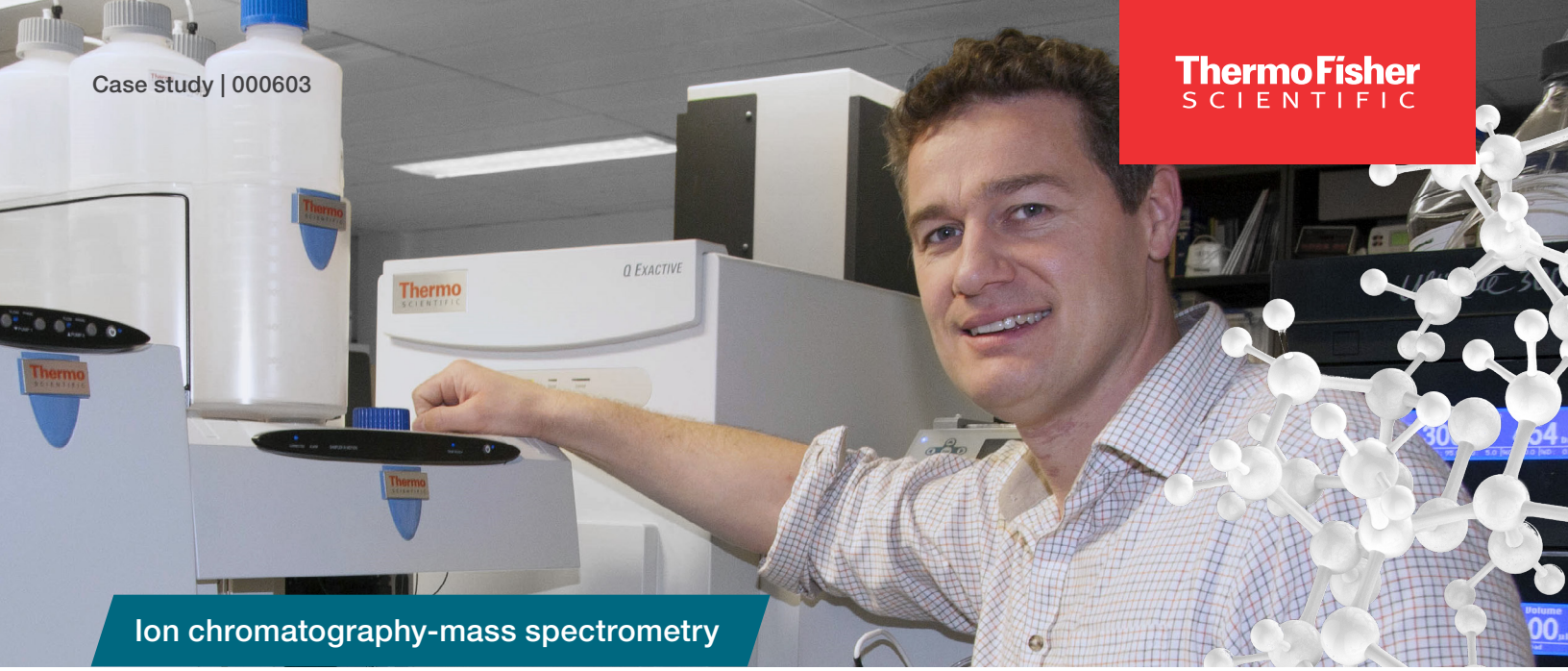

Advances in metabolomics using untargeted IC-MS

McCullagh Group, Department of Chemistry, University of Oxford, UK

"IC-MS revolutionized our capability for profiling changes in central metabolism associated with brain tumors. All the methods we had tried previously were not capturing relevant metabolites as effectively as we wanted. The broad coverage we obtained from IC-MS meant we could use a single analytical run to profile a wide range of compounds of interest."

—Dr. James McCullagh, Professor of Biological Chemistry, Director of the Mass Spectrometry Research Facility (SRF), Department of Chemistry, University of Oxford

Thermo Scientific IC-MS system expands untargeted metabolomics discovery

Metabolomics aims to comprehensively characterize and quantify metabolites in biological systems to understand altered biochemical states and functions. Untargeted metabolomics, in particular, focuses on obtaining a global comparison of as many metabolites as possible between experimental sample groups. However, robust, comprehensive untargeted analysis of the many thousands of chemically diverse metabolites present in complex biological extracts remains technically challenging.

Mass spectrometry (MS) is an efficient, sensitive, and selective technique for in-depth qualitative and quantitative metabolomics experiments. Though several chromatography techniques are available to separate sample components prior to MS analysis, ion chromatography (IC) with high-resolution accurate-mass (HRAM) MS uniquely allows robust, reproducible characterization of ionic and highly polar metabolites. Routine comprehensive characterization of these compounds is essential to understanding disease processes, especially those that affect central carbon metabolism as in cancers, diabetes, heart disease, viral and microbial infections, and immune response to disease. At the University of Oxford Mass Spectrometry Research Laboratory (SRF), Thermo Scientific™ IC-MS technology has become an indispensable tool enabling metabolomics discoveries produced across more than 50 collaborations to date.

thermo scientific

"With IC-MS, we've collaborated with an increasing number of scientists asking challenging analytical questions, where highly polar and ionic metabolite analysis is required. After we established IC-MS methods, collaborative applications snowballed. We have had over 50 collaborations leading to publications in the last few years."

—Dr. James McCullagh

Thermo Scientific™ Dionex™ ICS-5000⁺ Ion Chromatography System with the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer advances metabolomics research. Photo courtesy of the Mass Spectrometry Research Facility (SRF), Chemistry Research Laboratory, University of Oxford.

Untargeted metabolomics: analytical challenges

Understanding metabolic changes in biological systems and their pathway-level linkages requires overcoming three major challenges: obtaining comprehensive compound coverage, ensuring analytical reproducibility, and achieving high-confidence metabolite identifications. Because chromatographic separations are important for quantification and different chromatographic approaches are selective for different physiochemical properties, metabolites are difficult to analyze using a single method comprehensively. The application of multiple chromatography methods can provide a more comprehensive analysis. "Ideally, we want to access metabolomes with a small number of approaches, capturing the relevant metabolic pathways in a holistic way to have coverage of the connected pathways that change under different conditions," said McCullagh.

Untargeted metabolomics involves comparing of control and test groups to identify differences between their metabolite profiles, making analytical reproducibility and stringent data analysis essential. High analytical reproducibility increases confidence that data are a direct expression of biological variance. In addition, retention time stability is essential for compound identification when compounds have been run previously.

Following data comparison between experimental groups, the challenge remains to identify the potentially thousands of unknown compound features of interest. Accurate mass and isotope patterns can be used to predict a molecular formula. However, structural isomers, abundant in small molecules found in biology, can be challenging to differentiate by MS alone. Fragmentation information generated from tandem mass spectrometry (MS/MS) experiments can help but can be insufficient when structures are extremely similar. Fortunately, chromatographic retention time is often highly sensitive to structural differences between molecules, so a combination of these measurements can be beneficial for making identifications. Nonetheless, interpreting this complex combination of information is a challenge.

IC-MS offers a solution

To solve these challenges, the McCullagh group focused on IC-MS methodology as a new way to characterize the role of small molecules in complex biological systems. This approach has led to numerous collaborations in many research areas. Examples of the collaborative research are listed in Table 1.

"IC-MS is really powerful because, more than any other method, it enables us to obtain very broad coverage for polar and ionic metabolites. Except for calibration and routine maintenance, we continuously run a 40-minute untargeted method—with the same column and conditions—and apply it to a range of sample types, including cell and tissue extracts, across many different projects."

—Dr. James McCullagh

Table 1. Collaborative research discoveries powered by IC-MS.

Metabolic effects of isocitrate dehydrogenase 1 (IDH1) mutations in glioblastoma cells, revealing significant elevation of 2-hydroxyglutarate and depletion of 2-oxoglutarate as well as depletion of additional metabolites including previously unknown changes in lysine and tryptophan metabolism.[1]
Autophagy-mediated lipolysis produces free fatty acids to support a mitochondrial respiration pathway essential to neutrophil differentiation. Neutrophils are important but short-lived mediators of innate immunity which must be constantly replenished by the body.[2]
Identified circulating plasma metabolic biomarkers in Intrahepatic Cholangiocarcinoma (ICC), an aggressive cancer arising from the bile ducts that currently lacks methods for early disease stratification and diagnosis, and treatment options.[3]
Understanding the role of glucose metabolism and oxygen tension in regulating human retrovirus HTLV-1 proviral latency and reactivation from $CD4^+$ T lymphocytes, a process previously poorly understood.[4]
Discovery of the mechanism of butyrate-induced antimicrobial activity in intestinal macrophages in vivo. Intestinal butyrate could potentially bolster antimicrobial defense without tissue-damaging inflammation.[5]

IC-MS provides broad coverage for ionic and highly polar metabolites

Many of the metabolites associated with primary metabolic processes, such as energy transduction, nucleic acid metabolism, and carbohydrate metabolism, are highly polar or negatively charged at physiological pH (7.2). Most commonly, hydrophilic interaction liquid chromatography-MS (HILIC-MS), ion-pairing-MS (IP-MS), or derivatized gas chromatography-MS (GC-MS) have been used to analyze highly polar and ionic metabolites. However, achieving robust and reproducible coverage can be challenging due to method and sample preparation requirements. IC-MS offers broad coverage, highly reproducible analytical approach for analyzing these types of compounds. McCullagh explained, "To characterize all the compounds that we routinely analyze using IC-MS would require the deployment of multiple HILIC methods."

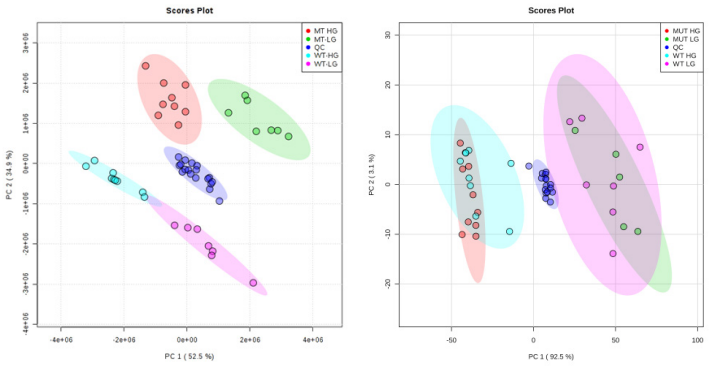

Figure 1. Principal component analysis (PCA) plot of four glioma cell lines and a QC sample analyzed using IC-MS (left) and HILIC-MS (right). Though in both plots the QC samples cluster nicely in the center, at the 95% confidence limit, only IC-MS produced data able to capture the metabolic differences between cell lines, providing comprehensive pathway coverage. Nature and Springer Nature Limited. Walsby-Tickle, J. et al. Anion-exchange chromatography mass spectrometry provides extensive coverage of primary metabolic pathways revealing altered metabolism in IDH1 mutant cells. Communications Biology. 2020, 3 (1), 1-12

"We need a metabolic pathways-relevant approach, and this is where IC-MS provides us with useful information. IC-MS has helped us understand in more detail and more holistically what happens to central carbon metabolism in disease, and in the basic biology of cellular processes. The Thermo Scientific™ Dionex™ ICS-6000 HPIC System with the Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer will enable us to explore other methods that will further open up our capabilities for metabolomics."

—Dr. James McCullagh

Thermo Scientific Reagent-Free IC (RFIC) capability and IC columns offer remarkable stability and reproducibility

Retention time stability is important for aligning chromatograms and allows larger numbers of replicates to improve statistics and thus confidence in measured differences. We found that IC-MS can deliver significantly higher retention time reproducibility than HILIC-MS (Figure 2). McCullagh continued, "We saw a contrast in the stability of the Thermo Scientific IC-MS system compared to HILIC-MS. When you run samples continuously 24/7 for several months, which we need to do to meet research demands, being able to handle that is impressive."

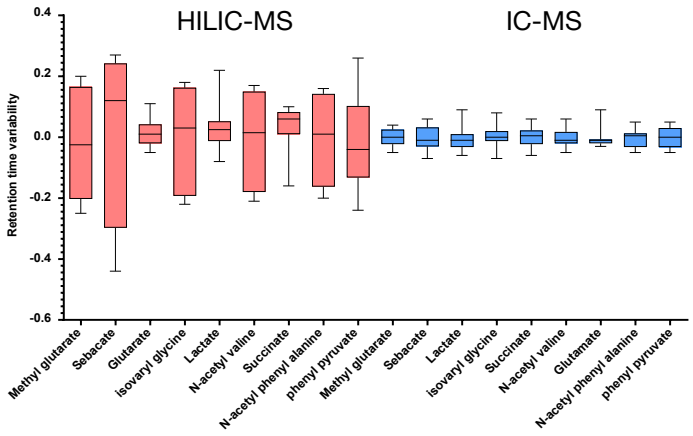

Figure 2. Comparison of retention time variability over three months for HILIC-MS and IC-MS for identical samples. The IC-MS system was the Dionex ICS-5000⁺ IC system coupled to the Q Exactive mass spectrometer. The Q Exactive instrument was also used for the HILIC-MS experiments. Nature and Springer Nature Limited. Walsby-Tickle, J. et al. Anion-exchange chromatography mass spectrometry provides extensive coverage of primary metabolic pathways revealing altered metabolism in IDH1 mutant cells. Communications Biology. 2020, 3 (1), 1-12

Behind the exceptional stability of the Lab's IC-MS results is the Thermo Scientific™ Dionex™ Reagent-Free™ Ion Chromatography (RFIC™) System, in which automated eluent generation (RFIC-EG™) and electrolytically regenerated suppressors create the eluents and regenerants required for IC-MS analysis for both isocratic and gradient methods. This provides an electrospray-compatible effluent that can be analyzed directly by the mass spectrometer. All that's needed is a source of high-purity de-ionized water and the IC system is always on and ready to run samples. Compared to manual eluent preparation, RFIC-EG provides superior performance and simplicity, higher sensitivity, and excellent reproducibility while eliminating labor, variability, and errors associated with manually prepared eluents. McCullagh explained, "We don't have to make up mobile phases manually where there is variability from one person or lab to the other—we just add water, and the mobile phase gradient is generated automatically. That's important when it comes to the reproducibility we want over long periods. It makes a significant difference."

Thermo Scientific ion exchange columns contribute to robust IC-MS retention time stability. "The columns we have tested are remarkably robust," commented McCullagh, "I'm constantly concerned that the number of complex biological extracts that we put through will have detrimental effects and shorten column lifetime, but we've had columns that have lasted a year running cell extracts 24/7 resolving thousands of components. We get within 2 or 3 seconds retention time reproducibility over weeks for our standards. It's quite incredible."

"When identifying compounds by IC-MS, accurate mass measurements enable chemical formula prediction for analytes, but structural information requires orthogonal information, including what is provided by the IC."

—Dr. James McCullagh

IC-MS enables confident compound annotation

Following a comparison between experimental groups, features of interest are annotated using retention time, accurate mass MS and MS/MS values, and isotope pattern. The experimental data is used to narrow down structural possibilities for identification, ideally to a single structural assignment.

The retention time stability provided by IC-MS is indispensable for identification when compounds, for example, standards, have been run previously. McCullagh noted, "We have found that IC retention times are more reproducible than some other types of chromatography we've used—meaning that we can rely on it to support compound identification." For compounds not previously run, IC retention time is highly selective for structure. Certain isomers are reliably resolved for subsequent identification, avoiding time-consuming sample derivatization and GC-MS analysis.

HRAM data increases certainty

HRAM data for MS ions, MS/MS derived fragments, and isotope patterns produced by the Q Exactive mass spectrometer facilitates confident determination of molecular formulas. High mass resolution makes the difference between detecting and not detecting analyte molecules at low concentrations due to the masking effect of isobaric matrix interferences. High mass resolution combined with accurate mass enables the user to directly depict fine structures, reducing the number of possible elemental compositions. Additionally, one of the useful characteristics of IC is that the technique produces peaks that are relatively broad. Broad peaks enable collecting multiple MS scans per peak and diagnostic fragment ions.

New capabilities facilitate what's next

Due to the success of combining the ICS 5000$^+$ chromatography system with the Q Exactive mass spectrometer, it is constantly in use supporting the lab's research. The system is now used more broadly for an increasing range of research projects across the department and beyond. As a result, the lab has obtained a second system—a Dionex ICS-6000 HPIC system coupled to an Orbitrap Exploris 240 mass spectrometer—as part of a collaboration with Thermo Scientific. "We had such success using the Q Exactive instrument with the IC that we haven't had time to do further method development work and explore the wide range of column types that are available or the breadth of coverage we get using IC-MS," said McCullagh. "This will also give us the opportunity to address application areas we haven't previously had instrument time for, including how IC-MS can be applied in isotopic tracer analysis to map the fluxes through central carbon metabolism with higher fidelity. We are also interested in early detection of disease, using multi-modal biomarkers not just related to small molecules, but combining metabolite changes with proteome and genome changes."

The Orbitrap Exploris 240 instrument will provide the lab with additional mass resolution and scan speed and the efficiency of Thermo Scientific™ AcquireX Intelligent Data Acquisition Workflow. AcquireX acquisition boosts sample profiling efficiency, generating highest-quality MS^2 data for component identification while reducing manual setup and subsequent data interpretation. McCullagh explained, "The Orbitrap Exploris 240 system is a significant step up in terms of mass resolution and speed. We are excited to find out whether AcquireX combined with the speed of the instrument will help us to be more selective with our coverage of the metabolome."

Conclusion

Untargeted metabolomics has been limited by traditional analytical capabilities, making new analytical methods capable of robust pathway coverage and metabolite identification essential to further development in the field. In the McCullagh Group, IC with HRAM MS is overcoming some of the challenges of established techniques such as HILIC-MS to provide a uniquely comprehensive perspective on the ionic and ionizable metabolites that are important in central carbon metabolism and other primary metabolic pathways. High-fidelity characterization of these pathways promises to improve understanding of disease processes and the identification of predictive, diagnostic, and therapeutic targets.

About James McCullagh

James McCullagh is Professor of Biological Chemistry and Director of the Mass Spectrometry Research Facility (SRF) in the Department of Chemistry at the University of Oxford, UK. His research group focuses on understanding the function of small molecules in biological, biomedical, and environmental systems, with emphasis on metabolism and metabolomics. He has 20 years of experience developing (bio)analytical chemistry techniques, in particular hyphenated MS techniques, for chemical, biological, and medical research. Highly collaborative, he works with academia and industry partners and has authored/co-authored over 100 journal publications, book chapters, articles, and a Mass Spectrometry textbook. He lectures on undergraduate and graduate analytical chemistry and runs workshops for post-graduate researchers in MS methods, metabolomics, chemometrics, and bioinformatics. He is Cell Metabolism section editor for the journal Metabolites and an editorial board member for the journal Scientific Reports.

About the Mass Spectrometry Research Facility (SRF)

The SRF houses over £10 million of analytical instrumentation with 20 mass spectrometer systems, supporting academic research. Its staff provide expert analytical services for the Department, wider University, and collaborators. Research focuses across a very broad range of chemistry-related subjects, from sustainable chemistry and net zero research through to chemical biology, medicinal chemistry, and high throughput screening.

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Additional resources

Ion-exchange chromatography in metabolomics: Exploring analytical performance and metabolome coverage

McCullagh Research Group

Mass Spectrometry Research Facility, Department of Chemistry, University of Oxford

Untargeted metabolomics Workflow

Learn more at **thermofisher.com/icms**