation when identifying metabolites	A lack of isotope simulation reporting should not be used to reject an identification. Isotope pattern matching should be performed manually against an isotope simulation performed manually using alternative software.	A few features
35	QC samples not reproducible	It is normal for the first one or two 'conditioning' QC samples in a sequence to be compositionally different (e.g. on PCA plot). If all QC are not clustering, however, or are linear, suspect bias such as intensity drift or insufficient re-equilibration. Investigate via a heatmap	Investigate heatmap and check for intensity drift over time (e.g. ESI getting dirty) or evidence of analytical hiatus. E.g. check the QC sample has not run out part way though the analysis or an error in vial position in the sequence. Rectify this and re-run all samples.	Uncommon

Table 4: Troubleshooting suggestions for AEC-MS/MS untargeted metabolomics.

FIGURE CAPTIONS

Figure 1: Benchmarking method validation parameters between HILIC-MS/MS and AEC-MS/MS for untargeted metabolomics analysis of authentic metabolite standards and cell extracts. The HILIC-MS/MS and AEC-MS/MS methods were run on separate chromatography systems coupled to the same Q Exactive™ mass spectrometer system. (a-b) Comparing chromatographic resolution for the analysis of a range of nucleic acid metabolite standards on the AEC-MS/MS and HILIC-MS/MS methods. (c-d) Plots showing the quantitative loss of phosphate for ATP and GTP metabolite standards analysed by AEC-MS/MS (n=5 for each concentration); error bars show standard deviation. Each repeated measurement is from the same sample. (e-h) Linearity plots for selected metabolite standards comparing AEC-MS/MS and HILIC-MS/MS methods (n=3 analytical replicates); error bars show standard error of the mean (SEM), concentrations are in µM. Each repeated measurement is from the same sample. (i-j) Principal Components Analysis (PCA) plots of sum normalised untargeted datasets from cell extracts representing four distinct metabolic groups comparing AEC-MS/MS and HILIC-MS/MS. Cell samples were mutant isocitrate dehydrogenase 1 (mutIDH1) and wild type IDH (wtIDH1) derived from the same isogenic LN18 glioblastoma cell line. Each cell line was grown on both high glucose media (20mM glucose, n=9) and low glucose media (5mM glucose, n=7) separately for comparison of the way they metabolised glucose. Each sample measurement is from a distinct sample. (k) Retention time reproducibility for 9 polar metabolites in repeat injections of an authentic standard analysed by AEC-MS/MS and HILIC-MS/MS (n=18 measurements over the course of 2 weeks with boxes spanning the inter-quartile range with a median line, whiskers are min to max).

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Figure 2: AEC-MS/MS metabolomics protocol. Schematic showing the main steps involved in performing untargeted or semi-targeted metabolomics using AEC-MS. Figure created using Biorender.com

Figure 3: Anticipated results for untargeted studies. (a) BPI chromatogram of authentic standards showing elution order reflects the strength of analyte charge and polarity. **(b)** compounds-feature intensity heatmap. **(c)** PCA plot of four experimental groups and QC samples. Each sample measurement is a distinct sample. **(d)** Untargeted pathways analysis pathways impact map (using the mummichog algorithm in MetaboAnalyst). **(e)** Typical metabolites characterised by AEC-MS indicating coverage across inter-connected pathways found in primary metabolism. Created with Biorender.com