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Semi-targeted analysis of metabolites using capillary-flow ion chromatography coupled to high-resolution mass spectrometry

Karl Burgess[1,2*], Darren Creek[1,2,3], Paul Dewsbury[4], Ken Cook[4] and Michael P. Barrett[1,2]

[1]Scottish Metabolomics Facility, University of Glasgow, Glasgow, UK
[2]Institute of Infection, Immunity and Inflammation, Wellcome Trust Centre for Molecular Parasitology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK
[3]Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Flemington Rd, Parkville, Victoria 3010, Australia
[4]Thermo-Fisher Scientific, Camberley, UK

This work describes a novel application of capillary-flow ion chromatography mass spectrometry for metabolomic analysis, and comparison of the technique to octadecyl silica and hydrophilic interaction chromatography (HILIC)-based mass spectrometry. While liquid chromatography/mass spectrometry (LC/MS) is rapidly becoming the standard technique for metabolomic analysis, metabolomic samples are extremely heterogeneous, leading to a requirement for multiple methods of analysis and separation techniques to perform a 'global' metabolomic analysis. While C18 is suitable for hydrophobic metabolites and has been used extensively in pharmaceutical drug metabolism studies, HILIC is, in general, efficient at separating polar metabolites. Phosphorylated species and organic acids are challenging to analyse and effectively quantitate on both systems. There is therefore a requirement for an MS-compatible analytical technique that can separate negatively charged compounds, such as ion-exchange chromatography. Evaluation of capillary flow ion chromatography with electrolytic suppression was performed on a library of metabolite standards and was shown to effectively separate organic acids and sugar di- and tri-phosphates. Limits of detection for these compounds range from 0.01 to 100 pmol on-column. Application of capillary ion chromatography to a comparative analysis of energy metabolism in procyclic forms of the parasitic protozoan *Trypanosoma brucei* where cells were grown on glucose or proline as a carbon source was demonstrated to be more effective than HILIC for detection of the organic acids that comprise glucose central metabolism and the tricarboxylic acid (TCA) cycle. Copyright © 2011 John Wiley & Sons, Ltd.

Metabolomics is a rapidly growing technique for the simultaneous analysis of the small molecule component of biological systems, the metabolome.[1] As such it underpins important research areas such as systems biology and systems medicine. In contrast to the relatively small chemical space produced by the 20 amino acid structures in proteomics, and the four nucleotide bases in genomics, the enormous heterogeneity of biological small molecules precludes comprehensive analysis of metabolites by a single generic technique. For this reason, multiple techniques are essential to obtain maximal coverage of the metabolome.[2] Liquid chromatography/mass spectrometry (LC/MS) has many advantages in the analysis of metabolites – the use of electrospray ionisation (ESI) as an ionisation technique emphasises the production of molecular ions,[3] and the variety of LC stationary phases available improves the number of different classes of compounds that can be separated.[4] Recent developments in accurate mass spectrometry have enabled determination of a limited set of potential empirical formulas for each detected molecular ion;[5] however, in-source fragments, contaminant ions and isomeric compounds often prevent metabolite identification and quantitation from MS data.[6] Isomeric compounds often have widely varying roles in biochemical pathways, and it can therefore be critical to distinguish between them by separating them chromatographically. The types of chromatography most commonly coupled to MS, and applied to metabolomics, are octodecyl silica (C18)[7–9] and hydrophilic interaction chromatography (HILIC).[10–13] These are especially effective for hydrophobic and polar metabolites, respectively, but there are important classes of metabolites that are poorly resolved or retained using these stationary phases with standard, MS-compatible buffers: di- and triphosphate sugars and organic acids, while critically important elements of metabolic pathways are often poorly resolved when separated using HILIC or not retained at all using reversed-phase chromatography.

While several methods have been described for detection of nucleotide phosphates and related compounds using novel ion-pairing reagents with C18 columns,[14–16] MS-compatible separation methods for organic acids are considerably less often described.[17,18] Both types of technique are generally used for targeted metabolomics approaches as they are rather specific for their metabolites of interest. A method that captures a broader range of these charged compounds as part of an untargeted methodology would therefore be beneficial.

* *Correspondence to:* K. Burgess, Scottish Metabolomics Facility, University of Glasgow, Glasgow, UK.
E-mail: karl.burgess@glasgow.ac.uk

We therefore investigated the use of reagent-free ion chromatography as an alternative method for resolution of a variety of difficult compounds. Ion chromatography is a powerful separation technique for charged compounds,[19] but its uptake in the field of metabolomics has been hampered by the high ionic concentration of the elution buffers, which lead to deleterious ion suppression when directly coupled to MS. Ion suppression technology allows post-column, in-line removal, of the elution counter-ions, while retaining the charge on the analytes. This technique has been used for targeted metabolomics applications,[20] although the high flow rate limits sensitivity of detection. In this paper, a novel, capillary flow system[21] has been applied to metabolomic analysis for semi-targeted, high-resolution analysis of metabolite standards. We also demonstrate its effectiveness for semi-quantitative analysis of samples of the protozoan parasite *Trypansoma brucei* grown using two different carbon sources.

EXPERIMENTAL

Sample preparation

Standard metabolites were obtained from Sigma-Aldrich (Dorset, UK), stock solutions were prepared at 10 mM or 100 mM in water or 50% aqueous ethanol (depending on solubility), and combined into three semi-complex mixtures to isolate interfering isomeric compounds. Mixes 1, 2 and 3 contained 52, 41 and 9 compounds detectable in negative ionisation mode, respectively.

Preparation of trypanosome extracts

Trypanosoma brucei strain 427 procyclic forms were grown in SDM-80 medium supplemented with either glucose or proline and 10% foetal calf serum as described.[22] A total of 4×10^7 cells (10 mL of cell culture) per replicate were quenched by rapid cooling to 4 °C while mixing vigorously to avoid freezing and possible cell lysis. After cooling, cells were centrifuged for 10 min at 1000 *g* at 4 °C and the supernatant was removed. The cell pellet was resuspended, transferred to a 1.5 mL tube and centrifuged for 5 min at 2500 *g* at 4 °C, after which the supernatant was removed. The pellet was suspended in 200 µL of chloroform/methanol/water (1:3:1) (at 4 °C), followed by vigorous mixing with a pipette. The resulting slurry was then vortexed on a cooled (4 °C) mixer for 1 h. The supernatant was clarified by centrifugation for 3 min at 13 000 *g* at 4 °C and then stored at −80 °C until analysis by LC/MS. Three biological replicates of each condition were obtained.

Chromatography

Ion chromatography was performed using an ICS-5000 system (Dionex Corporation, Sunnyvale, CA, USA). Columns used were IonPac AS-19 (standards) and IonPac AS-20 (trypanosome extracts) anion-exchange columns (Dionex Corporation). Both the AS-19 and AS-20 columns possess alkanol quaternary ammonium strong anion-exchange groups and low hydrophobicity. Their only distinction lies in their capacities, which are 2.4 and 3.1 µeq/L. The higher capacity AS-20 was thought to be appropriate for biological samples due to their greater variation of metabolite concentrations and larger potential for contaminants, and was therefore used for the trypanosome extracts, while the lower capacity AS-19 was retained for the initial analysis of standards. Flow rate was 10 µL/min supplemented post-column with a 10 µL/min methanol make-up flow. Gradient starting conditions were 8 mM KOH, rising to 40 mM in 15 min, then to 80 mM at 25 min, then 100 mM at 30 min. Concentration was held at 100 mM for 22 min, followed by a reduction to 8 mM for 8 min to re-equilibrate the column. Total run time was 60 min.

HILIC was performed on a U3000 RSLC system (Dionex Corporation). Column hardware consisted of a 4.6×150 mm ZIC-HILIC column (Sequant) coupled to a 2.1×50 mm ZIC-HILIC guard column. Flow rate was 300 µL/min and buffers consisted of 98% ACN, 1.92% H_2O, 0.08% formic acid and 98%H_2O, 1.9% ACN, 0.1% formic acid. The gradient ran from 80% ACN to 20% in 30 min, with a wash at 5% for 10 min and reequilibration at 80% for 6 min.

C18 chromatography was performed on a Summit system (Dionex Corporation). Column hardware consisted of a 2 mm 150 mm Acclaim column (Dionex). Flow rate was 200 µL/min and buffers consisted of 98% H_2O, 1.9% ACN, 0.1% formic acid and 98% ACN, 1.92% H_2O, 0.08% formic acid. The gradient ran from 2% ACN to 50% in 20 min, with a wash at 90% for 5 min, and reequilibration at 2% for 5 min.

Mass spectrometry

MS was performed using an Orbitrap Exactive (Thermo, Hemel Hempstead, UK) for all HILIC analyses, and an LTQ Orbitrap Velos (Thermo, Hemel Hempstead, UK) for all ICMS and C18 analyses. The Exactive was operated in switching mode with a positive voltage of 4.5 kV and a negative voltage of 3.5 kV. Resolution was set to 50 000. The Orbitrap Velos was operated in negative mode with a negative voltage of 3.0 kV and a resolution of 60 000.

Data analysis

Analysis of standards was performed using the ToxID software (Thermo, Hemel Hempstead, UK) for peak detection and integration. Chromatograms were selected by exact mass (±3 ppm) and confirmed by manual inspection.

Data analysis of trypanosome extracts was performed using XCMS[23] and mzMatch,[24] followed by post-processing in Microsoft Excel using in-house VBA scripts. Metabolites were positively identified on the basis of exact mass within 3 ppm, and further validated by concordance with standard retention times.

RESULTS AND DISCUSSION

Analysis of metabolite standards

ZIC-HILIC columns, which exploit a sulfobetaine stationary phase, have previously been reported to provide the broadest separation for untargeted metabolomic analysis using standard buffer conditions (pH ~2–4).[10] Unsatisfactory peak shapes, however, were observed for many critical compounds that play a major role in central metabolism and others were not observed at all, probably as a result of either ion suppression due to co-elution or strong binding to the column

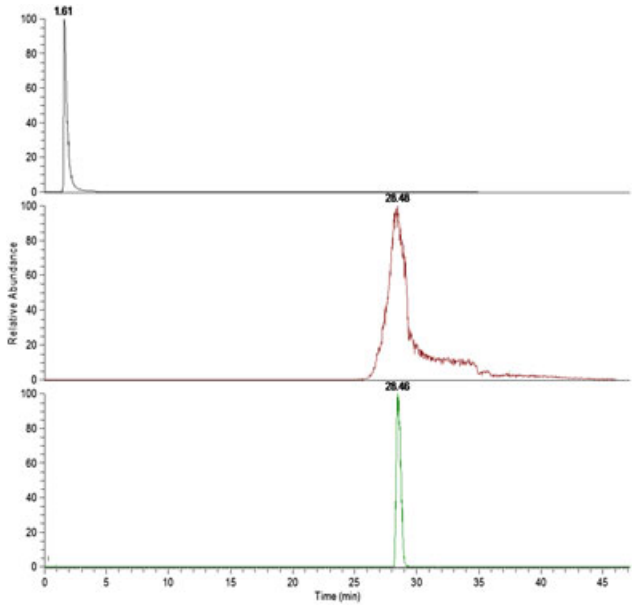

Figure 1. Comparison of the chromatography of ADP using C18, ZIC-HILIC and AS19 ion chromatography stationary phases. ADP elutes without being retained on the C18 column, ran with poor peak shape (FWHM peak width of 90 s) on the HILIC column, and produced a clear Gaussian peak with a FWHM peak width of 30 s under ion chromatography conditions.

resulting in failure to elute. Several reports have shown improved separation characteristics of high-pH buffering of HILIC columns,[25] but these have a deleterious effect on the robustness of the silica-based columns, and consequently are expensive to maintain. We assessed ion chromatography using standards in two ways, with a panel of 102 compounds covering the majority of central metabolism, and a smaller panel of compounds known to retain poorly on our HILIC system. A typical example of an important central metabolite, ADP, exhibiting poor chromatography using the HILIC system is shown in Fig. 1. On a C18 column, ADP is too polar to be retained, and elutes in the wash period, along with a large number of other polar compounds. Organic triphosphates, where the final phosphate is extremely labile, as shown in Fig. 2, are even less tractable. Co-elution of ADP and ATP would result in overlapping signals at 426.022, making identification and quantitation impossible. Separated by HILIC, ADP is retained but separates poorly, with a broad initial peak followed by significant tailing, making accurate quantification difficult. ATP does not produce a detectable peak on the HILIC platform. In contrast, by ion chromatography using an AS-19 or AS-20 column, ADP and ATP are clearly separated, with classical Gaussian peak shapes and FWHM peak widths of roughly 30 s. All common nucleotide di- and triphosphates run very poorly on ZIC-HILIC chromatography, but run well on ion chromatography (Fig. 3). Limits of detection were assessed for several common acidic and phosphorylated metabolites, and are presented in Table 1. The range of limits of detection is over 5 orders of magnitude, likely due to the heterogeneity of the chemical structures involved.

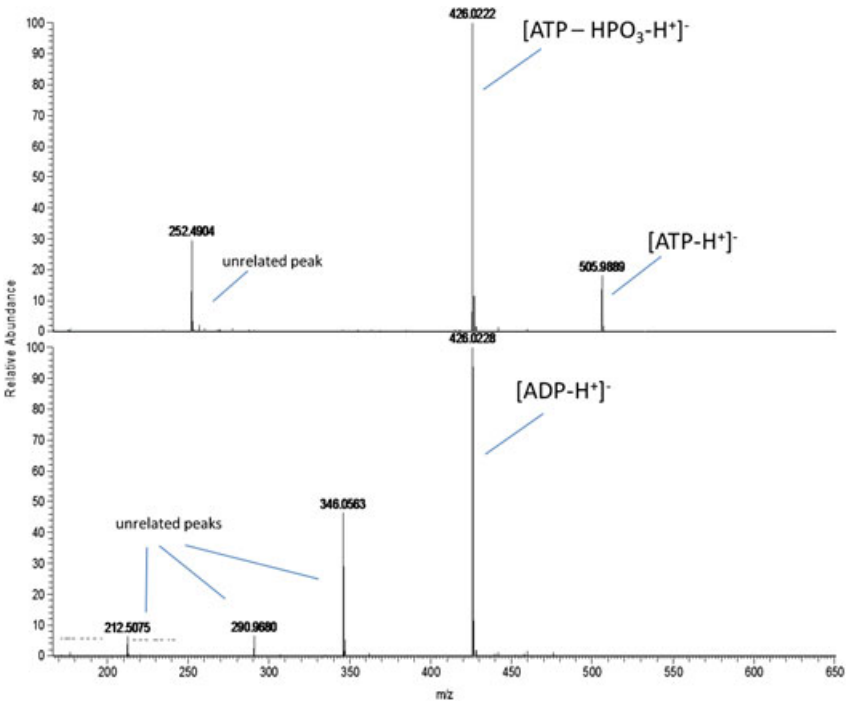

Figure 2. Spectra of ATP (top) and ADP (bottom). The peak at 505.9889 corresponds to the true mass of ATP. Loss of a phosphate group in source results in the dominant peak in the mass spectrum corresponding to ADP (426.0226).

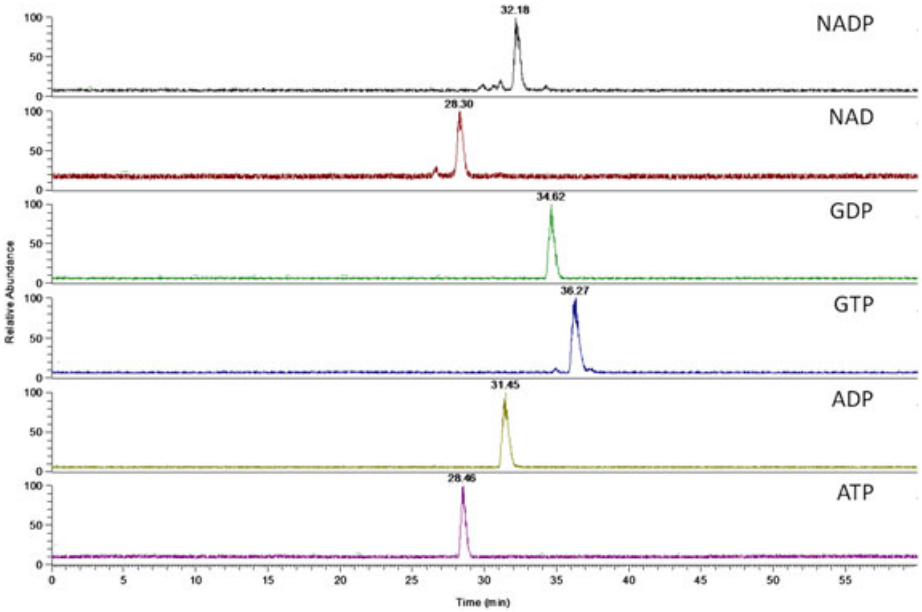

Figure 3. Separation of a group of compounds with poor retention on HILIC. These compounds are well retained using capillary flow ion chromatography and produce Gaussian peaks.

Table 1. Limits of detection (LODs) for 34 standard compounds assessed against a semi-complex background. LODs were determined by 10-fold dilution of standards from a 100 µM stock. W: elutes in wash-through (within 2.5 min of run start); B: elutes as a broad peak (>1 min FWHM), G: elutes as a clear, sharp peak. RT: retention time. ND denotes that the compound was not detected

Compound Name	Formula	LOD (pmol)	RT (min)	Presence in C18	Presence in HILIC
Fumarate	C4H4O4	100	21.83	W	G
Malonate	C3H4O4	10	16.47	W	B
Pyruvate	C3H4O3	0.01	8.19	W	B
D-Glucose	C6H12O6	10	4.97	W	G
cis-Aconitate	C6H6O6	10	25.04	ND	G
3′,5′-Cyclic-AMP	C10H12N5O6P	100	20.05	B	B
D-Gluconic acid	C6H12O7	1	6.81	W	B
UMP	C9H13N2O9P	100	26.87	W	G
IMP	C10H13N4O8P	10	32.18	W	G
CMP	C9H14N3O8P	100	16.59	ND	G
L-Cysteate	C3H7NO5S	0.1	18.37	W	G
D-Ribose 5-phosphate	C5H11O8P	100	18.49	ND	G
D-Glucose 6-phosphate	C6H13O9P	10	17.07	ND	B
Phosphoenolpyruvate	C3H5O6P	10	23.94	ND	B
6-Phospho-D-gluconate	C6H13O10P	10	22.09	ND	B
Succinate	C4H6O4	10	17.11	B	G
(*R*)-Lactate	C3H6O3	0.1	7.28	W	B
2-Oxoglutarate	C5H6O5	10	19.23	W	G
(*S*)-Malate	C4H6O5	10	16.60	W	G
Sucrose	C12H22O11	100	4.52	W	G
Orotidine	C10H12N2O8	100	23.18	W	G
Citrate	C6H8O7	100	24.2	W	B
3-Phospho-D-glycerate	C3H7O7P	100	22.88	W,B	B
ADP	C10H15N5O10P2	100	28.7	W, B	B
GDP	C10H15N5O11P2	100	34.7	W, B	B
GTP	C10H16N5O14P3	100	36.52	W, B	ND
CTP	C9H16N3O14P3	100	28.6	W, B	ND
ATP	C10H16N5O13P3	100	31.6	W, B	B
Maleic acid	C4H4O4	10	16.65	W	G
D-Fructose 6-phosphate	C6H13O9P	10	18.17	W	G
Pyrophosphate	H4O7P2	1	25.83	W,B	ND

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Application to a biomedical sample

Multiply phosphorylated sugars and organic acids are critical components of the energy metabolism cascade, and capillary flow ion chromatography was therefore applied to the analysis of trypanosome cellular extracts grown in medium containing either glucose or proline as a carbon source. Our standard conditions involve the use of an Orbitrap Exactive to scan both positive and negative ionisation modes of a HILIC separation. Assessing the combined output of both ionisation modes we attained coverage of only three compounds out of the 16 total compounds associated with the tricarboxylic acid cycle (Fig. 4). In contrast, data from negative ionisation mode ion chromatography/MS coverage increases to 7 out of 16 compounds, more than doubling the visible metabolites in this part of the metabolome (Fig. 4). When the whole of the data extracted is taken together, and profiled using principle component analysis (PCA), clear delineation may be made between the glucose-fed cells, proline-fed cells and the medium (containing either proline or glucose) (see Fig. 5), showing that this technique can be used both in targeted analysis of specific metabolites as well as in metabolite profiling for biomarker applications.

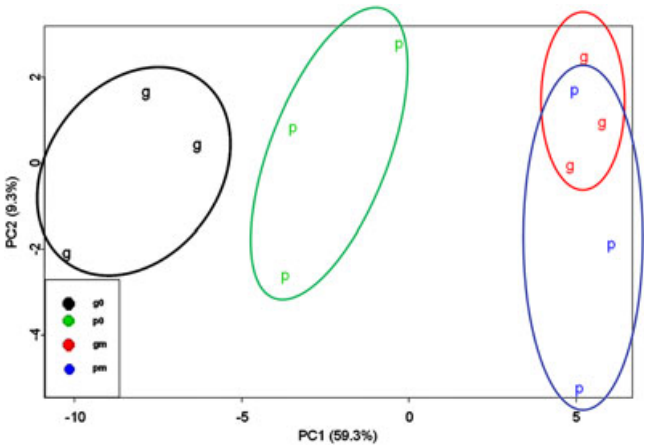

Figure 5. Principle component analysis (PCA) of the identified metabolites detected using ion chromatography. Samples (g0) comprising intracellular metabolites identified from trypanosomes grown on medium containing glucose are shown in black, while samples (p0) containing intracellular metabolites identified from trypanosomes grown on proline are shown in green. Extracts from fresh medium samples are shown in red (gm, glucose-containing medium) or blue (pm, proline-containing medium).

Technical benefits

In addition to the ability to separate and identify numerous organic acids and multiply phosphorylated compounds, a key benefit of the ion chromatography system is its 'always on' nature, due to the low consumption of both deionised water and reagent ion stocks. This makes it an ideal instrument for supplementary analysis if conventional separations are insufficient, as the set-up time is negligible. Additionally, as long as salt and pH are at appropriate levels, the buffer used to load the sample is largely irrelevant, unlike HILIC or C18 where high aqueous (in the case of the former) or high organic (in the case of the latter) concentrations lead to poor separation.

Technical limitations

In the anion-exchange system described, due to the action of the suppressor, which removes the cationic K^+ ions post-column, other cationic compounds are also removed. Detection is, therefore, limited to negative ions. Analysis of positively charged compounds can be performed, but would require a separate run on a cation-exchange system, which requires an additional sample load. More selective cation- or anion-exchange membranes would solve this problem and the use of ion chromatography systems in metabolomics will be enhanced by their anticipated arrival.

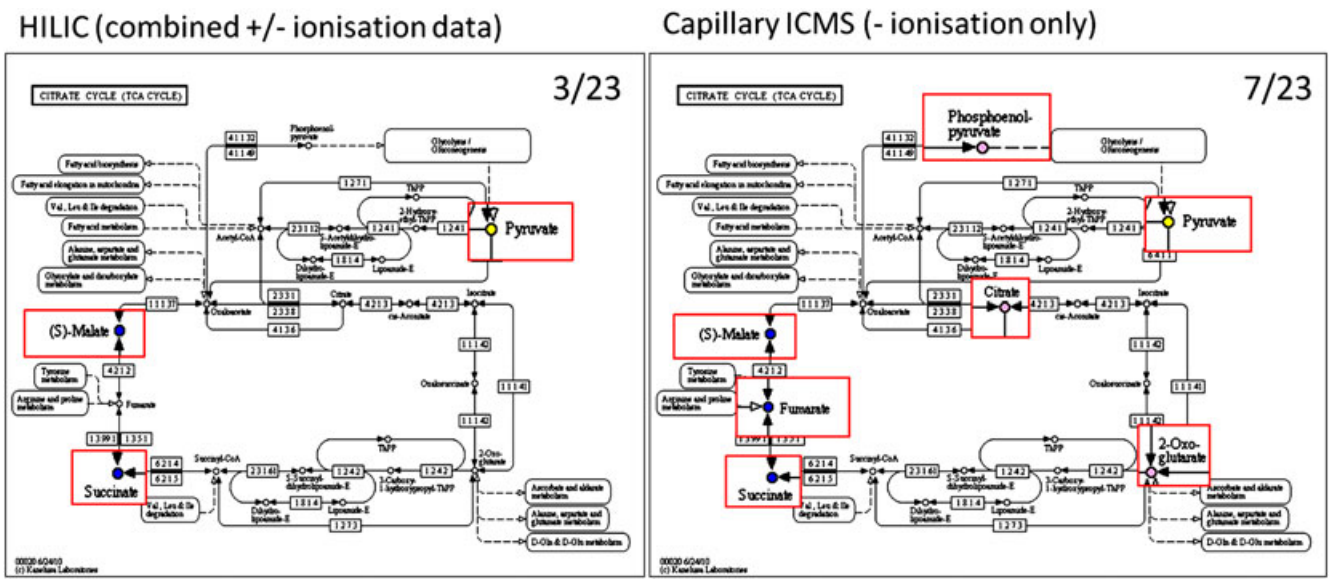

Figure 4. Identified metabolites mapped to the TCA cycle from KEGG using the *Pathos* software.[26] Combined data covering both positive and negative ionisation data from samples separated using ZIC-HILIC chromatography provided identification of three compounds of the 23 noted in the cycle. In contrast, using only negative ionisation mode data from samples separated using capillary ion chromatography, seven compounds were detected.

CONCLUSIONS

The capillary ion chromatography system, coupled with a high-resolution mass spectrometer, constitutes a powerful system for analysis of many metabolites, but especially di- and triphosphates and organic acids, many of which are poorly resolved with conventional metabolomics analytical techniques. These compounds are critical for their role in energy metabolism. We have demonstrated the value of the technique in the analysis of energy metabolism pathways in the parasite *Trypanosoma brucei*, where more than double the number of key metabolites were detected by ICMS, as compared to our conventional HILIC method.

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