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Investigating anti-tumour effects and associated metabolomic impacts of repurposed therapeutics for glioblastoma

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这篇论文献给我的父母，感恩他们无私的付出与始终如一的支持，是他们的理解与鼓励让我坚定前行。

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Abstract

Isocitrate dehydrogenase wild-type glioblastoma (GBM) is an aggressive brain tumour, requiring effective therapies against its progression. Drug repurposing offers a promising and efficient approach to accelerate drug development of GBM. In previous work, Afuresertib, Taxifolin and Caledonine were repurposed as candidates for GBM treatment.

First, their anti-tumour effects and associated metabolic impacts were investigated using primary GBM cells. Of these, Afuresertib was identified as the greatest potential, exhibiting strong anti-tumour activity *in vitro* and having a clinically favourable safety profile. Metabolomics analysis revealed that Afuresertib and Caledonine could disrupt amino acid metabolism, energy metabolism, and glycerophospholipid metabolism to exert anti-tumour function, while Taxifolin could affect histidine metabolism and nicotinate and nicotinamide metabolism, leading to tumour cell death. Additionally, metabolic heterogeneity was observed between cells derived from the tumour core and their counterparts from the invasive margin, with glycerophospholipid metabolism as a significant differentiating factor.

Next, to investigate the metabolic signatures associated with GBM invasion, single-cell metabolomics was conducted using 3D OrbiSIMS and AP-MALDI-MS. Glycerophospholipid metabolism was identified by both independent methods as a significantly changed pathway between invaded and non-invaded cells.

Finally, a capillary-flow LC-MS-based metabolite profiling method (1 μ L/min) was developed as a complementary approach to study the metabolic mechanisms underlying GBM invasion, capable of profiling metabolites from as few as 5,000 GIN31 cells. Glycerophospholipid metabolism was again identified as significant, further supporting its involvement in GBM invasion. Additional pathways suggested that invaded cells could reprogramme energy metabolism and remodel extracellular matrix, whereas non-invaded cells tend to maintain cellular homeostasis.

Key words: glioblastoma, drug repurposing, metabolomics, invasion, single-cell metabolomics, capillary-flow LC-MS

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May this mark the beginning of a new and hopeful journey.

List of Abbreviations

α KG	α -Ketoglutarate
2-HG	2-Hydroxyglutarate
3D OrbiSIMS	Three dimensional secondary ion mass spectrometry (coupled with Orbitrap analyser)
Acetyl-CoA	Acetyl coenzyme A
ACSS	Acetyl-CoA synthetase
AKT	Protein kinase B
AP-MALDI	Atmospheric pressure matrix-assisted laser desorption/ionisation
ARG1	Arginase 1
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CCS	Collisional cross section
CE	Capillary electrophoresis
CSF	Cerebrospinal fluid
CV	Coefficient of variation
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EIC	Extracted ion chromatogram
ESI	Electrospray ionisation
FC	Fold change
GBM	Glioblastoma
GC	Gas chromatography
GCE	Glioblastoma contrast-enhanced core
GCIB	Gas cluster ion beam
GDH	Glutamate dehydrogenase
GIN	Glioblastoma invasive margin
GLUT	Glucose transporter
GPC	Glycerophosphocholine
GS	Glutamine synthetase
GSC	Glioblastoma stem cell
HILIC	Hydrophilic interaction liquid chromatography
HK	Hexokinase
HPLC	High performance liquid chromatography
IDH	Isocitrate dehydrogenase
IDO	Indoleamine 2,3-dioxygenase
IMS	Ion mobility separation
LDLR	Low-density lipoprotein receptor
LESA	Liquid extraction surface analysis

m/z	Mass-to-charge ratio
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
MSI	Mass spectrometry imaging
MTA	5-Methylthioadenosine
MTAP	5-Methylthioadenosine phosphorylase
mTOR	Rapamycin complex
NAA	N-acetylaspartate
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares discriminant analysis
OXPHOS	Oxidative phosphorylation
PC	Phosphatidylcholine
PCA	Principal component analysis
PDK1	Pyruvate dehydrogenase kinase 1
PE	Phosphatidylethanolamine
PGK1	Phosphoglycerate kinase 1
PI3K/AKT/mTOR	Phosphatidylinositol 3-kinase-serine/threonine kinase-mechanistic target of rapamycin
PKM2	Pyruvate kinase M2
PRMT5	Protein arginine methyltransferase 5
PS	Phosphatidylserine
QC	Quality control
ROS	Reactive oxygen species
RPLC	Reversed phase liquid chromatography
RSD	Relative standard deviation
RTK	Growth factor receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
TCA	Tricarboxylic acid
TDO	Tryptophan 2,3-dioxygenase
TIC	Total ion chromatogram
TISMAN	Transcriptomics-informed Stoichiometric Modelling and Network analysis
ToF	Time of flight
VIP	Variables importance in projection

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Chapter 1

Introduction

Glioblastoma (GBM) is a devastating tumour of the central nervous system, demanding effective therapies to suppress its growth and spread. The incidence of GBM rises with increasing age, with over half of patients being diagnosed after the age of 65¹. GBM occurs more frequently in males than in females, with a male-to-female ratio of 1.6:1 observed in both paediatric and adult populations². GBM predominantly affects non-Hispanic White patients compared to other racial and ethnic groups³. Asian/Pacific Islanders and Hispanic Whites had lower mortality compared to non-Hispanic Whites⁴. However, the underlying reasons for these disparities remain unclear.

Metabolomics has been applied in identifying metabolic weaknesses of GBM through its unique biochemical profile. Targeting these vulnerabilities presents a promising opportunity for therapeutic innovation. In addition, drug repurposing can accelerate the discovery and development of effective therapeutics for GBM, many of which have been evaluated toxicologically for safe human use. This chapter mainly discusses the metabolic adaptations and genetic alterations of GBM, advances in metabolomics and related technologies, drug repurposing strategies for GBM, and the rationale for the proposed research.

1.1 Glioblastoma and advanced therapies

Although patients receive standard-of-care treatments including surgical resection, radiotherapy, and daily temozolomide chemotherapy, the prognosis of GBM remains

dismal with low overall survival (approximately 15 months) and poor quality of life throughout the progression of the disease⁵. Tumour treating fields therapy, which delivers low-intensity and intermediate-frequency electric fields to the tumour, was approved as a non-invasive strategy^{6,7}. Its combination with temozolomide chemotherapy increased 5-year survival to 13%⁸. However, the high expense largely limits its clinical application and the device was not adopted by the UK National Health Service⁹.

Developing effective GBM treatments has numerous challenges, including but not limited to inter- and intra-tumour heterogeneity, highly invasive nature, fortified location of tumour (shielded by the blood brain barrier (BBB)), strong local immunosuppression, and high recurrence driven by acquired drug resistance^{10–12}.

Various strategies have been investigated to overcome these challenges. Direct intra-tumour drug delivery at surgery mainly focuses on localised delivery of drugs to post-surgical residual disease cells and thus bypassing BBB, but these techniques fail to prevent brain damage¹³. Nanomedicine uses nanoparticle-based delivery systems, offering significant promise for GBM treatment¹⁴. However, no nanomedicine-based therapy has been clinically approved to date. Apart from challenges arising from the tumour itself, the effectiveness of nanomedicine-based therapy is also affected by the toxicity, stability, delivery efficiency, biocompatibility, and biodegradability of nano-formulations¹⁵. Additionally, cancer immunotherapy and anti-cancer vaccine are under investigation to either reverse immune suppression or boost the immune responses against GBM, such as nivolumab, ipilimumab, and DCVax[®]-L vaccine^{16,17}. Their clinical application was also limited, primarily due to the complex mechanisms of immune suppression within the GBM microenvironment¹⁸.

Molecularly targeted therapy has attracted significant research interest. It uses small molecules or monoclonal antibodies to inhibit genetic signalling or metabolic pathways involved in tumour progression¹⁹. Hence, a thorough understanding of genetic and metabolic alterations in GBM offers opportunities for innovative treatments.

1.2 Metabolic alterations of glioblastoma

GBM reprogrammes metabolisms to support growth and invasion, eventually giving rise to metabolic heterogeneity²⁰. These aberrant metabolisms present potential vulnerabilities through which pathways can be therapeutically targeted. Current research of GBM metabolism primarily focuses on glycolysis and oxidative phosphorylation (OXPHOS), tricarboxylic acid (TCA) cycle, amino acids metabolism, lipids metabolism, and nucleotides metabolism.

1.2.1 Glycolysis and oxidative phosphorylation

Biologically, glycolysis provides energy to the brain, converting glucose to lactate for the production of adenosine triphosphate (ATP). Due to mitochondrial dysfunction, cancer cells primarily depend on glycolysis to fuel tumourigenic behaviours, despite the presence of oxygen²¹. Higher levels of glycolytic metabolites, including glucose-6-phosphate, were observed in U87 GBM cells compared to healthy counterparts²². Similar results were evidenced in GBM tissues²³. Hexokinase (HK) triggers the first step of glycolysis by converting glucose to glucose-6-phosphate, and its isoform HK2 is found to be overexpressed in GBM²⁴.

To meet the increased energy requirement for survival, the glucose-starvation condition and hypoxia exist in GBM²⁵. Glucose transporter (GLUT) is mainly responsible for transferring glucose to cells²⁶. GLUT1 and GLUT3 are upregulated in GBM relative to normal brain cells, facilitating increased extracellular glucose uptake²⁷. Pyruvate kinase is involved in the final step of glycolysis to produce pyruvate and ATP. Pyruvate kinase M2 (PKM2) can inhibit apoptosis by phosphorylating the B-cell lymphoma 2 protein in mitochondria, promoting GBM progression²⁸. Moreover, hypoxia stimulates glycolysis via translocating phosphoglycerate kinase 1 (PGK1) to the mitochondria, followed by phosphorylation of pyruvate dehydrogenase kinase 1 (PDK1), leading to inhibited pyruvate dehydrogenase activity. Therefore, pyruvate cannot enter the mitochondria for further oxidation, promoting the conversion of pyruvate to lactate²⁹. In addition, macrophages can contribute to the regulation of glycolysis by secreting

interleukin-6 that enhances PGK1 phosphorylation, promoting glycolysis³⁰.

Inhibition of glycolysis suggests therapeutic value. The proliferation and angiogenesis of GBM were impaired when HK was depleted³¹. Due to the expression of HK1 isoform, HK2 inhibition could become ineffective. In addition, HK2 is present in various tissues, including skeletal muscle, heart, and adipose tissue, so its inhibition may raise concerns about potential systemic toxicity³². Furthermore, 2-deoxyglucose, the analogue of glucose, was used to inhibit the glycolytic pathway, but its efficacy and safety in GBM treatment needs further validation³³. Dichloroacetate, the inhibitor of PDK, shifts GBM cells from aerobic glycolysis to OXPHOS, depriving them of the metabolic flexibility needed for rapid proliferation, subsequently triggering tumour cell apoptosis³⁴.

For the mitochondrial GBM subtype, it was observed that they decreased glycolysis and highly relied on OXPHOS³⁵. Lovastatin has been demonstrated to impair mitochondrial respiration in the OXPHOS-dependent mitochondrial GBM subtype³⁶.

1.2.2 Tricarboxylic acid cycle

Alteration of TCA cycle has been discovered in GBM. Isocitrate is catalysed by isocitrate dehydrogenase (IDH) to generate α -ketoglutarate (α KG) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The depletion of NADPH by IDH1 knockdown sensitises GBM to radiation treatment³⁷. Juerchott *et al.* reported increased levels of fumarate and succinate, accompanied by a reduction in citrate levels in GBM²².

Acetyl coenzyme A (Acetyl-CoA) condenses with oxaloacetate to initiate the TCA cycle through the formation of citrate. However, in GBM, less than 50% of the acetyl-CoA pool is derived from glucose³⁸. GBM could alternatively consume other metabolites to support survival, including acetate, fatty acids and ketones. Mashimo *et al.* found that acetate contributes to TCA cycle in GBM, primarily through the action of acetyl-CoA synthetase 2 (ACSS2) that converts acetate to acetyl-CoA in the cytosol³⁹.

The dysregulated TCA cycle becomes a possible target for GBM treatment. IDH

is a vital enzyme, and strategies targeting on it will be discussed in the section 1.3.2.

1.2.3 Amino acids metabolism

Tryptophan metabolism has been proved to drive GBM progression. In addition to serving as a precursor for neurotransmitters, tryptophan is metabolised into kynurenine to produce NAD^+ modulated by two enzymes: indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO)⁴⁰. Further, kynurenine can be converted into quinolinic acid, picolinic acid, and kynurenic acid⁴¹.

Clinically, high IDO expression correlates with reduced survival in GBM patients⁴². Kynurenine alters DNA repair enzymes to permit DNA damage accumulation in gliomas, leading to genomic instability⁴³. Inhibition of IDO or TDO offers a promising strategy for GBM treatment. IDO inhibitors (PF-06840003, Navoximod and IDO-IN-1/2), as well as the TDO inhibitor (680C91), have been evaluated as monotherapies or in combination with other treatments^{44,45}. However, their therapeutic potential remains to be validated in clinical settings.

Glutamine participates in a wide range of metabolic processes. When both glycolysis and OXPHOS are impaired, glutaminolysis becomes the dominant energy-generating pathway to maintain GBM growth⁴⁶. The higher level of glutamine has been discovered in GBM compared to normal brain tissue⁴⁷. In IDH mutant GBM, glutamine can be metabolised into 2-hydroxyglutarate (2-HG) which accumulates at a higher level compared to IDH wild-type GBM and is associated with a relatively better prognosis⁴⁸.

Glutamate dehydrogenase (GDH) can catalyse the conversion of glutamate to αKG , which can then enter the TCA cycle. GDH is upregulated by amplified myelocytomatosis (*MYC*) oncogene to maximise the use of glutamine under glucose deprivation, which promoted the proliferation of SF188 GBM cells⁴⁹. GBM also expresses glutamine synthetase (GS) to either utilise circulating glutamine, or convert glutamate to glutamine when glutamine is depleted^{50,51}. GBM patients with lower GS levels show significantly better survival, suggesting that GS could serve as a reliable

prognostic biomarker although its role in GBM progression remains unclear⁵².

Targeting glutamine metabolism could have therapeutic benefits. Patients who carry the IDH mutation are better candidates for inhibition of glutaminolysis, and this will be discussed in Section 1.3.2. Glutaminase (GLS) converts glutamine to an ammonium ion and glutamate. Inhibition of GLS has been shown to reduce GBM proliferation^{47,53}.

Additional amino acids, such as arginine and aspartate, have also been dysregulated in GBM. Due to the higher abundance of arginine transporters, arginine metabolites are highly accumulated in GBM compared to normal cells²⁶. Physiologically, arginine serves as a substrate for arginase 1 (ARG1). In GBM, arginine depletion using a pegylated recombinant human ARG1 has been demonstrated to induce cytotoxicity⁵⁴. N-acetyl aspartate (NAA) is synthesised in neuronal mitochondria through the acetylation of aspartate by aspartate N-acetyltransferase, serving as a significant reservoir of acetate for lipid synthesis. The level of NAA is markedly lower in GBM than in healthy brain tissue, mainly due to neuronal and axonal loss, as it is primarily produced by healthy neurons⁵⁵.

1.2.4 Lipids metabolism

Lipids biologically contribute to structure integrity, cellular signalling, and energy metabolism. Dysregulated lipid metabolism in GBM has been observed across various lipid classes, including fatty acids, phospholipids, sphingolipids, and cholesterol.

Compared to normal brain tissue, the levels of free fatty acids are higher in GBM⁵⁶. To synthesise fatty acids *de novo*, GBM cells produce cytosolic acetyl-CoA through ATP citrate lyase acting on citrate, or through acetyl-CoA synthetase acting on acetate⁵⁷.

Phospholipid metabolism is markedly altered in GBM, with decreased phosphatidylcholine and increased glycerophosphocholine observed in IDH1-mutated U87 GBM cells⁵⁸. In addition, a high myo-inositol/glycine ratio has been identified in U87 GBM cells⁵⁹. Myo-inositol is involved in glycerophospholipid metabolism, its increased concentration may be explained by self-renewing properties of GBM⁶⁰.

Polymerase I and transcript release factor (PTRF) is a regulator of phospholipid metabolism in GBM. Huang *et al.* demonstrated that upregulated PTRF in GBM promoted cell growth and exosome secretion⁶¹. Yi *et al.* reported that PTRF promotes GBM survival by triggering a phospholipid remodelling pathway mediated by cytoplasmic phospholipase A2⁶².

In sphingolipid metabolism, sphingosine-1-phosphate (S1P) is important in driving GBM progression. The significance of the altered ceramide/S1P balance in GBM, favouring S1P over ceramide, promotes angiogenesis and tumour progression⁶³.

Cells obtain cholesterol through *de novo* biosynthesis or by importing it from their surrounding environment. Cholesterol synthesis via the *de novo* pathway is regulated by the hydroxymethylglutaryl-CoA reductase, which can be inhibited by statin drugs. Atorvastatin effectively suppresses the growth of GBM cells *in vitro* when combined with DNA damaging agents⁶⁴. In GBM, epidermal growth factor receptor (EGFR)-mediated activation of sterol regulatory element binding protein 1 (SREBP1) upregulates the expression of the low-density lipoprotein receptor (LDLR), a crucial regulator in mediating cholesterol uptake⁶⁵.

1.2.5 Nucleotides metabolism

Nucleotides are DNA/RNA structural components, and essential for energy metabolism and enzyme reactions. Cells generate nucleotides either by *de novo* synthesis or by recycling bases through the salvage pathway.

De novo purine synthesis is significantly up-regulated in glioblastoma stem-like cells (GSCs)⁶⁶. GSCs also increase the activity of *de novo* pyrimidine synthesis pathway, supporting their proliferative capacity⁶⁷. The purine salvage pathway enables cells to recycle purines (such as hypoxanthine, adenine, and guanine) from degraded nucleotides. GBM cells can take hypoxanthine from cerebrospinal fluid, allowing them to bypass *de novo* purine synthesis and resist anti-folate therapies⁶⁸.

Preliminary investigations have explored inhibitors of nucleotide metabolism in GBM. Gemcitabine, a cytidine analogue, disrupts DNA synthesis by incorporation into

DNA or by inhibiting ribonucleotide reductase to reduce deoxynucleotide production. Importantly, gemcitabine can cross the BBB and accumulate in GBM^{69,70}.

1.2.6 Other compounds metabolism

5-Methylthioadenosine (MTA) is normally catalysed by 5-methylthioadenosine phosphorylase (MTAP) to produce adenine (for purine metabolism) and 5-methylthioribose-1-phosphate (for methionine metabolism), supporting ATP generation. However, due to the loss of MTAP, it is demonstrated that MTA accumulates in GBM cells compared to normal astrocytes⁷¹. Consequently, GBM cells are more dependent on *de novo* purine biosynthesis for adenine production⁷². Thus, the inhibitors of *de novo* purine biosynthesis (6-mercaptopurine and 6-thioguanine) and methionine starvation (L-alanosine) could be potentially used against GBM⁷³.

Glutathione is a tripeptide composed of glutamate, cysteine and glycine. It has two forms: reduced glutathione (GSH) and oxidised glutathione, enabling glutathione to play a critical role in redox regulation and cellular protection against reactive oxygen species (ROS). Increased GSH levels in GBM contribute to tumour progression by improving cell resistance to ROS⁷⁴. In contrast, some research studies have reported lower levels of GSH in U87 GBM cells compared to normal cells, due to the high oxidative stress environment within tumour, leading to GSH depletion⁵⁸. Thus, the level of GSH relative to healthy tissue is not universally consistent. Moreover, GSH can be easily auto-oxidated during sample preparation, giving rise to false results⁷⁵.

1.3 Genetic alterations in glioblastoma

As downstream effects, metabolic adaptations in GBM are generally shaped by genetic alterations. Although understanding of genetic changes in GBM remains incomplete, there has been increasing interest in growth factor receptor tyrosine kinases (RTKs) and IDH.

1.3.1 Growth factor receptor tyrosine kinases

RTK-regulated growth factor signalling is essential for controlling cellular metabolism, and their alterations contribute to various tumour metabolic adaptations.

EGFR, a typical kind of RTKs, is amplified in GBM, with over 50% of GBM carrying an EGFRvIII mutation⁷⁶. EGFR activation in GBM promotes nucleus translocation of PKM2, which transactivates β -catenin, stimulating c-Myc-dependent glycolysis and cell growth⁷⁷. Activation of mutated EGFRvIII enhances the splicing of *MYC*-associated factor X, a key *MYC* partner protein, leading to increased glycolysis and reduced patient survival⁷⁸. Lipogenesis in GBM is regulated by SREBP1. EGFR enhances N-glycosylation of the SREBP cleavage activating protein, activating SREBP1 and promoting GBM growth^{79,80}.

Amplification of platelet-derived growth factor receptor alpha occurs in around 13% of GBM, with nearly half of these also presenting alterations in EGFR, while mesenchymal–epithelial transition factor amplification and fibroblast growth factor receptor mutations are found in about 2% of GBM⁸¹.

As downstream of RTKs, phosphatidylinositol 3-kinase-serine/threonine kinase-mechanistic target of rapamycin (PI3K/AKT/mTOR) pathway exerts a crucial function in regulating the energy metabolism in GBM, where mTORC1 and mTORC2 are aberrantly activated in GBM. mTORC2 regulates glycolytic metabolism in GBM through phosphorylation of class IIa histone deacetylases, which facilitates c-Myc release⁸².

Amplification of *MYC* and N-myc proto-oncogene (*MYCN*) is detected at high level in GBM. The expression of *MYC*, regulated by the PI3K/AKT/mTOR pathway, promotes glycolysis in EGFR-amplified GBM and upregulates the level of glutamine⁴⁹. Inhibition of glycolysis effectively eliminates *MYC/MYCN*-amplified GBM tumour spheres and prolongs the survival of mice carrying *MYCN*-amplified patient-derived xenografts²⁹.

EGFR, and particularly EGFRvIII, has therefore become a therapeutic target in GBM. However, EGFR inhibitors such as erlotinib, afatinib and ABT-414, showed

limited efficacy against EGFRvIII GBM in clinical trials^{83,84}. Rindopepimut, an EGFRvIII vaccine, showed preclinical and clinical promise, but did not improve overall survival in a phase III trial for EGFRvIII GBM⁸⁵. Rather than directly inhibiting RTKs, an alternative strategy is to disrupt their downstream effectors, especially the PI3K/AKT/mTOR signalling pathway. Drug repurposing efforts have introduced several potential candidates for this pathway in GBM treatment, which will be discussed in Section 1.5.1.

1.3.2 Isocitrate dehydrogenase

The distinction between IDH wild-type (IDHwt) and IDH mutant (IDHmut) GBM helps tailor therapeutic strategies. Biologically, IDH1 and IDH2 catalyse the interconversion of isocitrate and α KG in cytosol and mitochondria respectively, and they are essential for multiple cellular processes, including glucose metabolism, lipogenesis, and protection against ROS and radiation-induced damage^{86,87}.

In IDHwt GBM, the overexpressed IDHwt promotes tumour progression and therapy resistance. IDH1 supports antioxidant levels and lipid synthesis in IDHwt GBM, and its inhibition increases GBM cells sensitivity to therapy⁸⁸. Silencing IDH1 in IDHwt GBM cells depletes NADPH, deoxynucleotides, and antioxidants, weakening tumour's capacity against oxidative stress and enhancing sensitivity to radiotherapy³⁷. Collectively, these findings highlight the potential of targeting IDH1 as a promising therapeutic strategy for IDHwt GBM.

In IDH1mut GBM, α KG can be subsequently converted into 2-HG⁸⁹. 2-HG inhibits α KG-dependent histone demethylases, causing DNA and histone hypermethylation, followed by overexpression of GLUT1 and PDK1, enhancing GBM progression^{90,91}. Seltzer *et al.* discovered that IDH1mut GBM shifts toward glutamine-dependent energy pathways, especially under glucose depletion⁹². The accumulation of 2-HG suppresses the activity of branched-chain amino transaminase, forcing IDH1mut GBM cells to rely on glutaminase-driven glutaminolysis for glutamate biosynthesis. This dependency makes IDH1mut GBM cells more vulnerable to oxidative

stress compared to their wild-type counterparts⁹³. These findings suggest that inhibition of glutaminolysis may be a therapeutic strategy for IDHmut GBM.

Several small-molecule inhibitors of IDH have demonstrated preclinical activity, including AGI-6780, BAY-1436032, and G1-6780. Among them, AGI-5198, a selective IDH1 inhibitor, specifically impairs the growth of R132H-IDH1mut GBM, with no effect on IDHwt cells⁹⁴. IDH1 peptide vaccine (specifically designed for IDH1mut GBM) exhibited preclinical efficacy and is undergoing clinical investigation⁸³.

1.3.3 Additional genomic alterations

Numerous studies have identified additional genetic alterations in GBM.

Tumour protein 53 gene (*TP53*), one of the tumour suppressor genes, is mutated in approximately 30% of primary GBM and 65% of secondary GBM. In cancer, *TP53* mutations result in the complete inactivation of the p53 protein, promoting increased metastasis and enhanced glycolysis⁹⁵. However, its role in the progression of GBM requires further investigation.

MTAP is one of the frequently deleted genes in GBM, with its loss potentially caused by methylation of the *MTAP* promoter. As discussed in Section 1.2.6, purine deprivation therapy could be a promising strategy for treating *MTAP*-deleted GBM. Besides, *MTAP* deletion make cells more vulnerable to further inhibition of the protein arginine methyltransferase 5 (PRMT5). PRMT5 inhibitors, such as AMG 193 and MRTX1719, selectively block tumour cell growth, making PRMT5 inhibition a potential therapy for *MTAP*-deleted tumours^{73,96,97}. However, this idea should be further evaluated on GBM.

1.4 Metabolomics with glioblastoma

Metabolic and genomic alterations show that these changes are not simply byproducts of tumour progression but essential drivers of its aggressiveness. To dive into deeper understanding of GBM metabolism, metabolomics is a powerful tool to

draw the metabolic landscape and illustrate the mechanism of GBM response to various perturbations, such as genetic regulation, environmental stimulation, and drug treatment. In this section, advanced technologies in metabolomics are mainly discussed.

1.4.1 Metabolomics

Metabolomics is an analytical technique used to identify and quantify metabolites within cells, tissues, or biofluids⁹⁸. It bridges the gap between genotype and phenotype, linking genetic alterations, transcriptional changes, and protein activities to observed biological results⁹⁹.

Generally, approaches for analysing metabolites can be divided into four types: targeted metabolomics, untargeted metabolomics, stable isotope-resolved metabolomics (SIRM), and imaging metabolomics. Targeted metabolomics focuses on a set of metabolites that are biochemically defined and annotated¹⁰⁰. Untargeted metabolomics provides a global overview by profiling a variety of metabolites without pre-defined targets, allowing unbiased analysis of all detectable metabolites, particularly beneficial for identifying unexpected changes in metabolite concentrations and types, potentially leading to the generation of a new hypothesis¹⁰¹. SIRM can dynamically trace isotopically labelled metabolites involved in metabolic fluxes¹⁰². Imaging metabolomics investigates the spatial distribution of metabolites within samples, uncovering region-specific metabolic alterations, even at subregional, cellular, and subcellular levels¹⁰³.

1.4.2 Mass spectrometry and metabolomics

Analytical platforms commonly used in GBM metabolomics studies include magnetic resonance spectroscopy (MRS), nuclear magnetic resonance (NMR), and mass spectrometry (MS). MRS uses signals from hydrogen atoms to determine the relative concentrations of target metabolites. Its limited metabolite coverage hinders global metabolite profiling¹⁰⁴. NMR has the ability to identify the unique composition of metabolites by resolving carbon and hydrogen frameworks, but it has lower sensitivity

than MS¹⁰⁵.

MS has been widely applied in the molecular characterisation of GBM, detecting and identifying metabolites based on their exact mass and structural fragmentation¹⁰⁶. The critical step in MS analysis is to convert neutral molecules into charged ions. Once ionised, these molecules are separated and detected according to their mass-to-charge ratio (m/z)¹⁰⁷. MS offers remarkable sensitivity, speed, and accuracy, enabling the analysis of a broad range of metabolites from complex biological matrices.

1.4.3 LC-MS-based metabolite profiling

However, MS suffers from the presence of isobaric compounds and matrix effects (ion suppression/enhancement). To address these challenges, it is important to integrate multiple separation procedures prior to MS analysis, such as capillary electrophoresis (CE), gas chromatography (GC), and liquid chromatography (LC).

Although CE-MS offers high separation efficiency, its limited automation and stability issues (due to the need for specialised interfaces) challenge its routine application¹⁰⁸. GC-MS can analyse volatile and thermally stable metabolites, often requiring derivatisation steps, leading to complexed sample preparation and potential metabolite loss¹⁰⁹. Generally, LC-MS can handle diverse chemical properties without derivatisation and is highly compatible with automated high-throughput workflows, making it widely accepted in GBM metabolomics research.

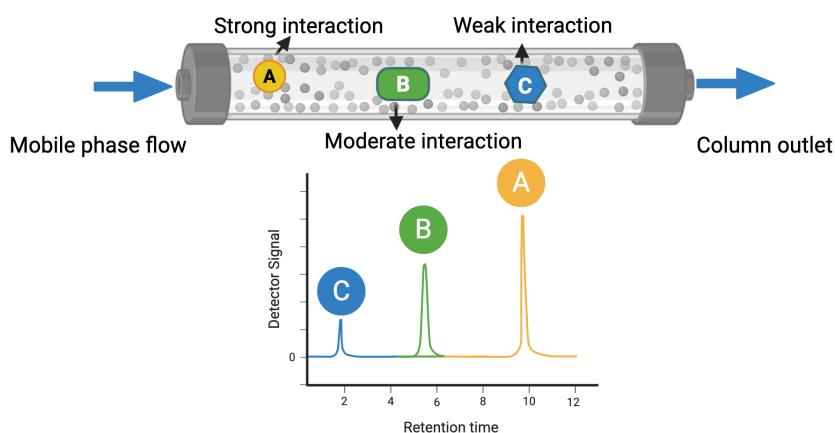


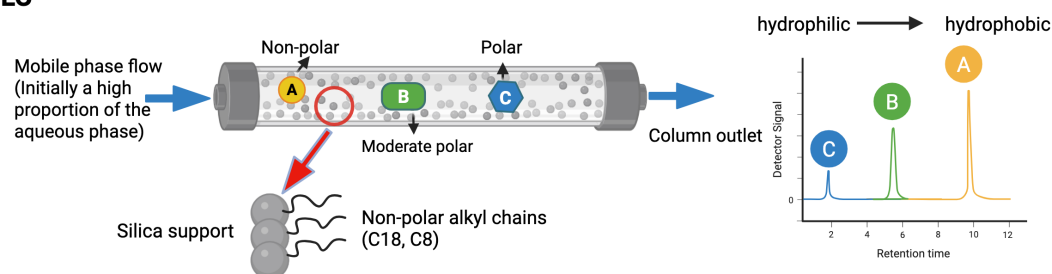
Figure 1.1. Analyte retention and separation in LC. The figure was created with BioRender.com.

As shown in Figure 1.1, analytes are retained within an LC column through interactions with the stationary phase while being transported by the mobile phase. As the mobile phase flows through the column, the analytes undergo repeated and reversible interactions with the stationary phase, resulting in different migration velocities along the column¹¹⁰. Separation occurs because analytes differ in their affinity for the stationary and mobile phases, a behaviour that can be described by a partitioning equilibrium governed by their physicochemical properties, such as polarity, charge, and functional groups¹¹¹. The separated analytes can subsequently be detected by MS.

1.4.3.1 Improved LC technologies

Improved LC technologies aim to enhance sample separation, increase sample throughput and metabolite coverage. The instrument has been updated from conventional high performance liquid chromatography (HPLC) to ultra-HPLC system (up to 1,500 bar), resulting in increased efficiency¹¹².

RPLC



HILIC

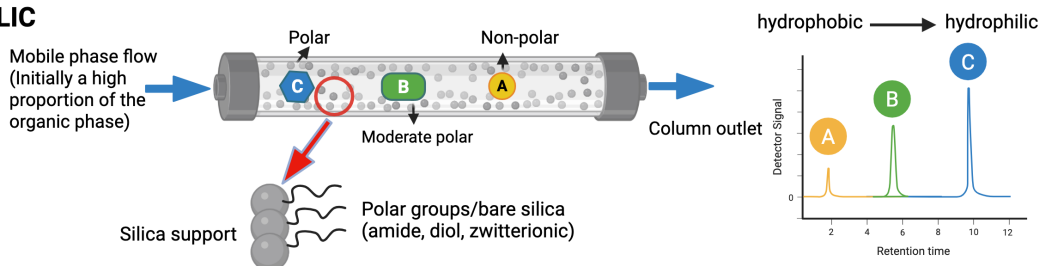


Figure 1.2. Schematic illustration of RPLC and HILIC. The figure was created with BioRender.com.

In addition to instrumental advancement, the chromatographic separation strategies have already evolved. In GBM metabolomics studies, reversed phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC)

are the most widely used separation techniques (Figure 1.2).

RPLC employs a non-polar stationary phase, typically composed of octadecyl (C₁₈) alkyl chains bonded to a silica support. Retention is driven by hydrophobic partitioning of analytes between the mobile phase and the stationary phase¹¹³. Generally, for gradient elution, the mobile phase initially contains a high proportion of the aqueous component. The elution sequence proceeds from the most hydrophilic to the most hydrophobic compounds¹¹¹. C₁₈ RPLC is highly effective in the separation of lipids, steroids and fatty acids. However, many metabolites are water soluble and polar, such as amino acids, organic acids and sugars, which are often poorly retained on C₁₈ columns¹¹⁴.

In such cases, HILIC offers improved retention and separation of polar metabolites. Unlike RPLC, HILIC employs a polar stationary phase, and retention is governed by mixed-mode mechanisms, including hydrophilic partitioning, hydrogen bonding, and electrostatic interactions¹¹⁵. Volatile buffers (2-20 mM ammonium acetate or ammonium formate) are commonly added to control pH, improve peak shape, and ensure MS compatibility. HILIC gradients begin with a high proportion of organic solvent (70-95%), typically acetonitrile. The elution sequence goes from the least to the most hydrophilic compounds¹¹⁶.

Several types of HILIC columns are commonly used in metabolomics. Bare silica columns provide strong hydrogen-bonding interactions, while amide-based columns offer improved stability and reproducibility¹¹⁷. Zwitterionic sulfoalkylbetaine-based columns (ZIC-HILIC) are another widely adopted option. In ZIC-HILIC columns, sulfoalkylbetaine groups are bonded to silica and strongly adsorb water, forming a water-enriched stationary phase that governs analyte retention¹¹⁸.

Šofranko *et al.* detected over 150 polar metabolites simultaneously by the developed HILIC method¹¹⁹. HILIC has been reported to suffer from poor reproducibility and retention-time drift in long batch analyses. Pičmanová *et al.* developed a HILIC method for untargeted metabolomics, achieving the relative standard deviations (RSD) of retention time below 1.8% and RSD of intensity within

20% over 200 injections, indicating acceptable reproducibility¹²⁰. Hence, given its effective retention of polar compounds and reliable reproducibility, HILIC serves as a complementary approach to RPLC in GBM metabolomics. Apparently, a single analytical method can not detect all metabolites in a biological system. Integrating HILIC and RPLC enhances metabolite coverage. Lee *et al.* utilised HILIC and RPLC in parallel to allow the detection of over 200 metabolites¹²¹.

To address applications requiring high sensitivity for the detection of limited biomass or low abundance analyte, low-flow LC has attracted great attention. Luo *et al.* developed a nano-flow method with the capacity of profiling metabolites down to 100 human breast cancer cells¹²². Low-flow LC remains relatively new to GBM metabolomics studies. Deng *et al.* reported a nano-LC-MS protocol for profiling metabolites in IDHmut human glioma cells¹²³.

As summarised in Table 1.1, the internal diameter of the column and the operating flow rate influence chromatographic efficiency and detection sensitivity¹²⁴. Reducing the column internal diameter and operating at lower mobile-phase flow rates can theoretically improve chromatographic sensitivity by minimising on-column dilution and band broadening, resulting in higher concentrations and increased peak heights for a injected amount¹²⁵. These advantages are particularly pronounced when coupled with advanced ionisation techniques, will be discussed in Section 1.4.3.3. However, columns with smaller internal diameters have reduced sample loading capacity, increasing the risk of column overloading and peak distortion. In addition, operation at very low flow rates requires precise flow control and careful system optimisation to ensure robust and reproducible performance¹²⁶.

Table 1.1. The definitions for HPLC techniques.

Flow mode	Typical column I.D.	Flow rate	Key features
Analytical	2.1-4.6 mm	0.1-2.0 mL/min	High robustness, high loading capacity
Micro	0.5-1.5 mm	10-100 μ L/min	Balance of sensitivity and robustness
Capillary	150-500 μ m	1-10 μ L/min	Reduced solvent use, improved MS sensitivity
Nano	10-150 μ m	10-1000 nL/min	Highest sensitivity, low loading capacity, lower robustness

“I.D.” refers to internal diameter. The information in the table is adapted from Reference 124.

1.4.3.2 Advanced ionisation sources and mass analysers

Followed by LC separation, metabolites undergo the ionisation process before being introduced into analyser. There are various types of ionisation sources, such as atmospheric pressure chemical ionisation, atmospheric pressure photoionisation, and electrospray ionisation (ESI). ESI is preferred in untargeted metabolomics of GBM because it is a soft ionisation technique, usually generating molecular ions with minimal fragmentation, thus preserving the structure information of metabolites¹²⁷.

In the ESI process, the analyte solution passes through a high-voltage capillary, generating charged droplets at the emitter tip. As these droplets travel through the atmospheric-pressure region, solvent evaporation increases their surface charge density until Coulombic repulsion triggers droplet fission. Through repeated cycles of evaporation and fission, gas-phase ions are ultimately generated and transferred into the mass analyser for detection¹²⁸.

The design of ESI can be varied from different manufacturers. In our laboratory, the Thermo Fisher Scientific Q-Exactive Plus hybrid mass spectrometer has been launched, equipped with a heated ESI (HESI). Compared to an unheated ESI, instead of using ambient gas, the HESI heats auxiliary gas where heated nitrogen improves the nebulisation process and promotes ion release from droplets, thereby increasing the ionisation efficiency. Lu *et al.* reported over 5-fold signal increases for 20 metabolite standards using HESI compared to unheated ESI¹²⁹.

When it comes to the mass analyser, there are various options from low resolving power (quadrupoles and ion trap) to high resolving power (time-of-flight (ToF) and Orbitrap). In untargeted metabolomics research, high-resolution mass spectrometry has been employed to enable accurate and global metabolite profiling. The ToF analyser measures the time that ions fly through a fixed distance and the flight time is proportional to the square root of their m/z ratio. It typically achieves a resolving power of around 40,000-60,000 with mass accuracy in the range of 5-10 ppm¹³⁰. In an Orbitrap mass analyser, ions are injected into an electrostatic field formed between a central spindle-shaped electrode and a coaxial outer electrode. The ions orbit the central

electrode while simultaneously oscillating along the axial direction. The frequency of this axial oscillation depends only on the m/z ratio of the ions and is detected as an image current on the outer electrodes. This signal is converted into a mass spectrum using Fourier transformation¹³¹. Compared with ToF, Orbitrap offers superior mass resolution (above 140,000 at m/z 200) and mass accuracy (1-5 ppm)¹³². Additionally, the integration of different/same types of analyser to form a tandem or hybrid mass spectrometer, such as triple quadrupoles, quadrupole-ToF, and quadrupole-Orbitrap, enables robust structural elucidation and precise targeted quantification.

1.4.3.3 Other novel techniques

Other novel techniques could be used to assist in metabolite identification and increase the sensitivity of the method, such as ion mobility separation (IMS) and low-flow ESI.

In addition to m/z value, IMS separates ions also based on shape and size, providing a unique parameter termed collisional cross section (CCS)¹³³. Integrating IMS with LC-MS helps distinguish isomeric metabolites through structural selectivity¹³⁴. Pičmanová *et al.* used CCS values to separate the co-eluting sucrose and maltose as distinct peaks in 2D IMS-MS spectra¹²⁰. Zhang *et al.* discovered a new dopamine isomer from the Parkinson's disease metabolome by IMS-MS¹³⁵.

As discussed in Section 1.4.3.1, downscaling the LC system offers improved sensitivity, but requires a compatible and stable ionisation at low-flow rate¹³⁶. Compared to conventional ESI, low-flow ESI generates smaller droplets with a higher surface-to-volume ratio, resulting in faster desolvation and the formation of more stable and focused ion stream, leading to increased sensitivity^{137,138}.

1.4.4 Imaging metabolomics

In addition to LC-MS-based metabolite profiling, imaging metabolomics by the application of surface analysis techniques, such as liquid extraction surface analysis (LESA), matrix-assisted laser desorption/ionisation (MALDI), and secondary ion mass

spectrometry (SIMS), has been employed in the GBM studies, particularly for mass spectrometry imaging (MSI).

1.4.4.1 Liquid extraction surface analysis

LESA, coupled with chip-based nano-ESI, enables the extraction of metabolites from sample surfaces and direct infusion to MS. After being analysed by MSI, samples can be further analysed by LESA-MS/MS for structure confirmation. Randall *et al.* used LESA-MS/MS to confirm long-chain acylcarnitines (myristoyl-l-carnitine, palmitoyl-l-carnitine, and stearoyl-l-carnitine) in patient-derived xenograft models of GBM after MALDI-MSI¹³⁹. He *et al.* used LESA-MS/MS to assist in the identification of 14 key metabolites discovered in GBM tissue after 3D OrbiSIMS analysis¹⁴⁰. Nevertheless, the spatial resolution of LESA is relatively low (around 1 mm), hindering its application in high-resolution MSI¹⁴¹.

1.4.4.2 Matrix-assisted laser desorption/ionisation

MALDI involves the co-crystallisation of the analyte with a suitable matrix, which absorbs laser energy and facilitates the desorption and ionisation of the analyte molecules. MALDI is also a soft ionisation technique, preserving molecular integrity through minimal fragmentation¹⁴². Compared with traditional MALDI, which requires samples to be placed in vacuum condition, atmospheric pressure-MALDI (AP-MALDI) allows ions to be generated at atmospheric pressure, increasing the ease of sample preparation. In Keller *et al.* study, the vacuum MALDI system detected the more number of features compared to the AP-MALDI system, while the AP-MALDI system offered faster acquisition speed¹⁴³.

MALDI-MSI has been applied for studying spatially-resolved metabolic profiles of GBM. Kampa *et al.* used MALDI-MSI to compare the metabolic profile between normal tissue and different subtype GBM. Antioxidants, such as taurine and vitamin C, showed decreasing levels with increasing distance from the tumour centre¹⁴⁴. Randall *et al.* identified elevated long-chain acylcarnitines in GBM edges using MALDI-MSI,

highlighting metabolic heterogeneity between tumour edge and core¹³⁹.

Advancements in MALDI system have greatly enhanced its spatial resolution (5-20 μm), enabling metabolite detection even at the single-cell level¹⁴⁵. Rappez *et al.* applied MALDI-MSI-based single-cell metabolomics to investigate metabolic differences between co-cultured HeLa and fibroblast cells¹⁴⁶. Buglakova *et al.* found that glioma-bearing mouse brains showed higher glucose-dependent synthesis of saturated fatty acids compared to healthy brains, by MALDI-MSI-based single-cell isotope tracing (spatial resolution 10 μm)¹⁴⁷. Zhang *et al.* captured the differential abundance of fatty acids and phospholipids between tumour and fibroblast cells at the single-cell level by MALDI-MSI (spatial resolution 20 μm)¹⁴⁸.

1.4.4.3 Secondary ion mass spectrometry

SIMS hits sample surface with a high-energy primary ion beam (such as Ar^+ , Ga^+ , and In^+), followed by analysis of ejected secondary ions. The primary advantage of SIMS is the high spatial resolution ($<1\text{ }\mu\text{m}$), while a main disadvantage is extensive fragmentation of molecules¹⁴¹.

The 3D OrbiSIMS combines SIMS with the high-resolution Orbitrap analyser, and incorporates a gas cluster ion beam, generating less fragmentation and improving the ability to profile metabolites and drugs in biological samples¹⁴⁹. He *et al.* applied 3D OrbiSIMS to investigate spatial distributions of metabolites within GBM tissues, highlighting intra-tumour heterogeneity¹⁴⁰. McCrorie *et al.* utilised 3D OrbiSIMS to detect chemotherapeutics (etoposide, olaparib, gemcitabine, vorinostat, and dasatinib) within brain tissue, assessing drug delivery systems for neurooncological applications¹⁵⁰.

Owing to its high spatial resolution, 3D OrbiSIMS also enables metabolite profiling at the single-cell level. Passarelli *et al.* developed a label-free 3D OrbiSIMS method to profile metabolites at subcellular lateral resolution ($<2\text{ }\mu\text{m}$) and applied it to conduct single-cell metabolomics on rat macrophages incubated with amiodarone¹⁴⁹. Suvannapruk *et al.* studied single-cell metabolic differences among

human monocyte-derived macrophages differentiated into naïve (M0), pro-inflammatory (M1), and anti-inflammatory (M2) phenotypes using 3D OrbiSIMS¹⁵¹.

1.5 Drug repurposing in glioblastoma

Achievements in GBM metabolism studies offer hope for new therapeutic approaches. Given the repeated failures to introduce new treatments for GBM, drug repurposing offers an alternative strategy, taking advantage of drugs with well-established pharmacokinetics, pharmacodynamics and safety profiles. Approaches for identifying repurposed candidates include computational bioinformatics, large-scale screening, and candidate-based approaches^{152,153}. However, repurposing drugs for GBM faces significant challenges, such as BBB penetration, limited drug distribution within tumours, the presence of resistance and recurrence, potential adverse effects, drug-drug interactions when combined with other medications, drug availability, and intellectual property conflicts^{154–156}. Furthermore, the heterogeneity of GBM complicates drug repurposing, emphasising the need to evaluate repurposed drugs across various GBM subtypes to broaden their clinical application¹⁵⁷.

RTKs/PI3K/AKT/mTOR signalling pathway has been a hot target for drug repurposing since it is one of frequently altered pathways in GBM. This section primarily summarises current repurposed drugs targeting this pathway.

1.5.1 Repurposed drugs targeting on RTKs/PI3K/AKT/mTOR pathway

In GBM, several repurposed drugs have shown potential in targeting RTKs, though they present poor clinical efficacy. Imatinib and dasatinib, originally developed for chronic myeloid leukemia, effectively reduce GBM proliferation by targeting the overexpressed platelet-derived growth factor receptors^{158,159}. Vandetanib, an EGFR inhibitor, shows strong effects in high-risk medulloblastoma when combined with PI3K inhibition, but has shown limited efficacy in GBM clinical trials^{160,161}.

As the downstream of RTKs, PI3K inhibitors have been proposed for GBM treatment. As PI3K inhibitor, Wortmannin (anti-fungal agent) and derivatives, like PX-866, have been evaluated in GBM. Wortmannin has been found to sensitise GBM cells to radiation¹⁶². Combined with gamitrinib, inhibition of PI3K with PX-886 can inhibit the growth of GBM¹⁶³. Voxtalisisib, dual PI3K/mTOR inhibitor, has been reported to be effective in GBM patients when combined with bevacizumab¹⁶⁴.

Inhibitors of AKT have been actively studied. Metformin, anti-diabetic drug, disrupts GBM metabolism and survival by suppressing the activity of AKT¹⁶⁵. Perifosfamine inhibits the phosphorylation and membrane localisation of AKT, leading to impaired GBM cell survival and enhanced efficacy in radio/chemotherapy¹⁶⁶.

Inhibitors of mTOR have shown potent anti-GBM function, but their efficacy must be evaluated in clinical trials. Chloroquine (anti-malaria) and sirolimus (immunosuppressive agent) have undergone several clinical research, presenting more therapeutic effects in GBM when combined with other anti-tumour agents^{167,168}. Temsirolimus combined with peritonise (AKT inhibitor) in a Phase I study, were found to be tolerable in heavily pretreated adults with recurrent malignant gliomas, including GBM¹⁶⁹. Another mTOR inhibitor, AZD2014 was found effectively depleted myeloid cell leukemia-1 (anti-apoptotic protein) in GBM cells. Combination therapy using AZD2014 with olaparib inhibited the growth of GBM xenografts¹⁷⁰.

1.5.2 Other repurposed drugs

In addition to repurposed drugs targeting on RTKs/PI3K/AKT/mTOR, multiple drugs have been tested on GBM. However, none of these agents has been translated into clinical studies.

The high permeability of antidepressants, antipsychotics, and neurocognitive agents across the BBB has led to their investigation as therapeutic candidates for GBM, such as fluphenazine, fluspirilene, and pimavanserin^{171,172}. Fluvoxamine, an antidepressant, inhibited the invasion of GBM cells¹⁷³.

IDH presents a potential target for drug repurposing in GBM. Clomiphen (a

fertility treatment) was found to interfere with mutant IDH1 to inhibit tumour growth by preventing the conversion of α -KG into 2-HG, thus disrupting the tumour-promoting effects of 2-HG¹⁷⁴. This must be validated in mutant IDH1 GBM.

GSCs exhibit a strong dependence on mitochondrial function, and mitochondria-targeted drugs, such as pyruvium pamoate and trifluoperazine, have demonstrated greater GSC-selective anti-proliferative activity than temozolomide *in vitro*, suggesting their potential as adjuvant therapies for treating temozolomide-resistant GBM¹⁷⁵.

Network analysis-based approaches facilitate drug repurposing in GBM. In our previous work, Transcriptomics-informed Stoichiometric Modelling and Network analysis (TISMAN) was developed, which integrates GBM transcriptomic data into a genome-wide metabolic model to identify key metabolic vulnerabilities. By searching drug–gene interaction databases, five candidates (Afuresertib, Taxifolin, Pyrogallol, Isorhamnetin, and Formononetin) were repurposed for GBM treatment¹⁷⁶. However, their efficacy and mechanism action require further investigation.

1.6 Rationale and objectives of the project

1.6.1 Anti-tumour and metabolomics studies of repurposed therapeutics for glioblastoma

As discussed above, the PI3K/AKT/mTOR pathway plays a critical role in the metabolic regulation of GBM progression. It is feasible to target this signalling axis for drug repurposing. Based on the results of TISMAN, among five candidates, Afuresertib and Taxifolin have been demonstrated to target the PI3K/AKT/mTOR pathway. Afuresertib, an AKT inhibitor, has been studied in other types of cancer (ovarian cancer, leukaemia, and gastric cancer) with strong anti-proliferative activities, but not yet been tested in GBM^{177–179}. Taxifolin has been reported to inhibit the activity of EGFR and PI3K in skin cancer, and dually inhibit PI3K and mTOR in GBM cells^{180,181}. Considering their reported anti-tumour effects, we hypothesise that

Afuresertib and Taxifolin would exhibit anti-GBM activity.

Additionally, Caledonine, provided by our collaborative team at the University of Nottingham, exhibited strong inhibition on brain tumour cell proliferation compared to other cancer types^{182,183}. While its biological target and the underlying mechanism remain unknown, Caledonine was included to explore its anti-tumour effects on GBM.

Cellular metabolism is a direct readout of functional changes within cells. LC-MS-based metabolite profiling is well-established in our laboratory, enabling the investigation of metabolic alterations of GBM in response to drug treatments.

This project aims to investigate the anti-tumour effects of three candidates on patient-derived GBM cells, and to uncover metabolic pathways associated with their anti-GBM function by the application of LC-MS-based metabolite profiling.

1.6.2 Metabolic signatures associated with glioblastoma invasion

The highly invasive nature of GBM complicates treatments. Understanding the metabolic mechanisms driving GBM invasion could aid in the development of effective therapeutics.

Although less physiologically relevant than 3D or *in vivo* models, the Transwell collagen barrier assay is a first-line tool for studying cell invasion, allowing analysis of the response of individual cells to controlled conditions. However, given the smaller number of cells from the invasion assay (approximately 1×10^4 cells), highly sensitive methods are required to capture metabolic changes within these samples.

As discussed in previous sections, the advances of low-flow LC-MS are beneficial for profiling metabolites in limited biomasses, showing the potential for analysing invasion samples. Furthermore, the high spatial resolution of AP-MALDI-MS and 3D OrbiSIMS, along with their applications in single-cell analysis, demonstrate the potential for detecting metabolites from invasion samples.

This project further aims to investigate metabolic signatures associated with GBM invasion using low-flow LC-MS, AP-MALDI-MS, and 3D OrbiSIMS. The metabolic pathways related to GBM invasion are expected to be characterised.

Chapter 2

Anti-tumour effects and associated metabolomic impacts of repurposed therapeutics for glioblastoma treatment

(The results related to Afuresertib and Taxifolin have been published in *Analyst*, <https://doi.org/10.1039/D5AN00461F>)

2.1 Introduction

Isocitrate dehydrogenase (IDH) wild-type glioblastoma (GBM) is a devastating central nervous system tumour¹⁸⁴. Substantial inter- and intra-tumour heterogeneity and the highly invasive nature are two crucial reasons for the lack of effective therapies for GBM¹¹. Both GBM growth and invasion involve complex interactions with their metabolic environment^{58,185}; however, this is not a well-understood area. To investigate metabolic alterations in GBM, metabolomics is capable of capturing the tumour's metabolic landscape and discovering metabolic vulnerabilities^{186,187}.

To translate these vulnerabilities into therapeutic advances, drug repurposing is an increasingly attractive proposition since it employs de-risked compounds (often with established patient safety profiles determined for certain indications), providing multiple advantages over developing an entirely new drug^{152,188}. Computational approaches

have primarily been employed in drug repurposing to gain meaningful interpretations for hypotheses generation¹⁸⁹. In the previous study, Transcriptomics-informed Stoichiometric Modelling and Network Analysis (TISMAN) was developed to repurpose compounds for GBM treatment. By constructing a genome-wide metabolic model of GBM using published transcriptomics datasets, TISMAN identified metabolic reactions of interest prior to screening a chemical-gene interaction database. As a result, Afuresertib, Taxifolin, Pyrogallol, Isorhamnetin, and Formononetin were prioritised¹⁷⁶.

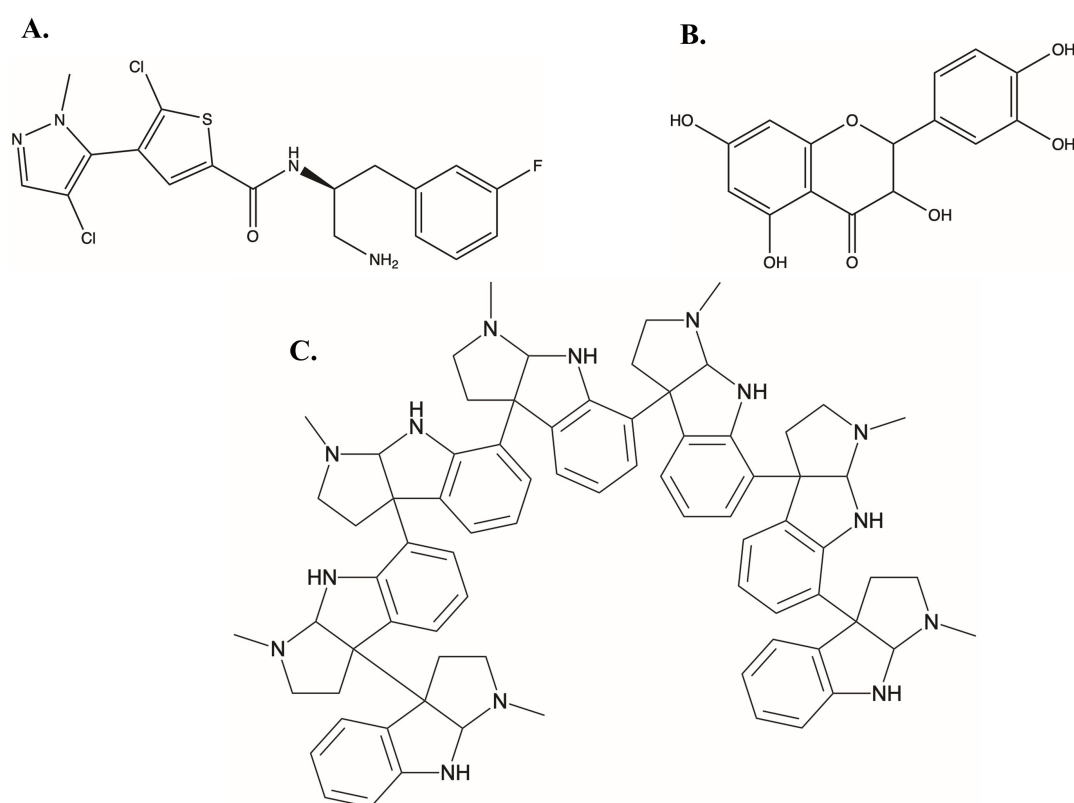


Figure 2.1. The chemical structure of Afuresertib (A), Taxifolin (B) and Caledonine (C).

The phosphatidylinositol 3-kinase-serine/threonine kinase-mechanistic target of rapamycin (PI3K/AKT/mTOR) pathway has been studied as one of the most frequently altered signalling cascades in GBM and related to metabolic regulation of GBM progression, thus representing a potential target for drug development and repurposing^{190,191}. Among the five candidates, Afuresertib and Taxifolin (Figure 2.1 A and B) have been reported to target on this pathway. Afuresertib, an AKT inhibitor, has shown potent anti-tumour effects across various cancer types, including multiple

myeloma, leukaemia, oesophageal cancer and ovarian cancer^{177–179}, but has yet to be evaluated on GBM. Taxifolin is a flavonoid, mainly exhibiting antioxidant and anti-inflammatory roles^{192,193}. Yao *et al.* reported that Taxifolin could dually target PI3K and mTOR to inhibit GBM growth¹⁸¹. However, it should be noted that commercial cell lines were used rather than primary patient-derived cells, and the ability to assess anti-invasion was not taken into consideration. Hence, we hypothesised that Afuresertib and Taxifolin could alter the metabolic regulation in GBM associated with their anti-tumour function.

In addition, Caledonine (Figure 2.1 C) is isolated from *Rubiaceae* root extraction by the collaborative team at the University of Nottingham^{182,183}. In their preliminary studies, Caledonine strongly inhibited brain tumour cell proliferation (U373V and U373M) than other types of cancer. Therefore, Caledonine was included to evaluate its anti-tumour effects on primary GBM cells.

As illustrated in Figure 2.2, the anti-tumour effects of Afuresertib, Taxifolin and Caledonine have been studied on patient-derived GBM cells and the drug mode of action has been inferred from a metabolomic perspective using liquid chromatography-mass spectrometry (LC-MS)-based metabolite profiling.

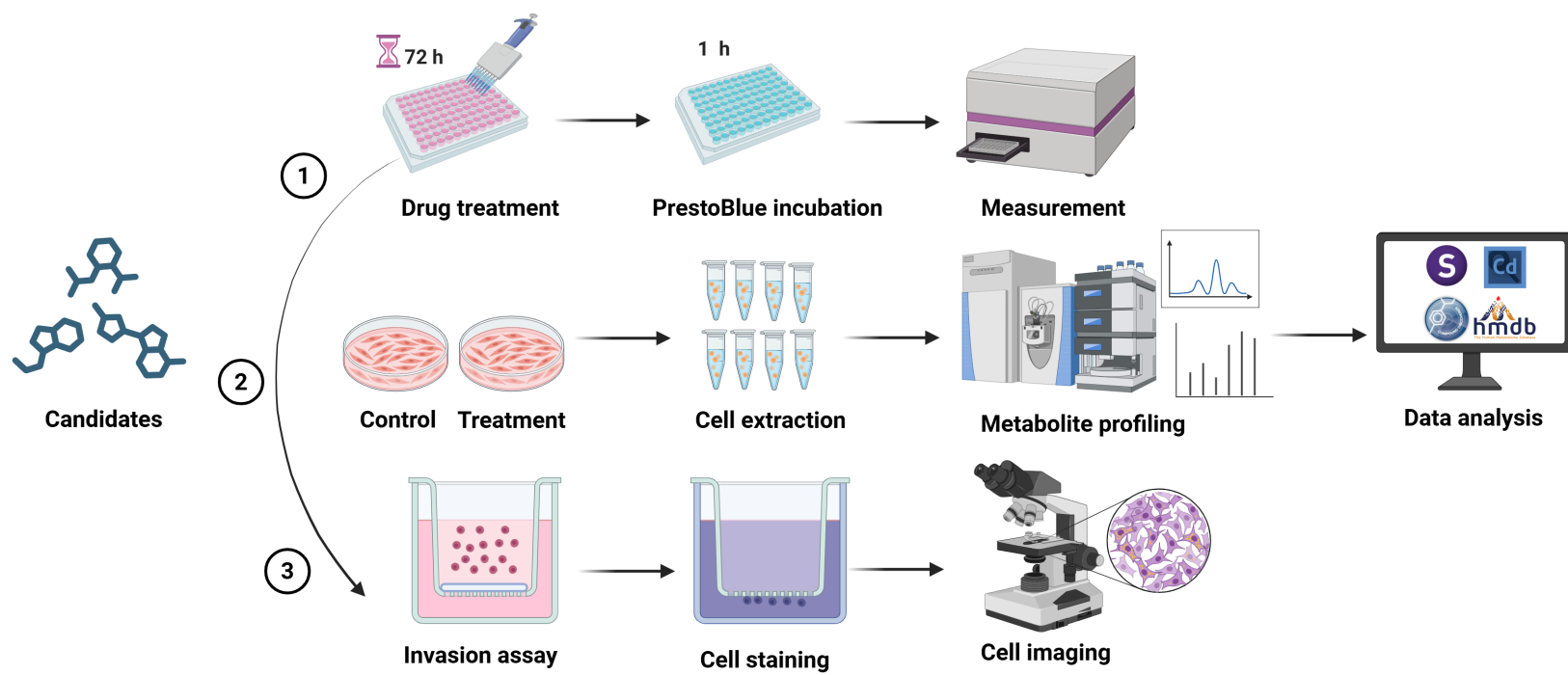


Figure 2.2. Anti-tumour and metabolomics studies of repurposed candidates. The figure was created with BioRender.com.

2.2 Materials and methods

2.2.1 Materials

Afuresertib (Lot No.: 01, purity: 99.26%) was purchased from Selleck Chemicals LLC (Berlin, Germany). Taxifolin (Lot No.: 23044, purity: 99.97%) was ordered from MedChemExpress LLC (Monmouth Junction, USA). Caledonine (10 mg/mL) was provided by the collaborative team.

PrestoBlue[™] cell viability reagent (Lot No.: 2342788), Dulbecco's modified eagle medium (DMEM, Lot No.: 2436216), and 96-well plates were purchased from Thermo Fisher Scientific Inc. (Leicestershire, UK). Dimethyl sulfoxide (DMSO, Lot No.: RNBM0113) and trypsin-EDTA 10X (0.5%, Lot No.: 2437235) were ordered from Sigma-Aldrich Co. LLC (Gillingham, UK). Bovine growth serum supplemented calf (FBS, Lot No.: AG29548253) was purchased from Cytiva (Utah, USA). The phosphate buffered saline solution (PBS) was provided by the laboratory.

Astrocyte medium (Lot No.: 34355), astrocyte growth supplement (Lot No.: 34032), 1 mg/mL poly-L-lysine (PLL, Lot No.: 33107), fetal bovine serum for astrocytes (Lot No.: 34604), trypsin neutralisation solution (Lot No.: 37673) and trypsin-EDTA (0.05% , Lot No.: 36762) were purchased from Caltag Medsystems Ltd. (Buckingham, UK).

Cultrex Mouse Collagen IV (Lot No.: 1578996) was purchased from Bio-Techne Corporation (Minneapolis, USA). 24-well ThinCert[™] 8 µm cell culture inserts (Lot No.: 23030116) were ordered from Greiner Bio-One International GmbH (Stonehouse, UK). Petri dishes (100×20 mm) and cell scrapers were ordered from Corning Incorporated (Flintshire, UK).

Ammonium carbonate (Lot No.: BCBS6405V) was purchased from Honeywell International Inc. (Berks, UK). LC-MS grade methanol and acetonitrile were supplied by VWR Chemicals, LLC (Lutterworth, UK). Deionised water was generated using a PURA-Q⁺ ultrapure water purification system (Scientific Laboratory Supplies, UK).

MirVan[™] miRNA isolation kit (Lot No.: 2651413) and RevertAid first strand

cDNA synthesis kit (Lot No.: 2968119) were ordered by Thermo Fisher Scientific Inc. (Hemel Hempstead, UK). IQ[™] SYBR[®] Green Supermix (Lot No.: 64582582) was purchased from Bio-Rad Laboratories, Inc. (Watford, UK). The primers were ordered from Sigma-Aldrich Co. LLC (Gillingham, UK).

2.2.2 Cell lines and culture conditions

Glioblastoma invasive margin (GIN28, GIN31) and glioblastoma contrast-enhanced core (GCE28, GCE31) cell lines were previously derived from intra-tumour regions of two tumours (patient 28 and patient 31) at Queen's Medical Centre. Ethics approval was obtained from the East Midlands-Derby Research Ethics Committee (Approval No.357723). All procedures were conducted in accordance with relevant guidelines and regulations.

All cells were cultured in antibiotic-free DMEM supplemented with 10% FBS and incubated under humidified growth conditions in 37 °C and 5% CO₂. Cortical astrocytes were purchased from ScienCell Research Laboratories. The culture process followed the supplier's instructions.

2.2.3 Metabolic viability assay

GBM cells were seeded at 5×10^3 cells/well. After 24 h, the culture medium was replaced with a drug-containing medium and incubated for another 72 h. Multi-point doses of drug treatment were applied, ranging from 1-200 μ M for Afuresetib, 1-2,000 μ M for Taxifolin and 0.414-8.28 μ M for Caledonine. These ranges were optimised based on literature reports and preliminary dose-response experiments to enable the construction of full dose-response curves¹⁷⁶. The appropriate vehicle (0.1% DMSO) and positive cell death controls (10% DMSO) were included. Fluorescence was measured at 544/590 nm (excitation/emission) on a microplate reader (FLUOstar[®] Omega, BMG Labtech) after 1 h of PrestoBlue[™] (1:10 dilution) incubation. The viability of drug-treated cells was determined by normalising fluorescence intensity against that of vehicle-only control.

Astrocytes were seeded at 1.6×10^3 cells/well in a PLL-coated plate. Multi-point doses were applied with 1-50 μM for Afuresertib, 1-500 μM for Taxifolin and 0.414-2.07 μM for Caledonine. Control groups, measurement and data analysis were performed as previously described for GBM.

For each dose point, four replicates were included, and two independent experiments were performed for each drug, resulting in a total of eight replicates used to generate the inhibition curves.

2.2.4 RT-qPCR analysis

MirVanTM miRNA isolation kit was used to extract total RNA from each cell line (1×10^6 cells). cDNA was synthesised from extracted RNA using the RevertAid First Strand cDNA Synthesis Kit. The concentration of cDNA was detected using a NanoDrop 2000/2000C spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). RT-qPCR was performed on the CFX384 Touch Real-Time PCR System (Bio-Rad Life Science, Watford, UK) for 40 cycles (94 °C for 20 s, 60 °C for 35 s and 72 °C for 35 s, per cycle). Reactions were run with 100 ng of cDNA and 200 nM primers in a total volume of 10 μL , with pre-added 5 μL IQTM SYBR[®] Green Supermix. Results were analysed using Bio-Rad CFX Manager v3.1. The sequences of primers are presented in Table 2.1. Two independent experiments were performed, and each condition was analysed using four technical replicates.

Table 2.1. Sequences of primers.

Gene	Primer	Sequence (5'-3')
AKT1	Forward	AAGGAGGGGATCAAGGACGG
	Reverse	GTTGTAGAAGGGCAGGCGAC
AKT2	Forward	AGCAGAATGCCAGCTGATGA
	Reverse	CAGGCAGCGTATGACAAAGGT
AKT3	Forward	GTCGAGAGAGCGGGTGTCT
	Reverse	TGTAGATAGTCCAAGGCAGAGACAA
HPRT1	Forward	GCTGAGGATTTGGAAAGGGT
	Reverse	GTAATCCAGCAGGTCAGCAA

HPRT1 is the human hypoxanthine phosphoribosyltransferase 1.

2.2.5 Invasion assay

Inserts were coated by mouse collagen IV (15 µg/insert) and then dried in a sterile environment. Before seeding cells, 100 µL of DMEM was added to dried collagen soaking for 1 h.

Drug-containing DMEM (100 µL) was added to the pre-soaked insert followed by adding 100 µL of cells suspended in DMEM. The seeding densities were 1.2×10^4 cells/insert for GIN31 and GCE31, and 1.4×10^4 cells/insert for GIN28 and GCE28. The bottom chamber was filled with 700 µL of DMEM supplemented with 10% FBS. Samples were incubated for 24 h to allow invasion to commence. For negative control, inserts contained the cells in DMEM supplemented with 10% FBS, while the bottom chamber was filled with DMEM.

When the incubation period ended, cells were fixed with ice-cold methanol before stained with 0.2% crystal violet. Finally, cells were imaged at four different fields of view using the multi-viewing microscope (Brunel, N-510) equipped with a 10X magnification len.

Invasion assays were conducted in two independent experiments, with three replicates per condition.

2.2.6 Metabolomics study

2.2.6.1 Sample preparation

Cells were cultured in Petri dishes and incubated with repurposed candidates for 72 h. The concentration of treatment is equal to the IC_{10} value for GBM and the IC_5 value for astrocytes. Each experimental group consisted of six biological replicates, and 1×10^6 cells were harvested for each replicate.

To extract intracellular metabolites, cells were quickly washed with pre-warmed PBS after the medium was removed. Then 1.0 mL of pre-cooled (-40°C) methanol was added to quench cells. Cell suspensions were collected and vortexed for 1 h at 4°C . Subsequently, the supernatant was dried in the vacuum centrifuge evaporator, before

being reconstituted with 70 μL of LC-MS grade methanol. Lastly, the reconstituted samples were centrifuged again (14,000 g/ 5 min), and the supernatant was transferred to LC-MS vials for analysis.

A pooled quality control (QC) sample was prepared by combining 10 μL aliquots from each reconstituted sample (excluding blanks) into a single vial.

2.2.6.2 LC-MS-based metabolite profiling

The reagent-blank samples were analysed prior to the experimental samples. The experimental samples were randomly assigned in sequences with QC samples ($n \geq 6$) analysed intermittently throughout the duration of the analysis. Five mixtures of authentic standard solutions (StdA-StdE, total 266 standards, details in <https://github.com/Rui-eebebeb/Standards-database-analytical-flow>) were analysed under the same conditions for metabolite identification.

Separation was performed on a ZIC-*p*HILIC column (5 μm , 4.6 $\times$ 150 mm) manufactured by Merck Sequant (Watford, UK) with a guard column, using a Dionex Ultimate 3000 HPLC system (Thermo fisher Scientific, Hemel Hempstead, UK). The mobile phases consisted of 20 mM ammonium carbonate in water (phase A) and acetonitrile (phase B), using a linear gradient (0-15 min: 20% A to 95% A, 15-17 min: 95% A to 20% A, 17-24 min: 20% A). The injection volume was 10 μL and the flow rate was 300 $\mu\text{L}/\text{min}$. The autosampler and column compartment temperature were maintained at 4 $^{\circ}\text{C}$ and 45 $^{\circ}\text{C}$ respectively.

Detection was conducted using a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) fitted with a HESI source. The instrument was set in a polarity-switching mode with spray voltages at +4.5 kV and -3.5 kV, respectively. Other settings were as follows: capillary temperature, 275 $^{\circ}\text{C}$; sheath gas flow rate, 40; aux gas flow rate, 5; sweep gas flow rate, 1; S-lens RF level, 55%. The spectra were recorded at a mass resolution of 70,000 at m/z 200, across an m/z range of 70-1,050 with an automatic gain control (AGC) of 3×10^6 . Data-dependent MS/MS (top 10) was performed on QC samples at a mass resolution of

17,500 with an AGC target 1×10^5 and stepped normalised collision energies (20, 30 and 40) for fragmentation.

2.2.6.3 Data processing and metabolite identification

The datasets were processed in Compound Discover 3.3 SP1 (Thermo Fisher Scientific, Waltham, MA, USA), applying an untargeted metabolomic workflow for peak picking, alignment (aligned window $m/z \pm 5$ ppm), metabolite identification, and gap filling. The data were not normalised to cell count due to the use of an equal number of cells in each group. Features exhibiting a blank-to-sample intensity ratio $\geq 20\%$ were excluded. Non-informative features were identified and removed if their relative standard deviations of intensity were $\geq 30\%$ in the technical replicates of the pooled QC samples¹⁹⁴.

Then ions were annotated by matching the accurate mass, retention time and fragmentation patterns to those of authentic standards, the exact mass and fragmentation patterns of metabolites in *mzCloud*[™] database, Thermo Scientific *mzVault* spectral library, and Human Metabolome Database v5.0¹⁹⁵.

Identified metabolites were classified into four levels of confidence according to the metabolomics standards initiative^{196,197}. Level 1 confidence was that the ions matched the accurate masses, retention times, and fragments of authentic standards. Level 2 confidence represented ions matched to the exact masses and fragments of metabolites in spectral/online libraries in the absence of standards. Level 3 confidence was that ion identification was based only on accurate mass matching with database entries, without supportive fragmentation information. Level 4 identification referred to ions only with or without predicted molecular formulas.

2.2.6.4 Pathway analysis

A list of all detected metabolites together with their corresponding intensities was subjected to pathway analysis using MetaboAnalyst v5.0¹⁹⁸. The module of pathway analysis consists of enrichment analysis and pathway topology analysis. These

complementary approaches not only list the components of biological pathways, but also characterise their interactions and relative importance within pathways. Based on existing knowledge of biological pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, metabolites are mapped to defined pathways, allowing interpretation of how they collectively function in biological systems^{199,200}.

The analysis parameters were set as follow: scatter plot for visualisation, global test for enrichment analysis, relative-betweenness centrality for topology analysis, the KEGG database as the pathway library.

2.2.7 Statistics

For viability assessment, invasion assay and RT-qPCR analysis, curve fitting of inhibition and statistical analysis were performed on GraphPad Prism v8.2.1 (GraphPad Software, San Diego, CA), with p value < 0.05 considered statistically significant. Images were processed with Fiji (ImageJ v1.54p). Venn diagrams were generated using TBtools-II²⁰¹.

For univariate analysis, the data were logarithmically transformed. Two-tailed p values from the t-test were adjusted using the Benjamini-Hochberg correction, with adjusted p value < 0.05 considered significant.

Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed on SIMCA-P v16 (Umetrics, Sweden). After logarithmic transformation, Unit Variance scaling was used for PCA to standardise variables, allowing unbiased exploration of overall variance across variables. Pareto scaling was applied for OPLS-DA as it reduces noise while preserving data structure and biological relevance.

2.3 Results and discussion

2.3.1 Metabolic viability

The viability results of cells upon acute exposure to repurposed compounds are shown in Figure 2.3 and Table 2.2.

Three candidates presented a dose-dependent impairment of metabolic viability. Afuresertib and Caledonine were identified as more potent for inhibiting GBM proliferation relative to Taxifolin. Moreover, cells derived from the invasive margin (GINs) were more sensitive to Afuresertib than their counterparts derived from the tumour core (GCEs). Astrocytes were more sensitive to all candidates than GBM cells.

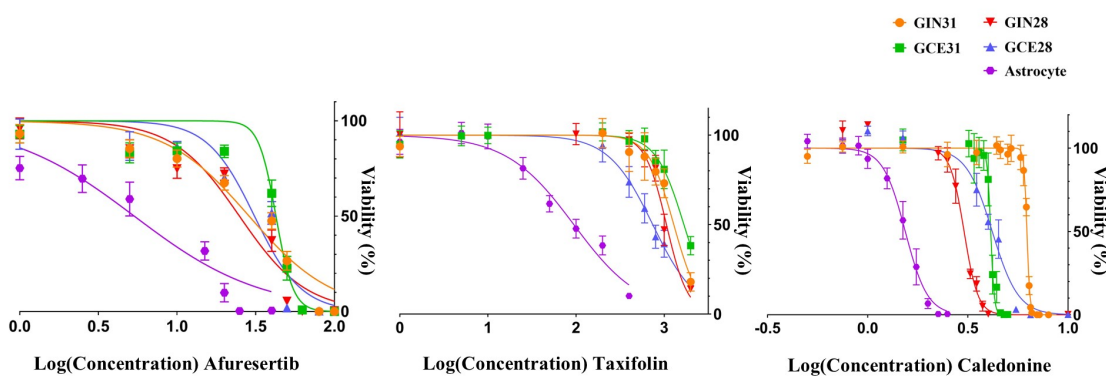


Figure 2.3. Metabolic viability of GBM cells and astrocytes exposed to Afuresertib, Taxifolin and Caledonine ($n = 8$). The concentration is $\log_{10}$ transformed.

Table 2.2. IC_{50} values of repurposed candidates on GBM cells and astrocytes (μM).

Compounds	Astrocyte	GIN31	GCE31	GIN28	GCE28
Afuresertib	5.353	28.75	42.54	24.95	31.09
Taxifolin	90.20	1287	1676	1067	742.8
Caledonine	1.298	5.164	3.407	2.509	3.467

IC_{50} represents half maximal inhibitory concentration.

2.3.2 RT-qPCR

Based on the results of the viability tests, GINs were more sensitive to Afuresertib than GCEs, and the mechanism underlying this difference is unknown. Given that

Afuresertib is an AKT inhibitor, to explore this further, the difference in AKT gene expression was measured between GINs and GCEs.

The results of the fold change (FC) showed that the heterogeneity of GBM was evident at the genetic level (Figure 2.4). AKT1 was more highly expressed in GIN31 than in GCE31, while it showed significantly higher expression in GCE28 than in GIN28. AKT2 and AKT3 were significantly expressed in GCE31 than in GIN31, while their expressions were at similar level in GCE28 and GIN28.

AKT1 and AKT2 are ubiquitously expressed, whereas AKT3 is primarily expressed in brain, lung, heart, kidney, and fat²⁰². It is unclear if three AKT isoforms are functionally redundant. Although the results did not directly explain the differences in sensitivity, they underscore the complexity of gene regulation, as gene expression does not necessarily correlate with protein levels. Further investigation of AKT protein expression combined with gene expression may better elucidate these mechanisms.

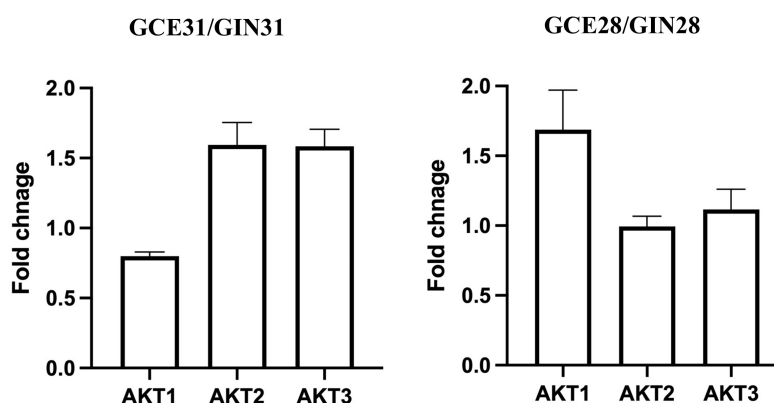


Figure 2.4. Fold change of AKT gene expression in GCEs vs. GINs. The cycle threshold value of three isoforms were measured. FC > 1.0 means higher expression, FC < 1.0 means lower expression. FC > 1.5 or FC < 0.5 is regarded as significant expression.

2.3.3 LC-MS-based metabolite profiling

After investigating the anti-tumour effects of candidates, LC-MS-based metabolite profiling was employed to explore the altered metabolites and metabolic pathways by drug exposure.

In the PCA scores plots (Figures 2.5 and 2.6), QC samples are clustered tightly toward the centre, indicating that satisfactory stability of the instrument was achieved.

Moreover, the clear clustering and separation for different drug treatments and control groups indicates that these were metabolic alterations resulting from drug exposure.

In the OPLS-DA scores plots (Figures B.2 to B.5), drug-treated groups and control group are clearly separated, indicating the presence of distinct metabolic profiles. The results of cross validation were excellent as the value of R^2Y and Q^2 in each model was ≥ 0.90 (Table 2.3), indicating good fitness and predictive ability. The permutation results suggested that these models were less likely to overfit dataset.

Subsequently, lists of variables importance in projection (VIP) were generated for each comparison. In addition, adjusted p value from the univariate analysis was combined to select significantly altered metabolites. In this study, metabolites with VIP > 1 and adjusted p value < 0.05 were considered differentially abundant.

Table 2.3. Cross-validation results of OPLS-DA models.

Cell line	Comparison	R^2Y	Q^2
–	GIN31 vs. GCE31	0.997	0.951
–	GIN28 vs. GCE28	0.999	0.927
GIN31	Afuresertib vs. Control	0.999	0.969
	Taxifolin vs. Control	0.994	0.885
	Caledonine vs. Control	0.999	0.984
GCE31	Afuresertib vs. Control	0.999	0.940
	Taxifolin vs. Control	0.999	0.908
	Caledonine vs. Control	0.999	0.987
GIN28	Afuresertib vs. Control	0.999	0.986
	Taxifolin vs. Control	0.999	0.921
	Caledonine vs. Control	0.991	0.969
GCE28	Afuresertib vs. Control	0.999	0.988
	Taxifolin vs. Control	0.999	0.900
	Caledonine vs. Control	0.998	0.978

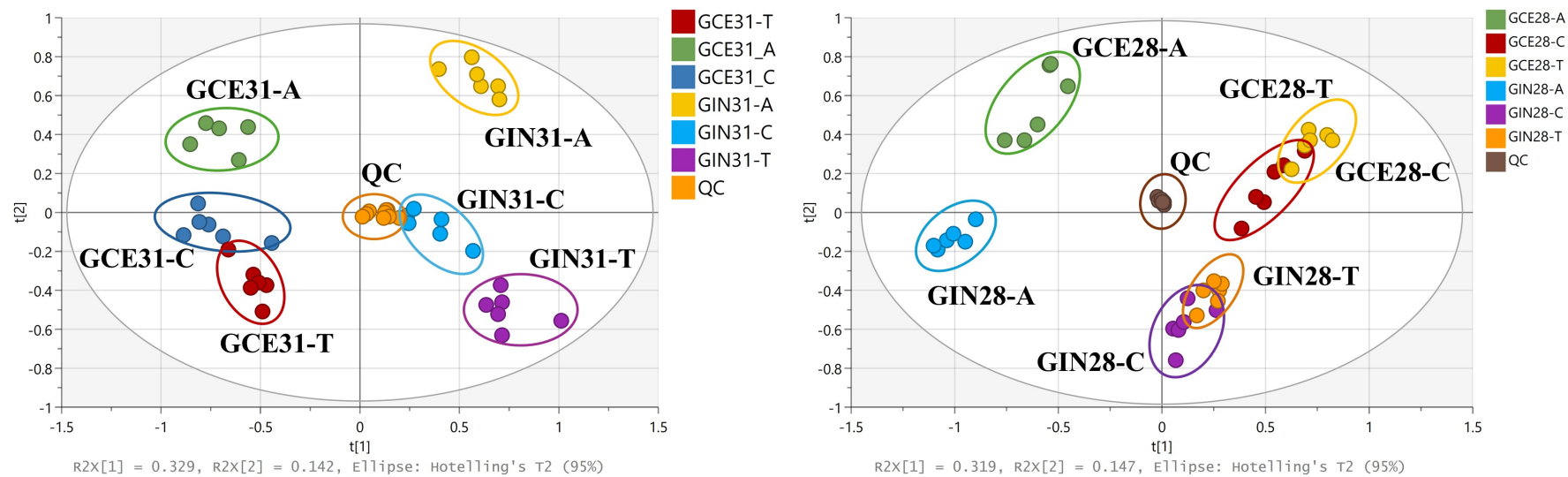


Figure 2.5. PCA scores plots of untreated and treated GBM cells (Afuresertib and Taxifolin).

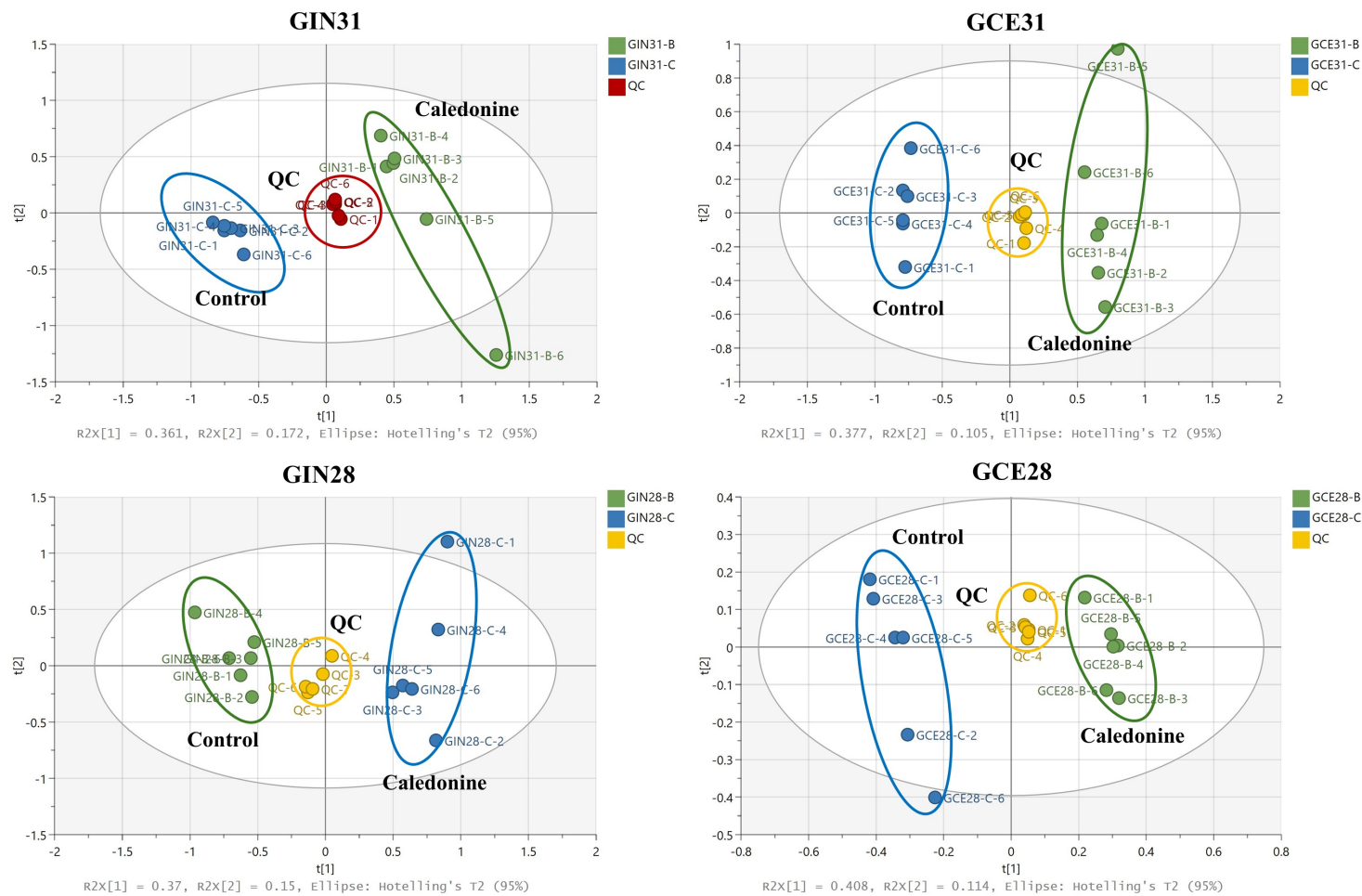


Figure 2.6. PCA scores plots of untreated and Caledonine-treated GBM cells.

2.3.4 Metabolic variations across untreated GBM cells from distinct intra-tumour regions

It has previously shown that cells from the GBM core exhibit a proliferative phenotype, whilst cells from the invasion margin blend into and infiltrate adjacent healthy brain parenchyma²⁰³. In this study, GINs and GCEs are clearly separated in the PCA scores plots, indicating that there are metabolic differences between respective two cell lines, despite being derived from the same patient's tumour.

The volcano plot visually presents significantly altered metabolites by combining the statistical p -values and FC results (Figure 2.7). The number of metabolites abundant in GIN31 (109) was greater than that in GCE31 (33). However, the number of metabolites abundant in GIN28 (45) was less than that in GCE28 (75). These results further suggested the presence of intra-tumour metabolic heterogeneity.

In well-constructed OPLS-DA models (Figures 2.8 and B.1), GINs and GCEs are distinctly separated, suggesting distinct metabolic differences. The metabolites contributing to the separation are shown in Tables A.1 and A.2.

The result of pathway analysis for GIN31 vs. GCE31 is shown in Figure 2.9 A. A total of 58 metabolic pathways were initially identified, which were then filtered to 23 significant metabolic pathways with adjusted p value < 0.05 and impact factor > 0.1 (Table 2.4). Among these 23 metabolic pathways, most metabolites related to amino acid metabolism and glycerophospholipids/sphingolipids metabolism were more abundant in GIN31. Only metabolites mapped to taurine and hypotaurine metabolism were more abundant in GCE31. Hence, compared to GCE31, GIN31 cells had more active metabolic pathways which were associated with higher demand in energy production and consumption, amino acid metabolism, and phospholipid metabolism.

The pathway analysis result of untreated GIN28 vs. GCE28 is shown in Figure 2.9 B and Table 2.5. In contrast to GIN31 vs. GCE31, GCE28 cells were more metabolically active than GIN28 cells, with metabolites mapping to pathways including lysine degradation, alanine, aspartate and glutamate metabolism, nicotinate and nicotinamide metabolism, arginine and proline metabolism, glycine, serine and threonine metabolism.

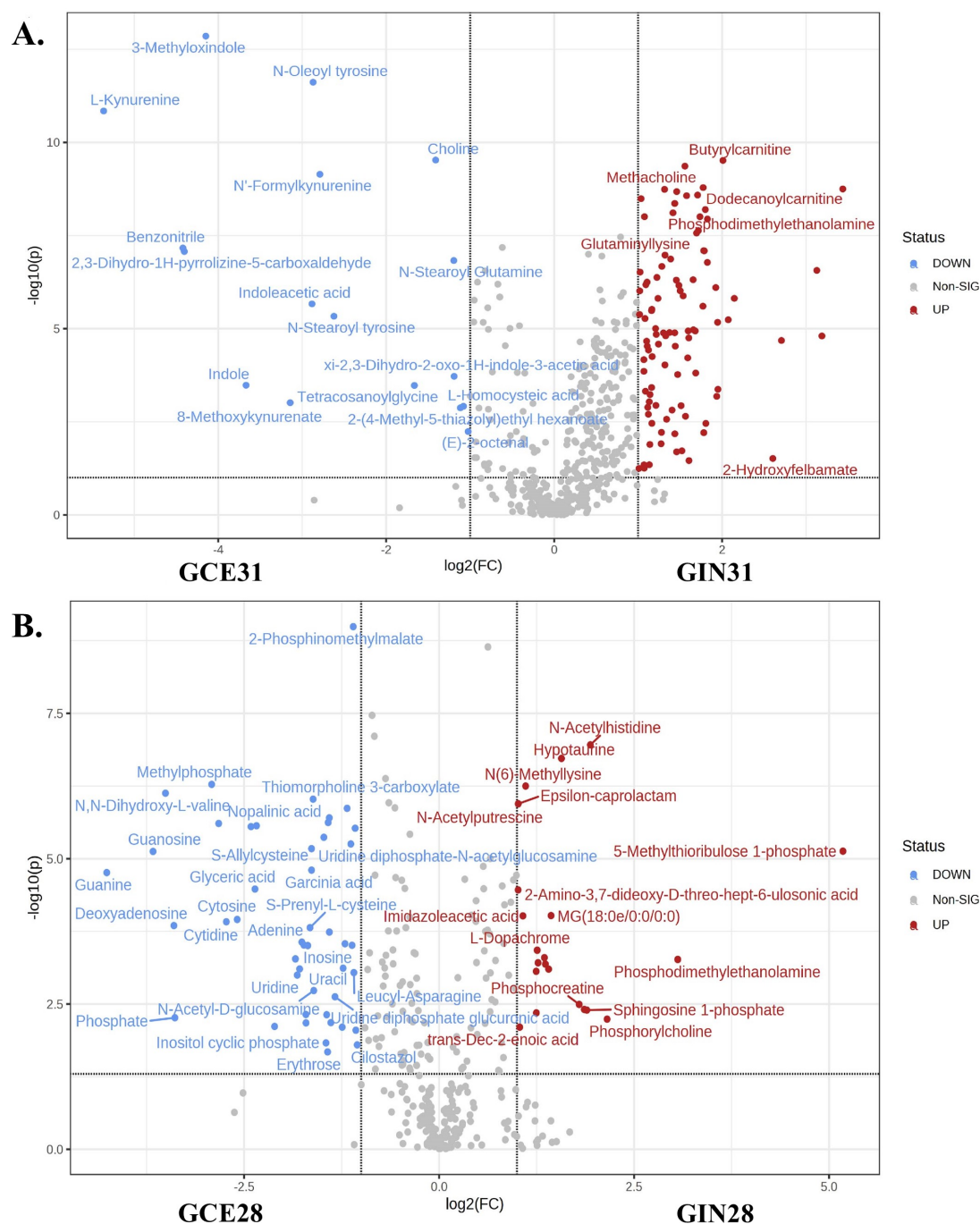


Figure 2.7. Metabolic variations between GINs and GCEs. (A) and (B) are the volcano plots of GINs vs. GCEs derived from each patient. The comparison direction is GINs relative to GCEs. Metabolites are highlighted with fold change > 2 and adjusted $p < 0.05$.

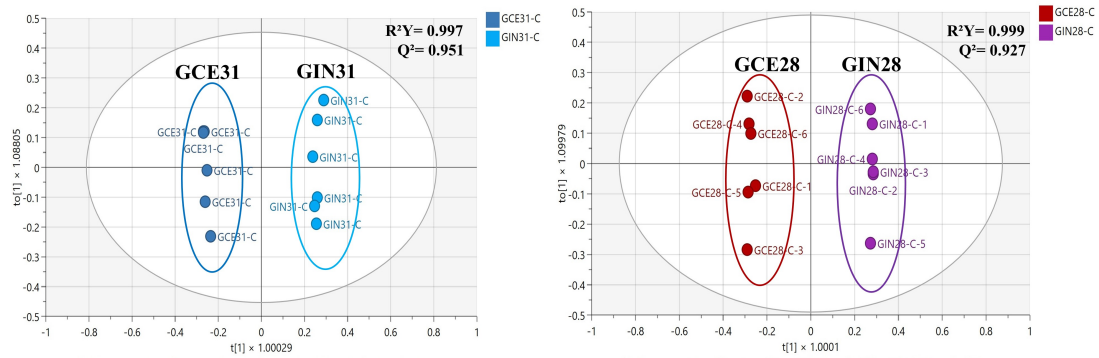


Figure 2.8. OPLS-DA models of GINs vs. GCEs.

These findings revealed significantly altered metabolites between GINs and GCEs, enriching the understanding of the intrinsic metabolic profiles of GBM cells propagated *in vitro*. Moreover, combined with RT-qPCR results, the heterogeneity of GBM was evident at both the genetic and metabolic levels.

As shown in Figure 2.10, twelve shared significant pathways were identified in two comparisons. Even though the main metabolic patterns of GINs vs. GCEs were distinct between two patients, metabolites mapped to glycerophospholipid metabolism showed a similar trend. These metabolites associated with phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), exhibited higher abundance in GINs, except for choline phosphate, shown in Figure 2.11. Additionally, other metabolites mapped onto glycerolipid metabolism and sphingolipid metabolism, were more abundant in GINs, including serine, sphinganine, and sn-glycerol 3-phosphate.

Changes in glycerophospholipids and their metabolites are closely related to cell membrane fluidity, adhesion, apoptosis, proliferation, invasion, angiogenesis, and lymphoangiogenesis^{204,205}. In cancer cells, increased PC synthesis affects cellular proliferation and programmed cell death²⁰⁶. Moreover, the large amount of PS and PE exposed on the surface of GBM cell membranes compared with healthy cells, increases the negative charges on the outer surface of the membrane, inducing mutual repulsion and promoting migration of tumour cells^{204,207}. Collectively, the high abundance of PE, PC, PS and their metabolites could be associated with GINs being derived from infiltrative margin of tumour tissue, indicating their potential as putative metabolic biomarkers for discriminating GINs and GCEs.

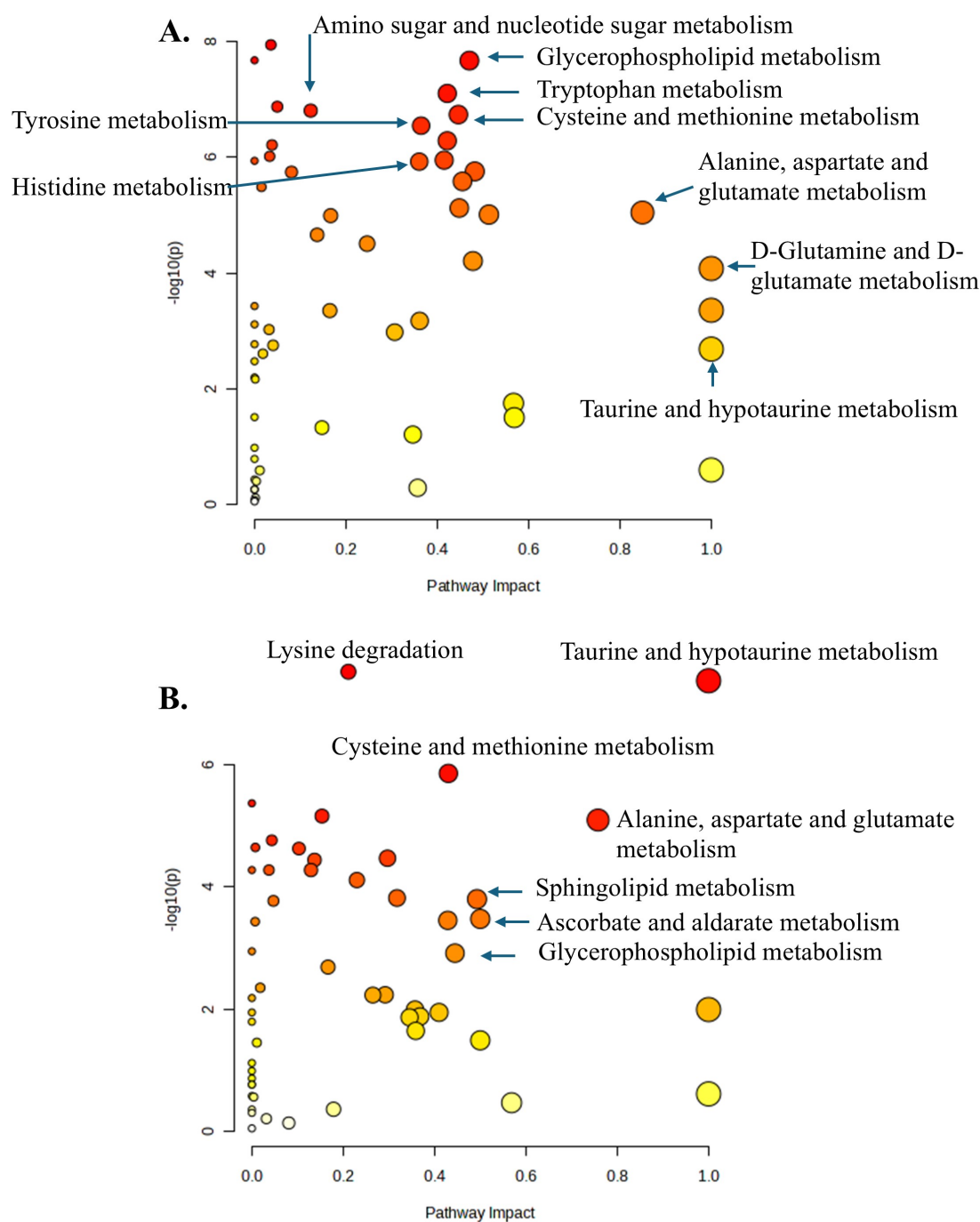


Figure 2.9. Pathways analysis of untreated GINs vs. GCEs. (A) and (B) are the pathway analysis results of GIN31 vs. GCE31 and GIN28 vs. GCE28 respectively. The size of bubble represents the number of compounds mapped on a particular pathway. The colour represents the degree of significance: white (non-significant) to red (statistically significant). The impact factor represents the importance of an identified pathway within the whole metabolic network.

Furthermore, phosphatidylinositols are another type of glycerophospholipids which are important mediators of several signalling pathways²⁰⁸. It has been reported that the 1-phosphatidyl-1D-myo-inositol-4-phosphate metabolic pathway is related to cell metastasis²⁰⁹. Supporting this notion, its downstream metabolite, myo-inositol, was identified at higher abundance in GIN28 compared with GCE28, although not detectable in either GIN31 or GCE31.

Table 2.4. Significantly changed metabolic pathways of GIN31 vs. GCE31 (adjusted p value < 0.05 and impact factor > 0.1).

Pathway name	Total compounds	Hits	Raw p	Adjusted p	Impact factor
Glycerophospholipid metabolism	36	10	2.14E-08	1.20E-06	0.47
Tryptophan metabolism	41	7	7.88E-08	4.33E-06	0.42
Amino sugar and nucleotide sugar metabolism	37	8	1.56E-07	8.27E-06	0.12
Cysteine and methionine metabolism	33	11	1.82E-07	9.49E-06	0.45
Tyrosine metabolism	42	5	2.84E-07	1.45E-05	0.36
Pentose and glucuronate interconversions	18	4	5.17E-07	2.59E-05	0.42
Arginine and proline metabolism	38	12	1.12E-06	5.25E-05	0.42
Histidine metabolism	16	7	1.19E-06	5.34E-05	0.36
Arginine biosynthesis	14	8	1.75E-06	7.68E-05	0.48
beta-Alanine metabolism	21	6	2.60E-06	1.09E-04	0.46
Sphingolipid metabolism	21	5	7.49E-06	3.00E-04	0.45
Alanine, aspartate and glutamate metabolism	28	14	8.96E-06	3.50E-04	0.85
Glycine, serine and threonine metabolism	33	10	9.71E-06	3.69E-04	0.51
Aminoacyl-tRNA biosynthesis	48	19	1.01E-05	3.75E-04	0.17
Glycerolipid metabolism	16	2	2.17E-05	7.82E-04	0.14
Pantothenate and CoA biosynthesis	19	7	3.09E-05	1.08E-03	0.25
Glutathione metabolism	28	8	6.16E-05	2.09E-03	0.48
D-Glutamine and D-glutamate metabolism	6	4	8.29E-05	2.74E-03	1.00
Ascorbate and aldarate metabolism	8	2	4.35E-04	1.35E-02	1.00
Pyrimidine metabolism	39	8	4.42E-04	1.35E-02	0.16
Lysine degradation	25	8	6.66E-04	1.93E-02	0.36
Glyoxylate and dicarboxylate metabolism	32	8	1.04E-03	2.71E-02	0.31
Taurine and hypotaurine metabolism	8	4	2.04E-03	4.69E-02	1.00

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted p is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

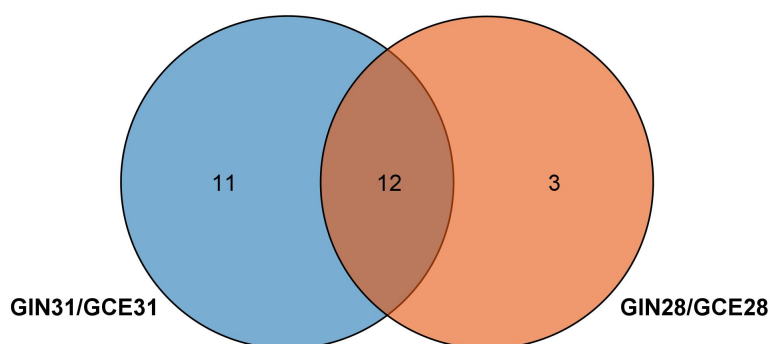


Figure 2.10. Shared pathways between GIN31 vs. GCE31 and GIN28 vs. GCE28.

Table 2.5. Significantly changed metabolic pathways of GIN28 vs. GCE28 (adjusted p value < 0.05 and impact factor > 0.1).

PathwayName	Total compounds	Hits	Raw p	Adjusted p	Impact factor
Lysine degradation	25	7	2.98E-08	1.58E-06	0.21
Taurine and hypotaurine metabolism	8	5	4.18E-08	2.17E-06	1.00
Cysteine and methionine metabolism	33	7	1.38E-06	7.03E-05	0.43
Glyoxylate and dicarboxylate metabolism	32	5	6.89E-06	3.38E-04	0.15
Alanine, aspartate and glutamate metabolism	28	12	8.01E-06	3.84E-04	0.76
Amino sugar and nucleotide sugar metabolism	37	5	2.35E-05	1.06E-03	0.10
Pentose and glucuronate interconversions	18	2	3.39E-05	1.49E-03	0.30
Glycerolipid metabolism	16	2	3.61E-05	1.55E-03	0.14
Inositol phosphate metabolism	30	1	5.29E-05	2.22E-03	0.13
Arginine and proline metabolism	38	8	7.72E-05	3.01E-03	0.23
Glycine, serine, and threonine metabolism	33	9	1.52E-04	5.78E-03	0.32
Sphingolipid metabolism	21	6	1.58E-04	5.86E-03	0.49
Ascorbate and aldarate metabolism	8	2	3.34E-04	1.17E-02	0.50
Nicotinate and nicotinamide metabolism	15	3	3.53E-04	1.20E-02	0.43
Glycerophospholipid metabolism	36	9	1.22E-03	3.77E-02	0.44

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted p is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

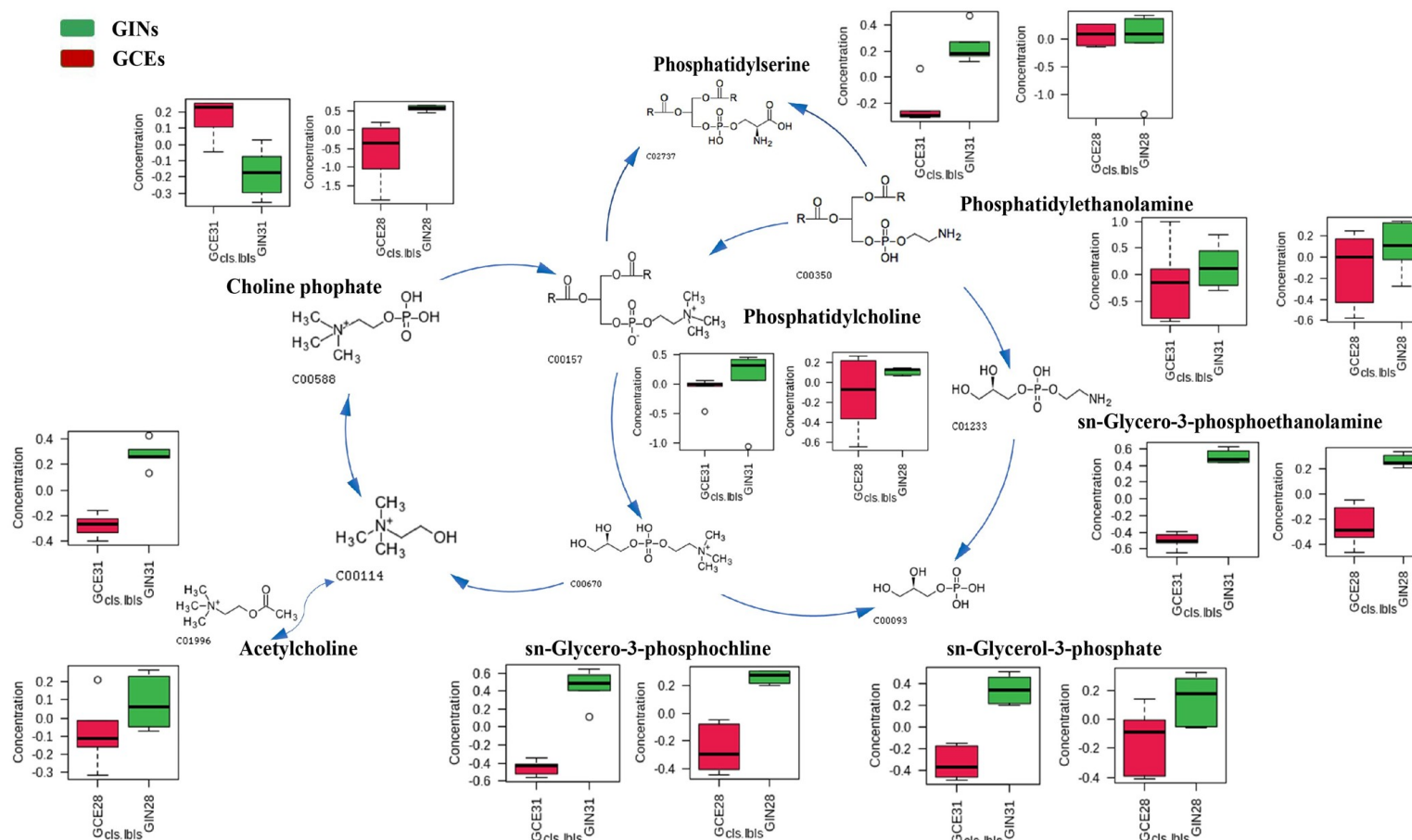


Figure 2.11. Metabolites identified in glycerophospholipid metabolism and their abundance between GINs and GCEs. The normalised abundances of PC, PE, PS and their metabolites are shown for GCE cells in red and GIN cells in green. The abundance of metabolites was exported from MetaboAnalyst v5.0, and reflects transformed and scaled data for visualisation.

2.3.5 Metabolic pathways altered in GBM cells upon Afuresertib treatment

To investigate metabolic pathways affected by treatments, the results of pathway analysis for each cell line after treatments are summarised in Tables A.3 to A.6.

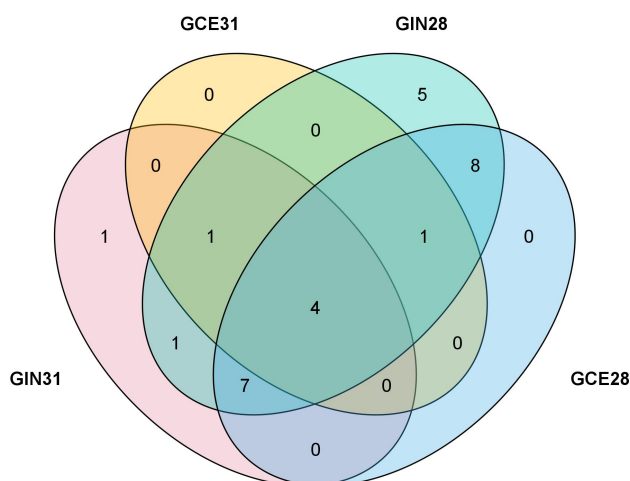


Figure 2.12. Shared pathways affected in GBM cells after Afuresertib treatment.

As shown in Figure 2.12, the comparative analysis between the untreated and Afuresertib-treated groups revealed 14 significantly altered pathways for GIN31 and 6 pathways for GCE31. These pathways showed high similarity as 5 pathways were shared in both cell lines. A similar phenomenon was observed in GIN28 and GCE28, indicating that Afuresertib could exert a similar downstream effect in cells derived from different tumour regions of same patient. Four metabolic pathways were shared by all GBM cell lines upon the Afuresertib exposure, including alanine, aspartate and glutamate metabolism, histidine metabolism, cysteine and methionine metabolism, and glycerophospholipid metabolism.

Upon Afuresertib treatment, the identified pathways where the metabolite abundance in treated cells was lower than untreated cells, were related to amino acid metabolism, energy production and consumption, and glycerophospholipid metabolism (Figure 2.13). Taken together, these results supported that Afuresertib altered amino acid metabolism, energy production and consumption, and glycerophospholipid metabolism, which are putatively associated with anti-tumour effect.

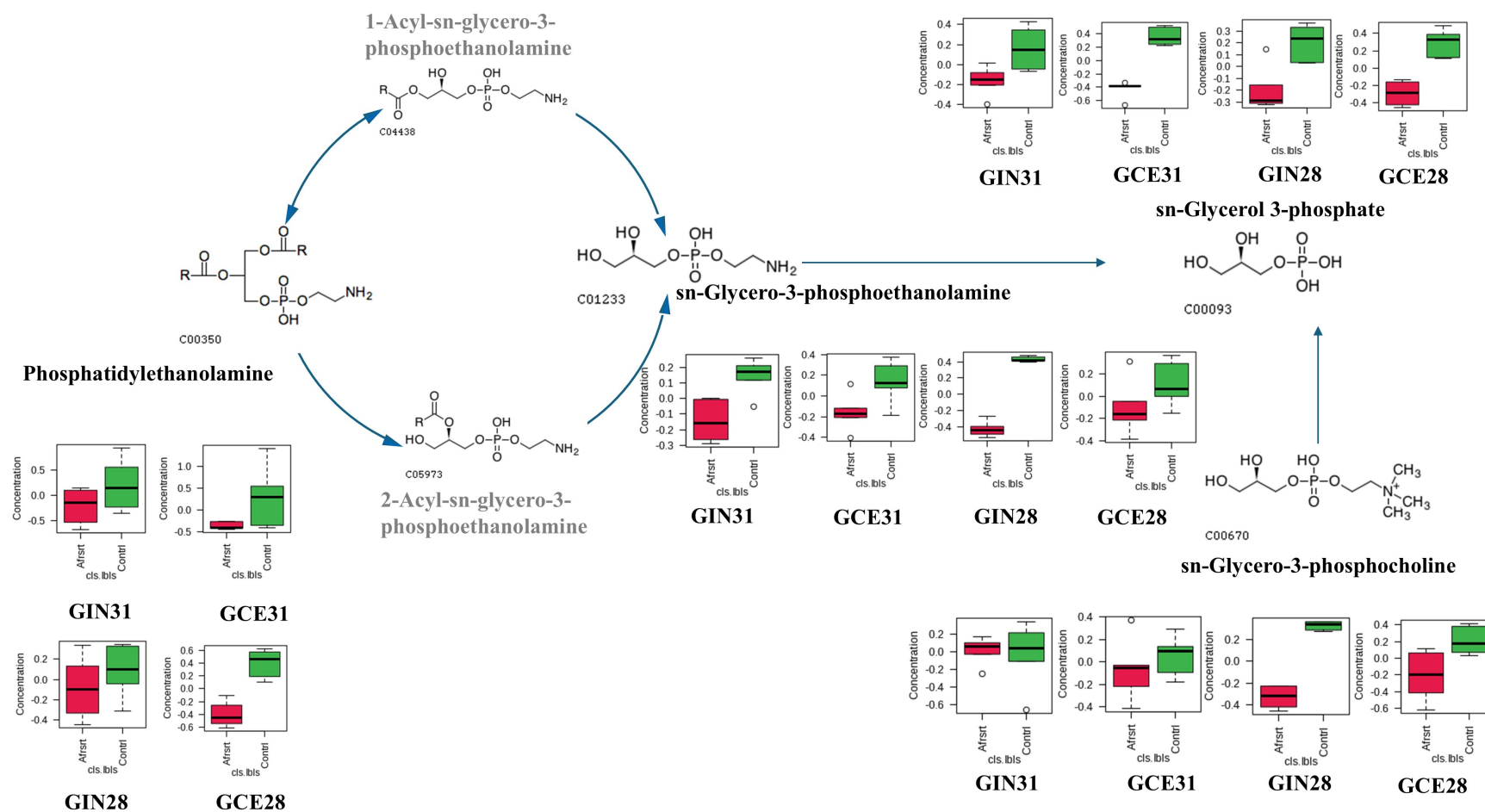


Figure 2.13. Metabolites mapped onto glycerophospholipid metabolism and their abundance in GBM cells upon Afuresertib treatment. The normalised abundances of identified metabolites are shown in Afuresertib-treated cells in red and untreated cells in green. The abundance of metabolites was exported from MetaboAnalyst v5.0, and reflects transformed and scaled data for visualisation.

2.3.6 Metabolic pathways altered in GBM cells upon Taxifolin treatment

As shown in Figure 2.14, only 3 pathways were shared for all GBM cell lines after Taxifolin exposure (histidine metabolism, nicotinate and nicotinamide metabolism, and glycerophospholipid metabolism).

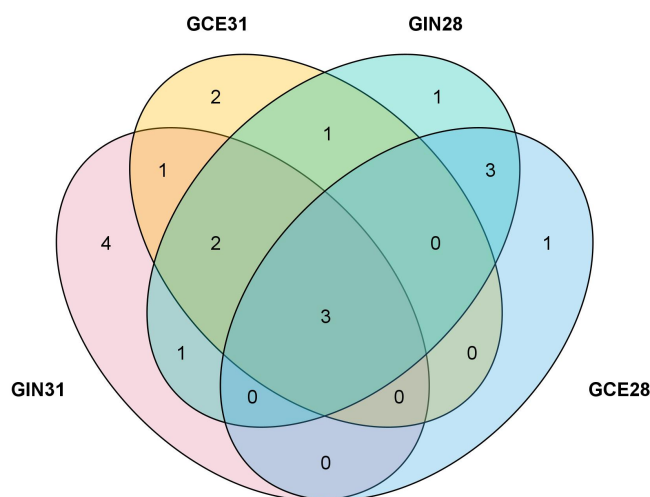


Figure 2.14. Shared pathways affected in GBM cells after Taxifolin treatment.

Compared to untreated cells, the abundance of metabolites involved in histidine metabolism was lower in Taxifolin-treated cells, including histidine, histamine, aspartate and glutamate. Apart from that, the abundance of metabolites mapped onto nicotinate and nicotinamide metabolism were at lower levels in drug-treated cells (Figure 2.15). Nicotinamide adenine dinucleotide (NAD^+) and its degradation products have been considered as key regulators in energy metabolism such as glycolysis, oxidative phosphorylation and TCA cycle²¹⁰. The results suggested that Taxifolin may perturb histidine metabolism and nicotinate and nicotinamide metabolism, associated with impaired metabolic activity.

Glycerophospholipid metabolism was also identified as a significant pathway in GBM cells exposed to Taxifolin. In contrast to Afuresertib treatment, the abundance of metabolites in this pathway were identified at higher levels in treated cells (Figure 2.16). The results indicated that Taxifolin may not inhibit glycerophospholipid metabolism.

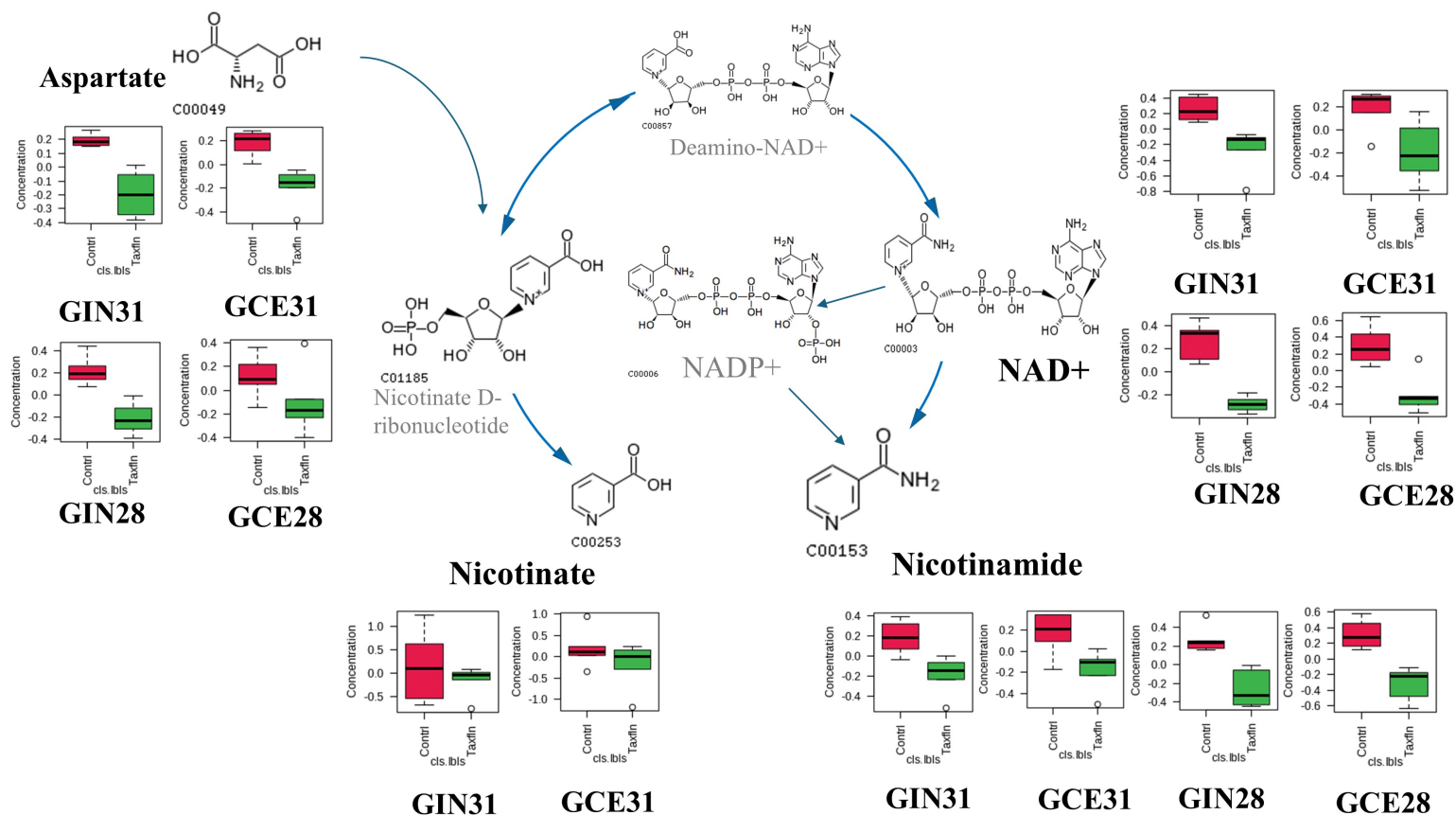


Figure 2.15. Metabolites mapped onto nicotinate and nicotinamide metabolism and their abundance in GBM cells upon Taxifolin treatment. The normalised abundances of identified metabolites are shown for untreated cells in red and Taxifolin-treated cells in green. The abundance of metabolites was exported from MetaboAnalyst v5.0, and reflects transformed and scaled data for visualisation.

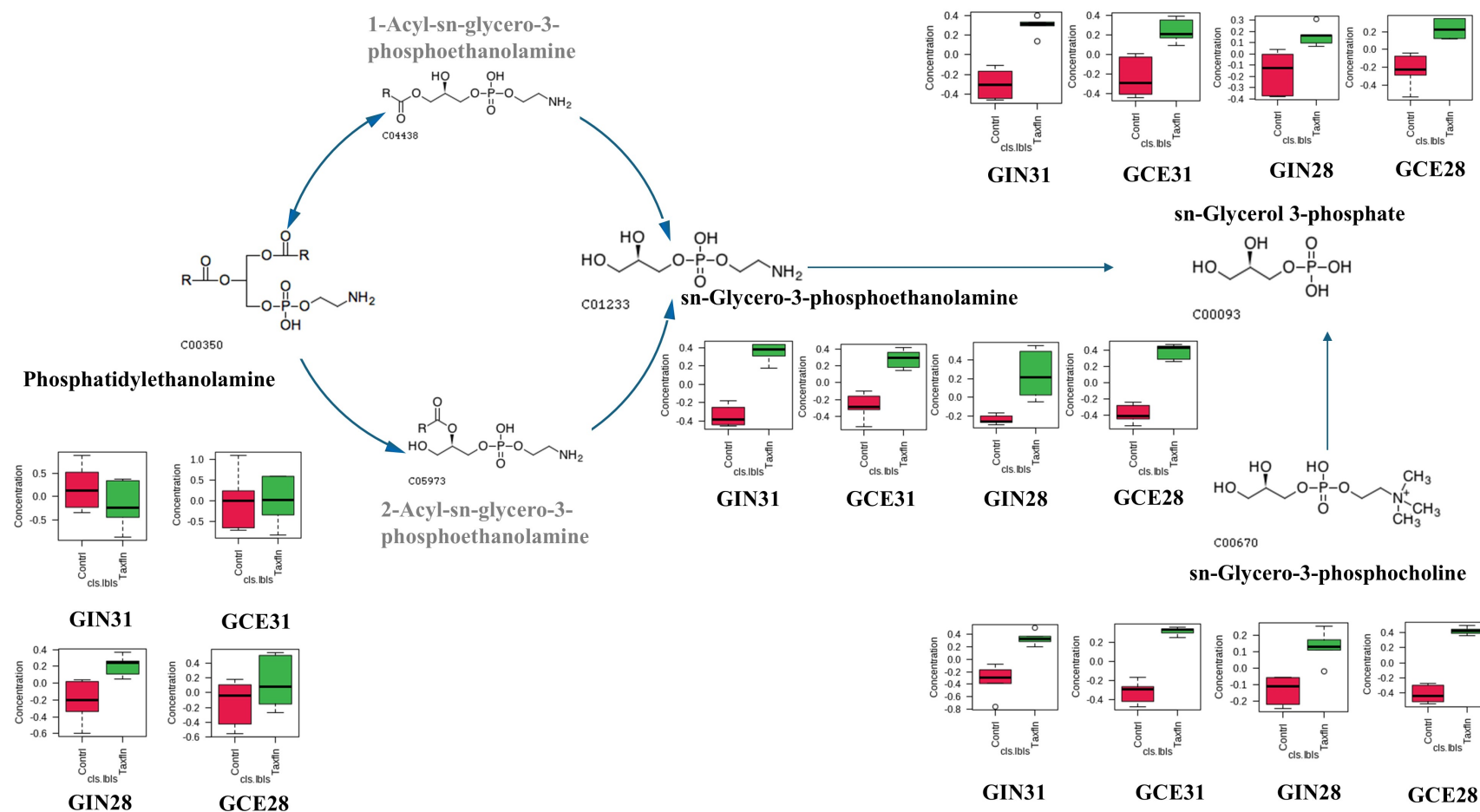


Figure 2.16. Metabolites mapped onto glycerophospholipid metabolism and their abundance in GBM cells upon Taxifolin treatment. The normalised abundances of identified metabolites are shown for untreated cells in red and Taxifolin-treated cells in green. The abundance of metabolites was exported from MetaboAnalyst v5.0, and reflects transformed and scaled data for visualisation.

2.3.7 Metabolic pathways altered in GBM cells upon Caledonine treatment

As presented in Figure 2.17, four metabolic pathways (taurine and hypotaurine metabolism, cysteine and methionine metabolism, sphingolipid metabolism, and glycerophospholipid metabolism) were shared by GBM cells exposed to Caledonine. The abundance of metabolites involved in cysteine and methionine metabolism and sphingolipid metabolism were identified at lower levels in drug-treated cells, indicating that these pathways are associated with anti-cancer effects induced by Caledonine.

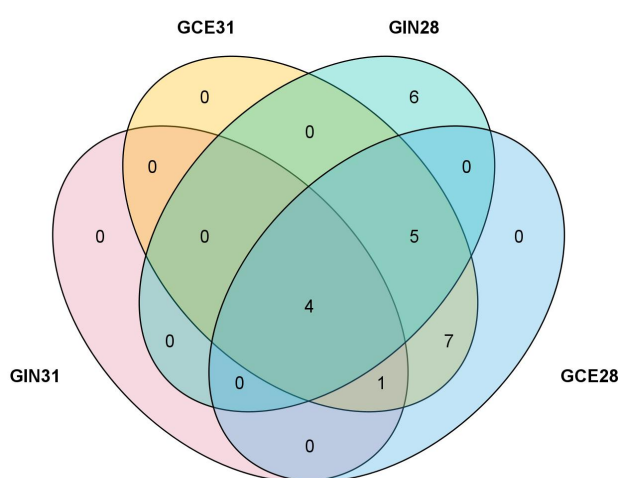


Figure 2.17. Shared pathways affected in GBM cells after Caledonine treatment.

Regarding glycerophospholipid metabolism, a similar phenomenon upon Caledonine treatment was observed, where the abundance of metabolites associated with phosphatidylethanolamine metabolism in the drug-treated group was less than that in control group (shown in Figure 2.18).

Based on the current results, Caledonine may be against GBM by perturbing amino acid, sphingolipid and glycerophospholipid metabolisms.

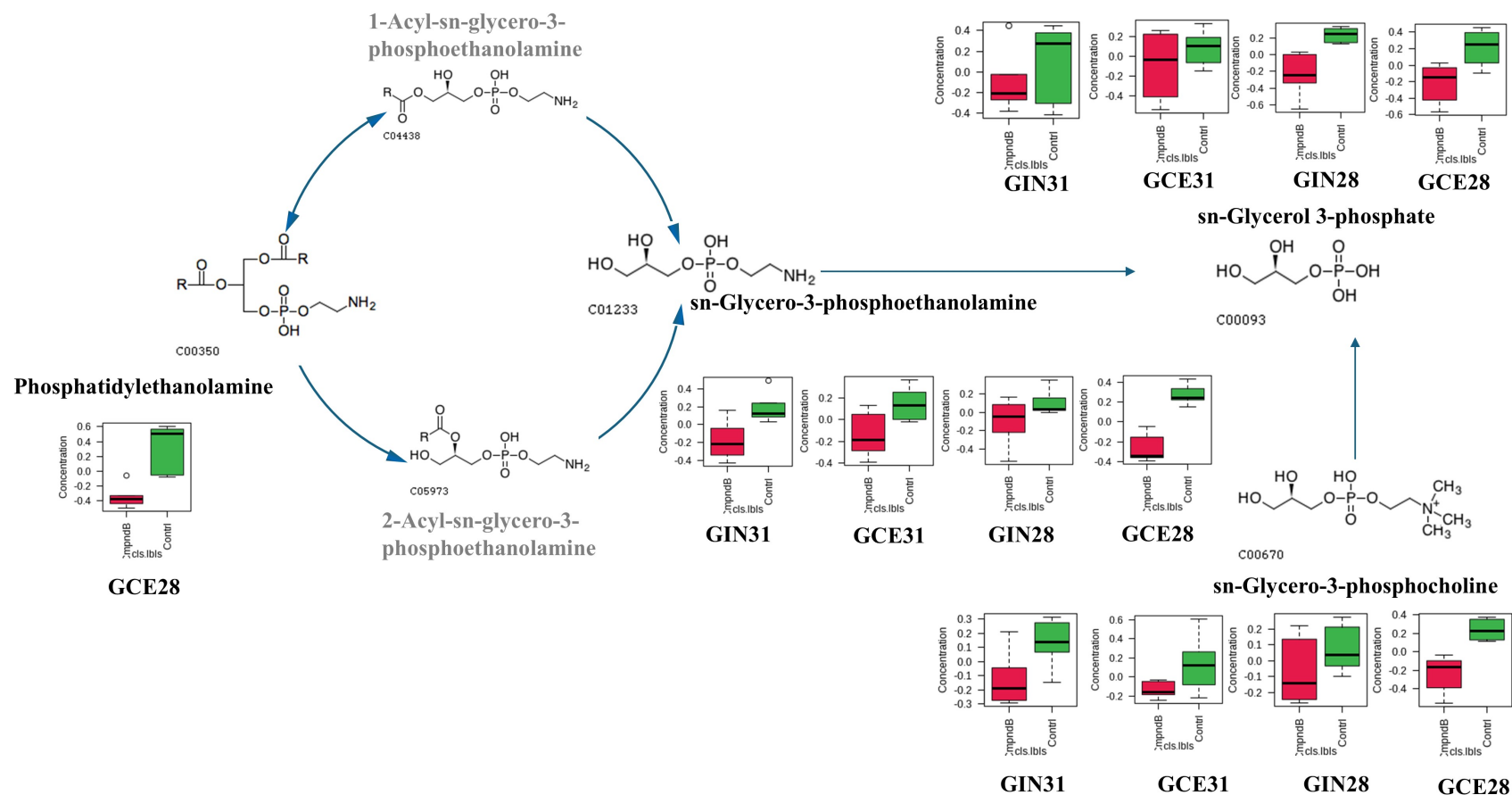


Figure 2.18. Metabolites mapped onto glycerophospholipid metabolism and their abundance in GBM cells upon Caledonine treatment. The normalised abundances of identified metabolites are shown for Caledonine-treated cells in red and untreated cells in green. The abundance of metabolites was exported from MetaboAnalyst v5.0, and reflects transformed and scaled data for visualisation.

2.3.8 Distinct metabolic pattern between untreated GBM cells and astrocytes

To understand whether these identified metabolic pathways affected by three candidates are specifically related to GBM disease, experiments were repeated to compare the metabolic difference between untreated GBM cells and astrocytes. The GBM cells were first successfully adapted to astrocyte medium.

Astrocytes and GBM cells were clearly separated in OPLS-DA scores plots (Figures B.6 and B.7), indicating distinct metabolic differences between GBM cells and astrocytes. Six metabolic pathways (adjusted p value < 0.05 and impact factor > 0.1) were shared across the comparisons of astrocytes vs. GBM cells: alanine, aspartate and glutamate metabolism, sphingolipid metabolism, glutathione metabolism, glycerophospholipid metabolism, TCA cycle, taurine and hypotaurine metabolism.

As shown in Figure 2.19, compared to astrocytes, the abundance of metabolites mapped onto glycerophospholipid metabolism were at higher levels in GBM cells ($p < 0.01$), indicating that this pathway is more active in GBM. The level of hypotaurine was also higher in GBM cells compared to astrocytes, consistent with previous studies reporting significantly higher hypotaurine levels in tumour tissue relative to adjacent control tissue^{211,212}. In addition, a higher level of glutathione was observed in GBM cells. Emerging evidence has revealed a positive association between increased glutathione and chemo-resistance in GBM^{213,214}.

These findings proved that the metabolic pathways affected by the three candidates are closely related to GBM pathology, including energy metabolism, amino acid metabolism, glycerophospholipid and sphingolipid metabolism.

The metabolic differences between GINs and GCEs were also observed when cells were cultured in astrocyte medium (data not shown). The results showed a similar trend with that generated from DMEM medium, providing supportive evidence that adapting GBM cells into astrocytes medium did not change the metabolic pattern between GBM cells.

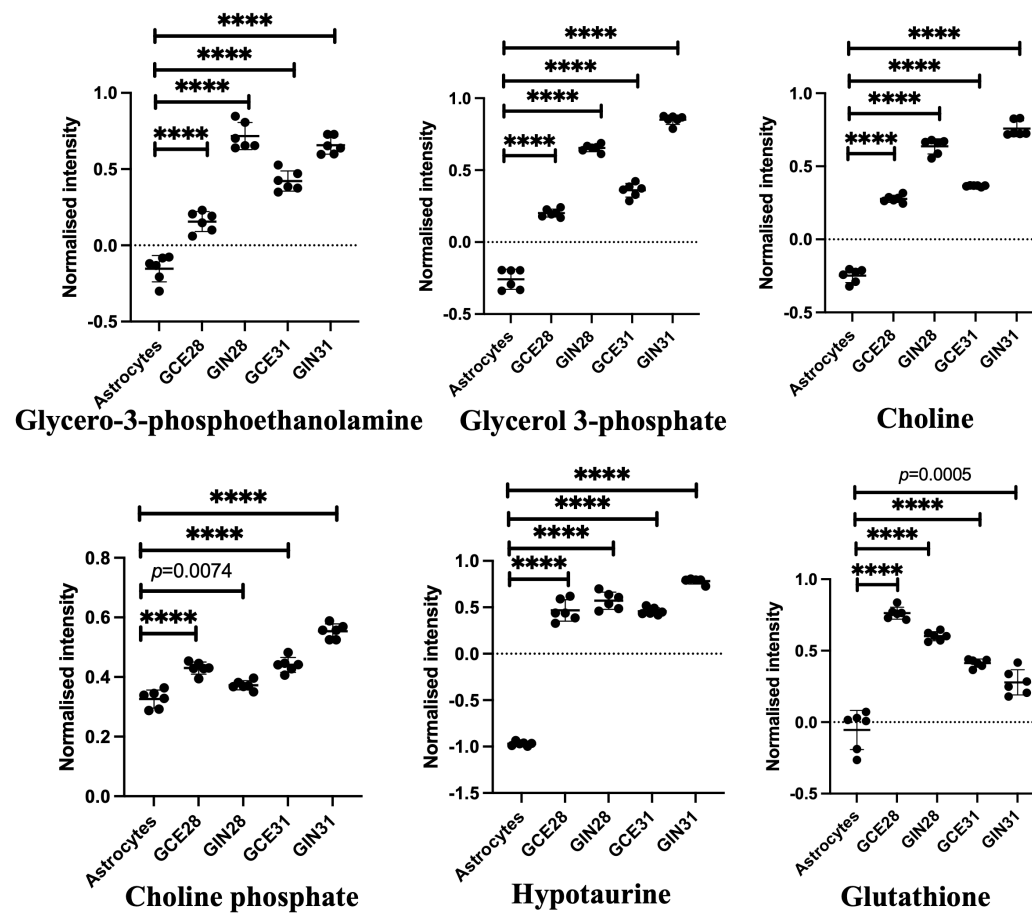


Figure 2.19. The normalised abundance of identified metabolites in astrocytes and GBM cells. The abundance of metabolites were transformed and scaled. The symbol of **** means $p < 0.0001$.

2.3.9 Metabolic pathways altered in astrocytes upon drug treatment

Drug treatment in astrocytes aimed to further explore whether the previously identified pathways affected by the three drugs were specifically associated with anti-tumour functions.

The untreated and treated astrocytes were clearly separated in PCA and OPLS-DA scores plots (Figure B.8), indicating distinct metabolic alterations caused by drug exposure.

As previously mentioned, after Afuresertib treatment, four metabolic pathways were commonly affected across all GBM cell lines. Of these, three pathways (alanine, aspartate and glutamate metabolism, histidine metabolism, and glycerophospholipid metabolism) were also altered in astrocytes with treatment.

Similarly, two pathways affected in GBM cells upon Caledonine exposure were also identified in astrocytes: glycerophospholipid metabolism, and cysteine and methionine metabolism. Three pathways affected in GBM cells by Taxifolin exposure were also identified in astrocytes.

Metabolites (except aspartate) involved in nicotinate and nicotinamide metabolism presented lower abundance in Taxifolin-treated group compared to controls. Additionally, metabolites mapped onto glycerophospholipid metabolism displayed lower abundance in the Afuresertib-treated and Caledonine-treated group, while higher abundance in the Taxifolin-treated group, compared with controls (Figure 2.20).

These findings suggested that the metabolic pathways altered by these drugs were not tumour specific as drug-induced metabolic responses in astrocytes were consistent with those observed in GBM cells. Additionally, the consistent drug response observed in both GBM cells and astrocytes supported the proposed mechanism of action from a metabolomics perspective.

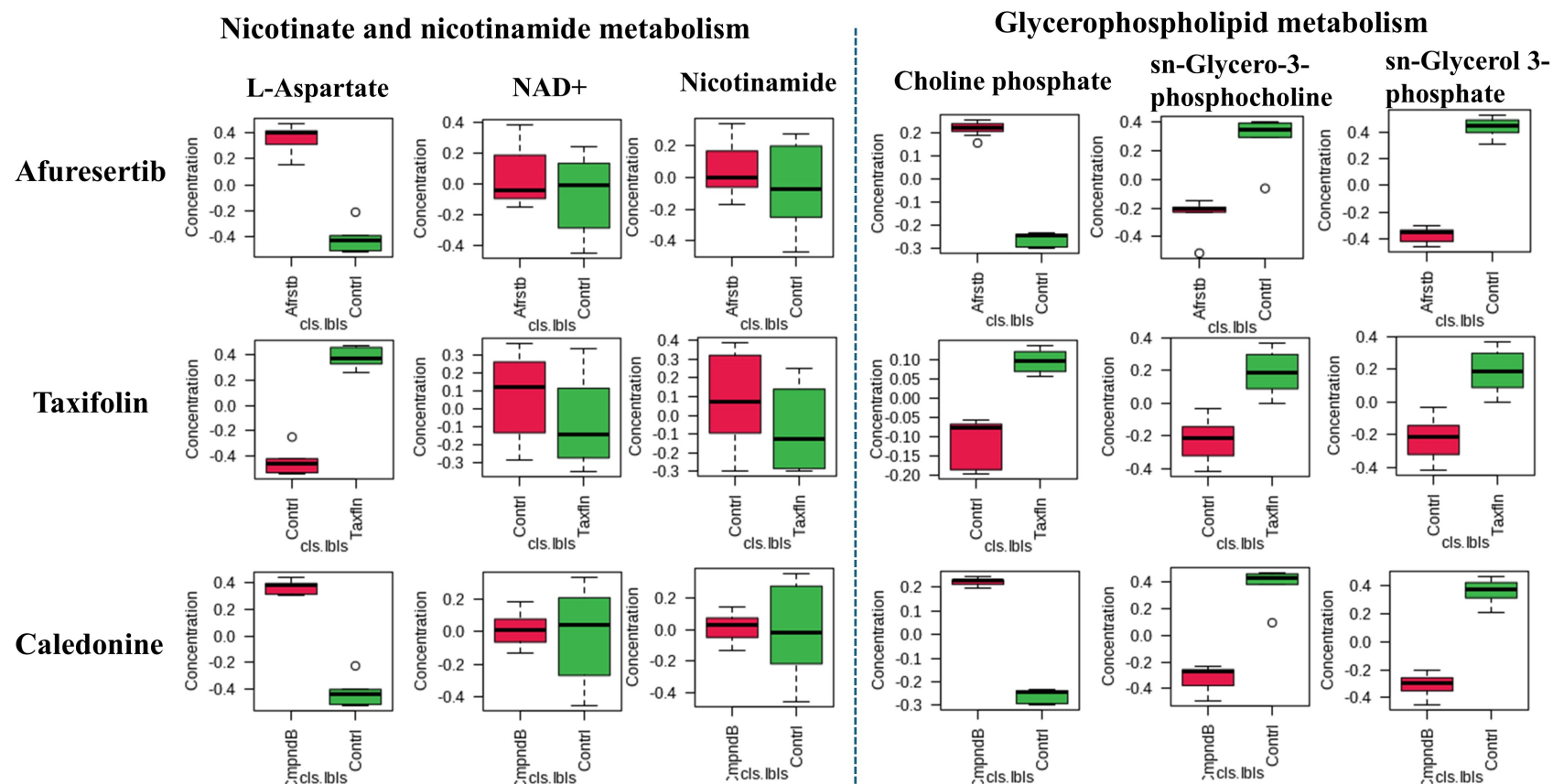


Figure 2.20. Metabolites and their abundance in untreated and treated astrocytes. The normalised abundances of metabolites are shown with Afuresertib-treated or Caledonine-treated cells in red and untreated cells in green, while the normalised abundances of metabolites are shown with untreated cells in red and Taxifolin-treated cells in green. The abundance of metabolites was exported from MetaboAnalyst v5.0, and reflects transformed and scaled data for visualisation.

2.3.10 Invasion assay

As discussed previously, changes in glycerophospholipids and their metabolites are closely related to cell membrane fluidity, adhesion, and invasion. Based on the results of metabolomics study, the metabolites involved in glycerophospholipid metabolism showed lower abundance upon Afuresertib or Caledonine treatment but not upon Taxifolin treatment. Hence, the ability of these candidates to inhibit GBM invasion was next investigated.

Concentrations equal to the IC_{50} values determined from metabolic viability assays were selected for treatment. When applied for 24 h, these concentrations did not affect cell proliferation, indicating that proliferation was not a confounding factor in the invasion assay. For Afuresertib, both IC_{50} and IC_{25} concentrations were tested to evaluate dose-dependent effects. For Taxifolin, 500 μ M was used for most GBM cell lines, as this concentration was below the IC_{25} value, with the IC_{25} concentration additionally tested in GCE28 cells. For Caledonine, only the IC_{50} concentration was tested due to limited sample availability.

The results are presented in Figures 2.21 to 2.24. Considering the comparison of the number of invaded cells between control and drug-treated group, Afuresertib significantly inhibited the ability of GBM cells invading through the collagen barrier in a concentration-dependent manner. Caledonine also exhibited strong inhibition on GBM invasion. No statistical differences were observed between the Taxifolin-treated and control groups across all cell lines, indicating that Taxifolin did not inhibit GBM invasion within this experimental context.

2.4 Further discussion

To develop an effective therapeutic for GBM, its pharmacokinetics profiles, molecular mechanisms, and safety need to be fully investigated. Clinically, single-agent Afuresertib showed a favourable pharmacokinetic and safety profile, with a maximum tolerated dose of 125 mg/day²¹⁵. However, its ability to penetrate the blood-brain

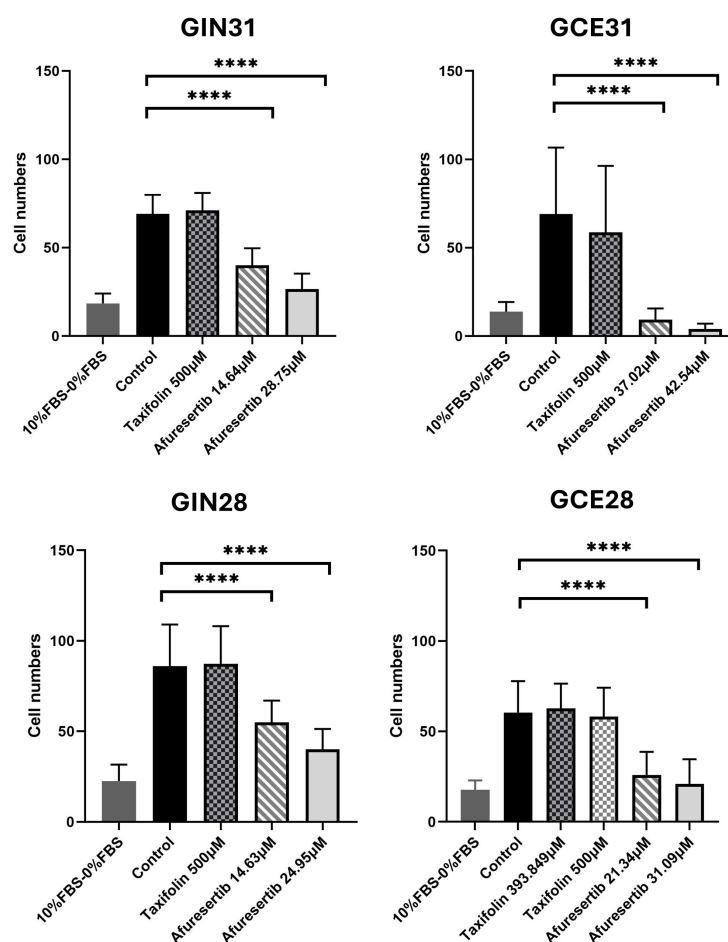


Figure 2.21. Assessment of putative anti-invasion effects on GBM cells upon Afuresertib and Taxifolin treatment. Two concentrations of Afuresertib (IC_{25} and IC_{50}) were tested on GBM cells, while only 500 μ M Taxifolin was tested, except for GCE28 where the IC_{25} concentration of 393.85 μ M was also included. The symbol of **** means $p < 0.0001$.

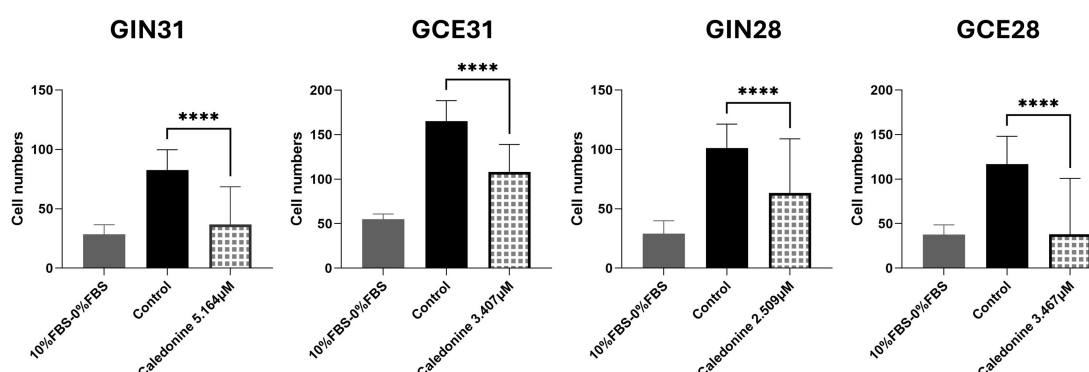


Figure 2.22. Assessment of putative anti-invasion effects on GBM cells upon Caledonine treatment. GBM cells were treated with IC_{50} concentration of Caledonine. The symbol of **** means $p < 0.0001$.

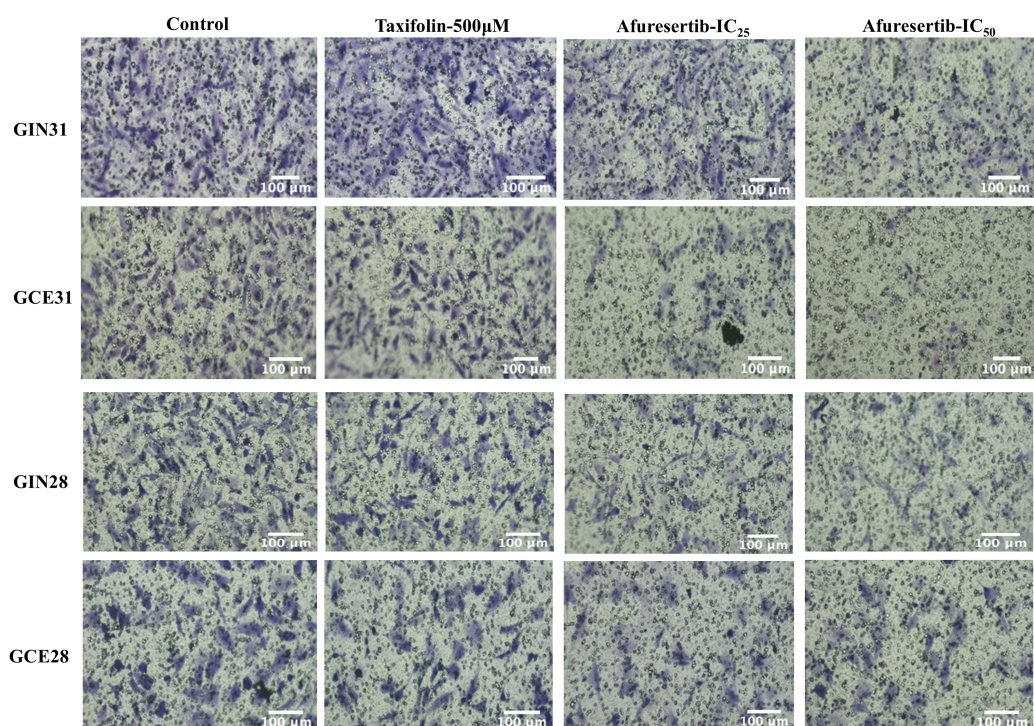


Figure 2.23. Light microscopy of invasion assays performed on GBM cells exposed to Afuresertib and Taxifolin. Each GBM cell line was treated with 500 μ M of Taxifolin, IC₂₅ and IC₅₀ concentrations of Afuresertib. Cells were stained with 0.2% crystal violet. Images from four fields of view were taken under a bright-field microscope with 10X magnification len.

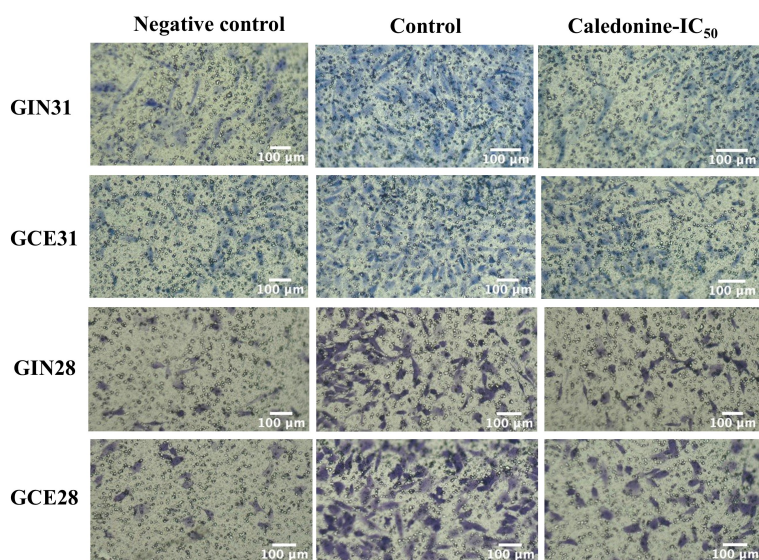


Figure 2.24. Light microscopy of invasion assays performed on GBM cells exposed to Caledonine. Each GBM cell line was treated with the IC₅₀ concentration of Caledonine. Cells were stained with 0.2% crystal violet. Images from four fields of view were taken under a bright-field microscope with 10X magnification len.

barrier has not been reported, raising uncertainty about its therapeutic potential in brain tumours *in vivo*. The safety of Taxifolin has been well studied and toxic effects from overdose have not been reported, including mutagenicity, embryotoxicity and immunotoxicity²¹⁶. Taxifolin was detectable in rat brain²¹⁷. However, its low bioavailability (approximately 0.17%) presents a main limitation for clinical use²¹⁸. In terms of Caledonine, unfortunately, no pharmacokinetics and safety data are reported. Hence, given our *in vitro* findings, clear molecular mechanism, and clinical safety, Afuresertib is identified as the most promising anti-GBM candidate among those investigated.

Although *in vitro* metabolic viability and metabolomics results showed no selectivity of drugs between tumour and healthy cells, further investigation remains warranted. Since laboratory-cultured astrocytes are required to be in a progenitor state (to permit propagation), this does not reflect the majority of astrocytes in the GBM tumour microenvironment, which are terminally differentiated (postmitotic) mature cells and therefore likely to be less sensitive to intracellular drug uptake²¹⁹. This suggests that clinically, there may be a therapeutic window that has not been replicated *in vitro*. Therefore, the predominance of post-mitotic astrocytes in a patient-derived rodent xenograft model will be a more accurate to determine a tolerated dose and a potential therapeutic window.

This study revealed intra-tumour metabolic heterogeneity in GBM at the cellular level. In the previous work on metabolic heterogeneity in IDH wild-type GBM primary tissue, regional heterogeneity in glycolysis and TCA cycle was observed: invasive regions were rich in D-glucose and citrate, while non-invasive areas showed higher levels of ATP and NADH²²⁰. These patterns were not observed in the current study, likely due to differences in patient samples and the use of cell lines rather than primary tumour tissue.

Glycerophospholipid metabolism was identified as more active in GINs relative to GCEs, indicating potential variability in underlying genetic or epigenetic mutations within GINs and GCEs. From the perspective of metabolomics, our results add

to the knowledge base and rationale supportive of deriving GBM cell lines from different locations, with particular appreciation of the clinically relevant invasive margin. Admittedly, the number of included patients where the cells were derived from, was limited in this study, and our results can be further validated by including additional GIN/GCE cell lines.

2.5 Conclusion

This study evaluated the potential of repurposed Afuresertib, Caledonine, and Taxifolin for GBM treatment *in vitro*, and revealed their potential anti-tumour metabolic mechanisms. Observed anti-tumour phenotypes upon these drug exposure provide evidence that these compounds are sufficient to induce these phenotypes *in vitro*, despite being agnostic to whether they are necessary *in vivo*. Further assessment of drug efficacy in GBM xenograft models will be required to validate these findings.

We cannot conclude that our *in vitro* findings fully represent GBM tumour tissue, unless metabolic changes are associated with underlying genetic or epigenetic mutations which are maintained with high fidelity in culture conditions. Moreover, our results did not determine what proportion of observed metabolic profiles in untreated GBM cells are related to *in vitro* propagation and which are representative of *in situ* tumour metabolism. Future work will need to cross-validate these *in vitro* profiles against primary tumour infiltrative margin tissue.

Chapter 3

Metabolic characterisation of glioblastoma invasion at the single-cell level using 3D OrbiSIMS and AP-MALDI-MS

3.1 Introduction

Based on the results of previous metabolomics studies and invasion assays (Chapter 2), two hypotheses are raised: (1) the glycerophospholipid metabolism pathway contributes to GBM invasion; (2) Afuresertib and Caledonine inhibit GBM invasion *in vitro* by disrupting glycerophospholipid metabolism, compared to Taxifolin. To test these hypotheses requires the detection of metabolites from cells during invasion process. However, the number of invaded cells from Transwell inserts is relatively small (approximately 1×10^4 cells), making it unsuitable for conventional LC-MS-based metabolite profiling, which typically consumes around 1×10^6 cells. The impact of sample loss during conventional metabolite extraction can be significantly magnified at this scale. Moreover, the use of mobile phases in LC-MS-based metabolite profiling introduces an additional sample dilution, reducing analyte concentration. Given these

constraints, a highly sensitive analytical approach is required.

Three-dimensional secondary ion mass spectrometry (3D OrbiSIMS) and atmospheric pressure matrix-assisted laser desorption/ionisation mass spectrometry (AP-MALDI-MS) are surface analytical techniques that allow for the direct detection of metabolites from biological samples without the need for conventional extraction procedures. They have been widely applied in metabolic profiling of GBM tissues. Randall *et al.* employed MALDI-MS to investigate the distribution of metabolites in GBM tissues, identifying increased long-chain acylcarnitines at tumour edge, highlighting metabolic differences between the core and edge¹³⁹. He *et al.* revealed the distinct spatial distributions of metabolites in GBM using 3D OrbiSIMS, demonstrating significant intra-tumour heterogeneity¹⁴⁰.

Improvements in spatial resolution, reaching $< 1\ \mu\text{m}$ for 3D OrbiSIMS and 5–20 μm for AP-MALDI-MS, have enabled their application in single-cell metabolomics. Suvannapruk *et al.* differentiated the metabolic signatures of human monocyte-derived macrophages (naïve, pro-inflammatory, and anti-inflammatory phenotypes) at the single-cell level using 3D OrbiSIMS¹⁵¹. Rappez *et al.* applied AP-MALDI-MS-based single-cell metabolomics, distinguishing co-cultured HeLa cells and fibroblasts by their metabolic profiles¹⁴⁶.

Given their capability of minimising sample loss and performing single-cell metabolomics, it was hypothesised that 3D OrbiSIMS and AP-MALDI-MS could be applied for analysing invasion samples. As illustrated in Figure 3.1, the samples collected from the Transwell inserts were successfully analysed using 3D OrbiSIMS and AP-MALDI-MS at the single-cell level, revealing distinct metabolic features associated with GBM invasion.

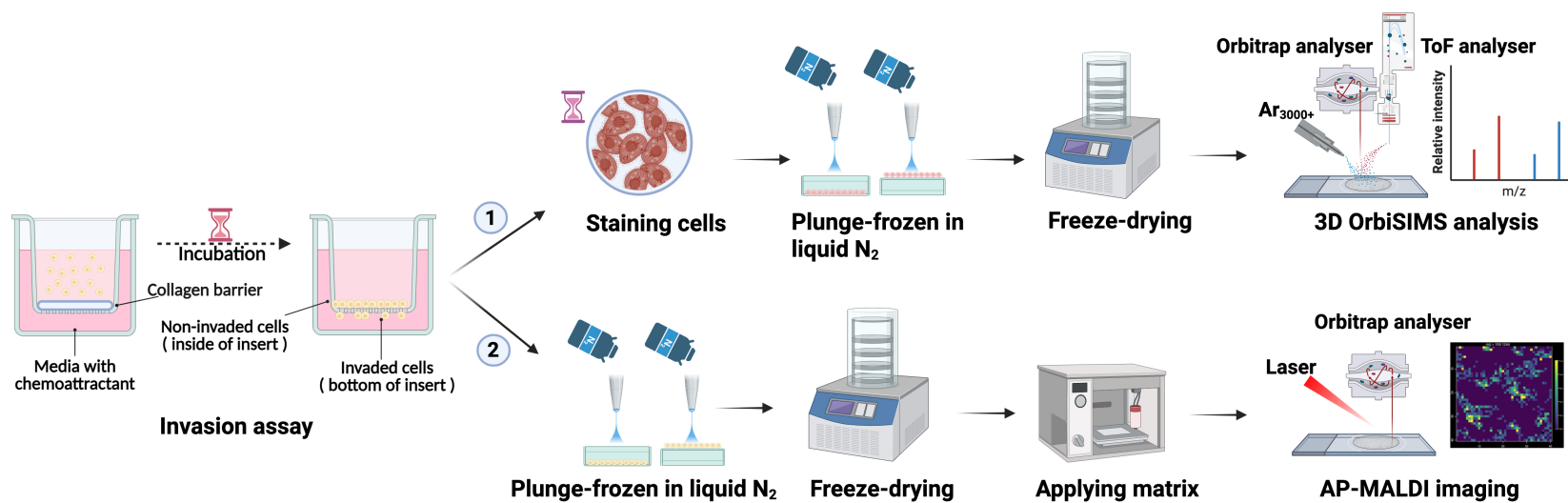


Figure 3.1. Analytical workflow for 3D OrbiSIMS and AP-MALDI-MS analysis. The figure was created with BioRender.com.

3.2 Material and method

3.2.1 Materials

The materials related to cell culture and the invasion assay have been described in Chapter 2.2.1. GIN31 cells were cultured under the conditions described in Chapter 2.2.2.

Diamidino-2-phenylindole (DAPI, Lot No.: YK4114512) was purchased from Fisher Scientific Inc.(Leicestershire, UK). Neutral red solution (0.33%, Lot No.: RNBL8329) and 2,5-dihydroxybenzoic acid (DHB, Lot No.: WXBD0438V) were ordered from Sigma-Aldrich Co. LLC (Gillingham, UK). LC-MS grade ammonium formate (Lot No.: NX749402) was supplied by VWR Chemicals, LLC (Lutterworth, UK).

3.2.2 Sample preparation

3.2.2.1 Invasion assay

The invasion assay was performed following the steps described in Chapter 2.2.5. Briefly, GIN31 cells suspended in DMEM (1.2×10^4 cells/insert) were seeded in pre-soaked inserts. The bottom chambers were filled with 700 μ L of DMEM supplemented with 10% FBS. The samples were further incubated for 12 h to allow the invasion to start.

3.2.2.2 Samples for 3D OrbiSIMS analysis

One hour before sample collection, 5 μ L neutral red solution was gently added to each chamber followed by another 1 h of incubation.

When incubation was concluded, for non-invaded samples, the medium inside the inserts was removed, and the cells were gently washed with pre-warmed PBS. The cells at the bottom of the inserts were removed. The inserts were washed with 150 mM ammonium formate before being plunge-frozen in liquid nitrogen.

For invaded samples, after the removal of non-invaded cells, the inserts were gently washed with pre-warmed PBS and 150 mM ammonium formate. Then, the inserts were placed upside down and plunge-frozen in liquid nitrogen.

Blank samples were inserts without cells and underwent the same steps of invasion assay and staining.

Subsequently, all samples were placed in the freeze dryer (Heto PowerDry PL3000, Thermo Fisher Scientific) over 12 h.

After the samples reached room temperature, the membranes were carefully cut off before being mounted on the sample plate for 3D OrbiSIMS analysis.

3.2.2.3 Samples for AP-MALDI-MS analysis

There was no staining procedure. After freeze drying, the membranes were carefully cut off and adhered to a glass slide with a double-sided tape. Samples were then sprayed with 7.5 mg/mL of DHB solution (70% acetonitrile) for 18 layers using the SunCollect system (SunChrom v1.11). Finally, samples were put into the instrument for analysis.

3.2.3 3D OrbiSIMS analysis

Analysis was performed using a hybrid SIMS instrument (IONTOF, GmbH). To analyse the regions of interest, a 20 keV Ar_{3000}^{+} gas cluster ion beam (GCIB) with a diameter of 20 μm was operated with a cycle time of 200 μs on the Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). The duty cycle was set to 4.4% and continuous GCIB current of 240 pA over an area of $200 \times 200 \mu\text{m}$. The electron flood gun was operated with an energy of 21 V and an extraction bias of 40 V for charge compensation. The pressure in the main chamber was maintained at 8.7×10^{-7} mbar. The Orbitrap analyser was operated in positive mode and negative mode with a mass resolution of 240,000 at m/z 200. The mass range was set to m/z 75 to 1,125.

3.2.4 AP-MALDI-MS imaging

Imaging was performed by an AP-MALDI UHR ion source (MassTech Inc., Columbia, MD, USA), coupled to a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). The laser energy was set to 2.3 μJ per pulse, using a 355 nm Nd:YAG laser operating at a repetition rate of 1000 Hz and a spot size of approximately 10 μm . The samples were raster scanned with a step size of 10 μm to match the spatial resolution and the area of imaging was $400 \times 400 \mu\text{m}$. Spectra were recorded at a mass resolution of 140,000 at m/z 200, with mass range m/z 70 to 1,050.

3.2.5 Data analysis

Images were processed with Fiji (ImageJ v1.54p). Statistical analysis was performed on GraphPad Prism v10.3.0 (GraphPad Software, San Diego, CA), with p value < 0.05 considered statistically significant. Orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed using SIMCA-P v18 (Umetrics, Sweden).

3D OrbiSIMS data were analysed using in-house MATLAB R2024a scripts (The MathWorks, Inc., Natick, MA) and IonTOF SurfaceLab 7 (IONTOF GmbH, Münster, Germany). Peaks (intensity > 1000 and signal-to-noise ratio > 10) were extracted from the spectra, and TXT files were exported for each sample. Then, background subtraction, peak alignment, and imputation of missing value, t-test, and fold change (FC) were performed using internal scripts: <https://github.com/Rui-eebebeb/3D-OrbiSIMS>. Briefly, multiple blank samples were averaged prior to background subtraction. The peaks were aligned with a mass tolerance of 5 ppm. The presence threshold was set to filter out the features existing less than 80% samples. The method of k nearest neighbour ($k=10$) was applied to impute the missing value.

AP-MALDI-MS data were analysed by Shiny Cardinal v0.3.5^{221,222}, including spectra pre-processing, image visualisation, data-driven segmentation, and data export (m/z and corresponding intensity). The exported data from the region of interest were

further analysed using the scripts provided.

The ions were annotated using SIMS-MFP software and the Human Metabolome Database v5.0^{195,223}. $[M-H]^{-1}$ was the adduct type for negative ions, while $[M+H]^{+1}$, $[M+Na]^{+1}$ and $[M+NH_4]^{+1}$ were the adduct type for positive ions. Pathway analysis was performed on MetaboAnalyst v6.0²²⁴.

3.3 Results and Discussion

3.3.1 Optimal sampling window

The invasion assay typically lasts 24 to 48 h to allow cells to fully complete the process. However, over 24 h incubation may be too long, as invaded and non-invaded cells could adapt to a stable state under the same culture conditions, making it challenging to capture metabolic differences related to invasion.

As shown in Figure 3.2, the number of invaded cells increased over the measurement time, reaching its peak at 12 h, which was comparable to 24 h (no statistical significance), indicating that the invaded cells may begin to stabilise metabolic activity after 12 h. These findings suggested that sampling at 10–12 h may provide the optimal window to capture metabolic changes between invaded and non-invaded cells.

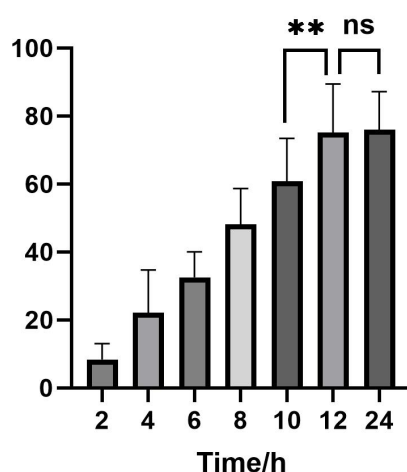


Figure 3.2. Optimal sampling window. Cells were stained by 0.2% crystal violet, and imaged using a multi-viewing microscope (Brunel, N-510) equipped with a 10X magnification len. The ** represents $p < 0.05$ and "ns" indicates no statistical significance.

3.3.2 Invaded and non-invaded cells attached to the membrane

In a preliminary study, cells were unstained for 3D OrbiSIMS analysis. When cells were directly cultured and fixed on a glass slide, they appeared as black dots in high-resolution pictures¹⁵¹. However, numerous black dots were observed in our high-resolution pictures, and the blank membrane itself had a similar black-dot pattern, making it difficult to distinguish between cells and background. Moreover, no cells were visible under the bright-field microscope without staining.

To determine whether the cells detached from the membrane after plunge freezing, the cells were stained as presented in Figure 3.3. The results proved that both invaded and non-invaded cells remained attached on the membrane after preparation procedures.

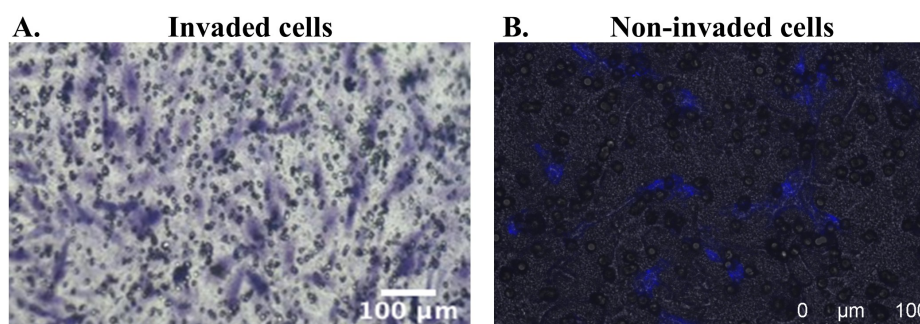


Figure 3.3. Invaded and non-invaded cells attached to the membrane. (A): Invaded cells were stained with 0.2% crystal violet, and imaged using a multi-viewing microscope (Brunel, N-510) equipped with a 10X magnification len. (B): Non-invaded cells were stained with 1 µg/mL DAPI, and imaged by a confocal laser scanning microscope (Leica, DMI4000B) with a 20X magnification len (fluorescence at 360/470 nm).

3.3.3 Neutral red staining

To make samples detectable for 3D OrbiSIMS, cells were stained with neutral red. Neutral red could penetrate the cell membrane and concentrate in the lysosomes of viable cells²²⁵. Repetto *et al.* successfully stained living SH-SY5Y human neuroblastoma cells with neutral red²²⁶.

Neutral red was added to the Transwell chamber 1 h before sample collection, with three concentrations (8.25, 16.5 and 33 µg/mL) evaluated. Two concentrations of neutral red (16.5 and 33 µg/mL) provided clear visualisation of cells on the insert

membrane (Figure 3.4).

As shown in Figure 3.5, the PrestoBlue assay confirmed that neutral red (16.5 $\mu\text{g/mL}$) was non-toxic to GIN31 cells, indicating that cellular metabolic activity was not impaired. This finding was further validated in other GBM cell lines, including GCE31, GIN28, and GCE28, where 16.5 $\mu\text{g/mL}$ neutral red also did not show cytotoxicity up to 4 h of treatment (data not shown).

Based on these findings, the optimised staining protocol was adding 16.5 $\mu\text{g/mL}$ neutral red to the Transwell chamber at 11 h of incubation.

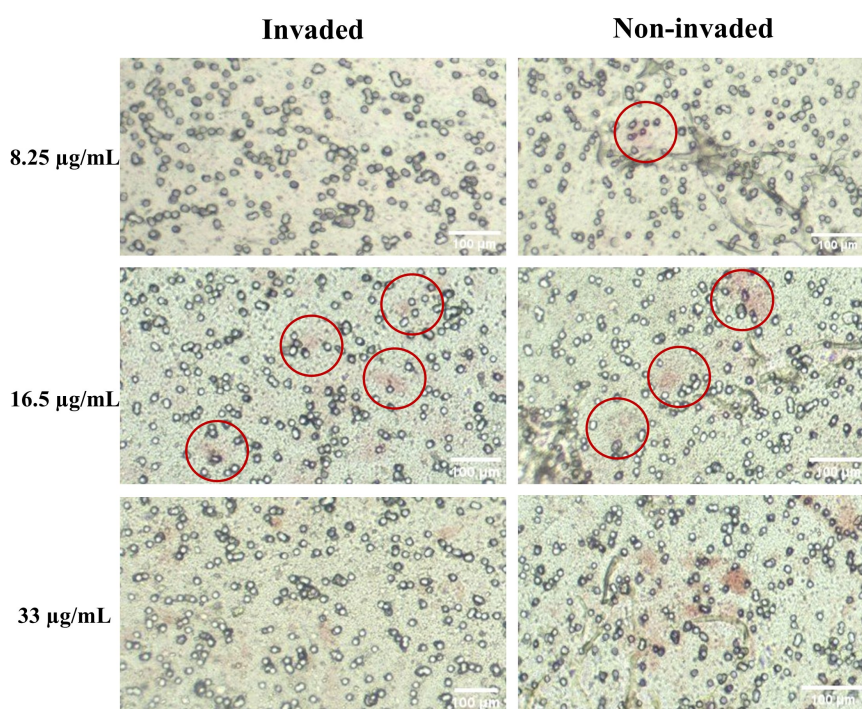


Figure 3.4. Optimal concentration of neutral red staining. The invaded and non-invaded cells were incubated with 8.25, 16.5 and 33 $\mu\text{g/mL}$ neutral red for 1 h in the chamber. The membranes were imaged using a multi-viewing microscope (Brunel, N-510) equipped with a 20X magnification len. The red circles highlight single cells stained with neutral red.

3.3.4 Metabolic differences between invaded and non-invaded cells detected by 3D OrbiSIMS

Representative spectra in positive and negative modes are presented in Figure 3.6. A distinction was observed between the spectra of invaded and non-invaded cells in positive mode, indicating metabolic differences between the two groups. However, the

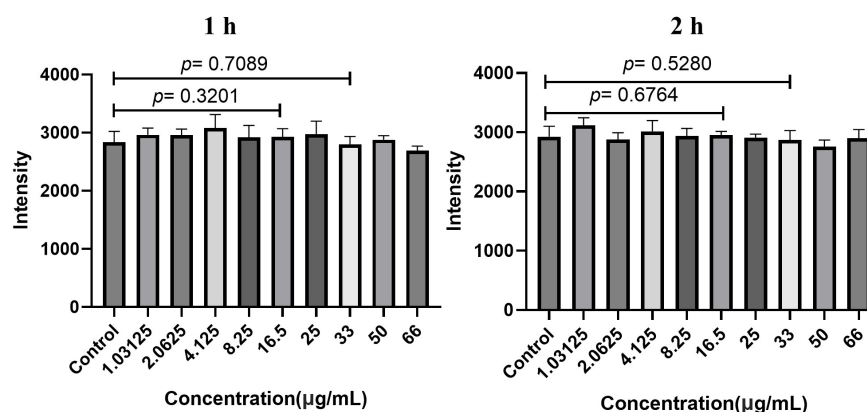


Figure 3.5. Cytotoxicity of neutral red. Neutral red was tested on GIN31 cells at concentrations ranging from 1 to 66 µg/mL, and fluorescence measurements of PrestoBlue assay were conducted after 1 and 2 h treatments.

spectra generated in negative mode did not present sufficiently distinct patterns.

Volcano plots (Figure 3.7) present the metabolic differences between invaded and non-invaded group, with a greater number of positive ions more abundant in non-invaded cells compared to invaded cells. Although negative ions presented large fold changes (FC), none reached statistical significance. These findings indicated that metabolic reprogramming may accompany GBM invasion.

In OPLS-DA scores plots (Figures 3.8 and B.9), clear separation of invaded and non-invaded groups also suggested distinct metabolic profiles. Positive ions with variable importance in projection (VIP) value > 1 and adjusted p value < 0.05 were considered significantly altered, shown in Table 3.1. Significantly altered negative ions were selected based on VIP value and FC results, presented in Table 3.2.

For pathway analysis (Figure 3.9), there were no statistically significant pathways in negative mode. For the positive mode, only glycerophospholipid metabolism met the threshold of adjusted p value < 0.05 and impact factor > 0.1 , shown in Table 3.3.

Four metabolites were assigned to the glycerophospholipid metabolism, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), choline phosphate, and choline. As shown in Figure 3.10, the abundance of PE, PC, and choline phosphate was higher in invaded cells compared to non-invaded cells. In contrast, choline was more abundant in non-invaded cells.

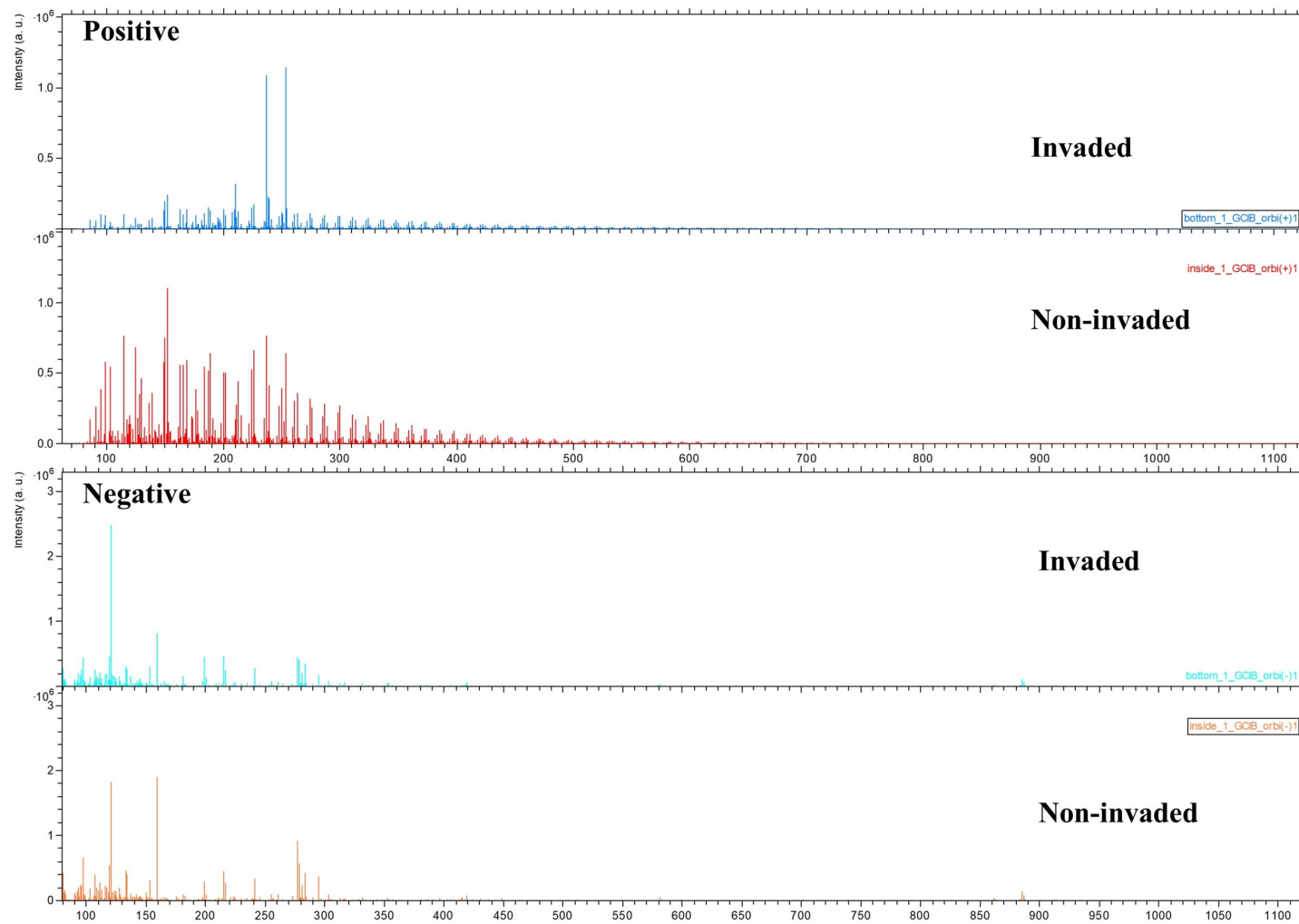


Figure 3.6. Representative spectra acquired from 3D OrbiSIMS.

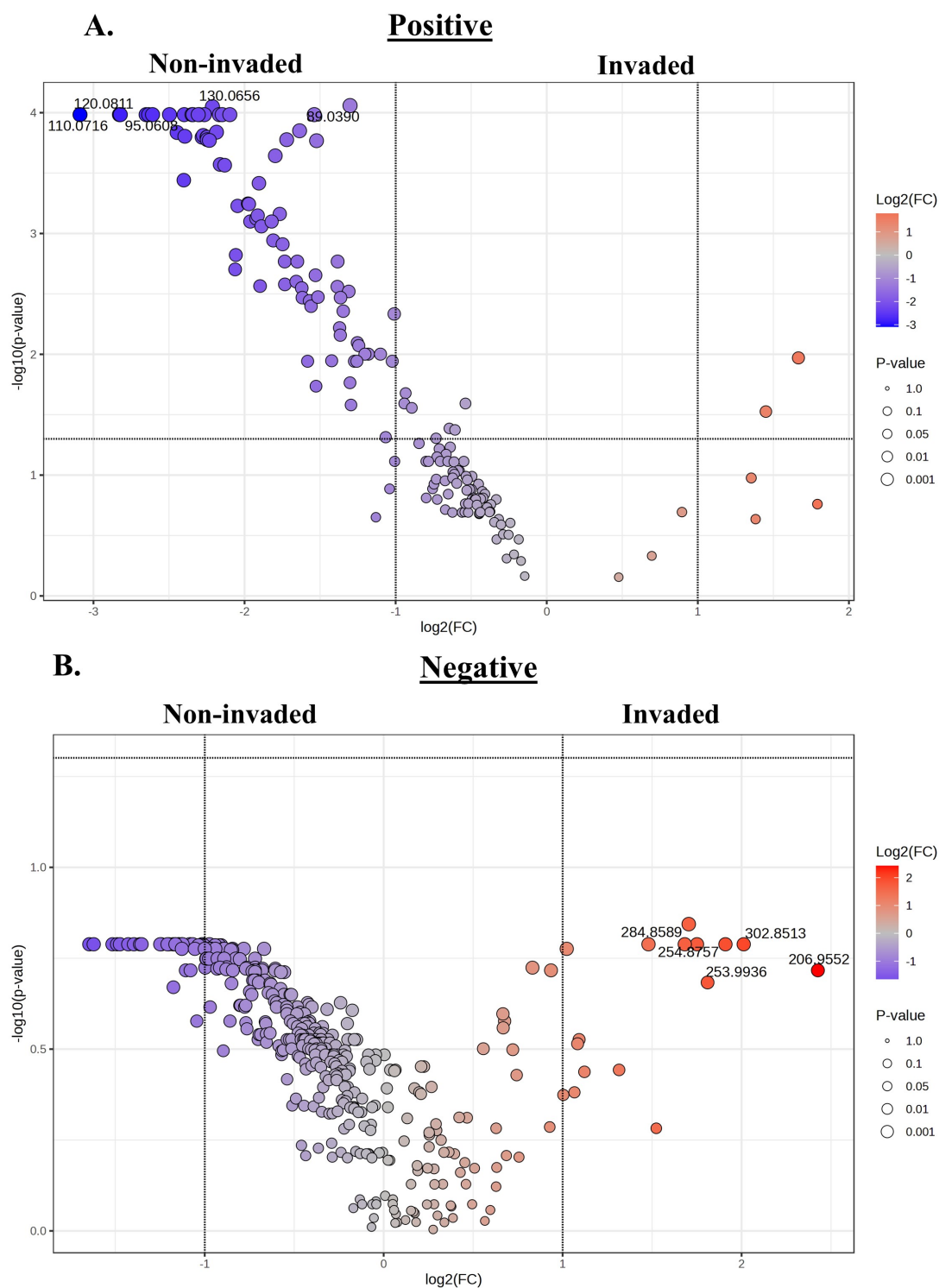


Figure 3.7. Metabolic variations between invaded and non-invaded cells analysed by 3D OrbiSIMS. (A) and (B) are volcano plots showing metabolic differences between invaded and non-invaded cells in positive and negative modes, respectively. Ions are highlighted with fold change > 2 and adjusted $p < 0.05$.

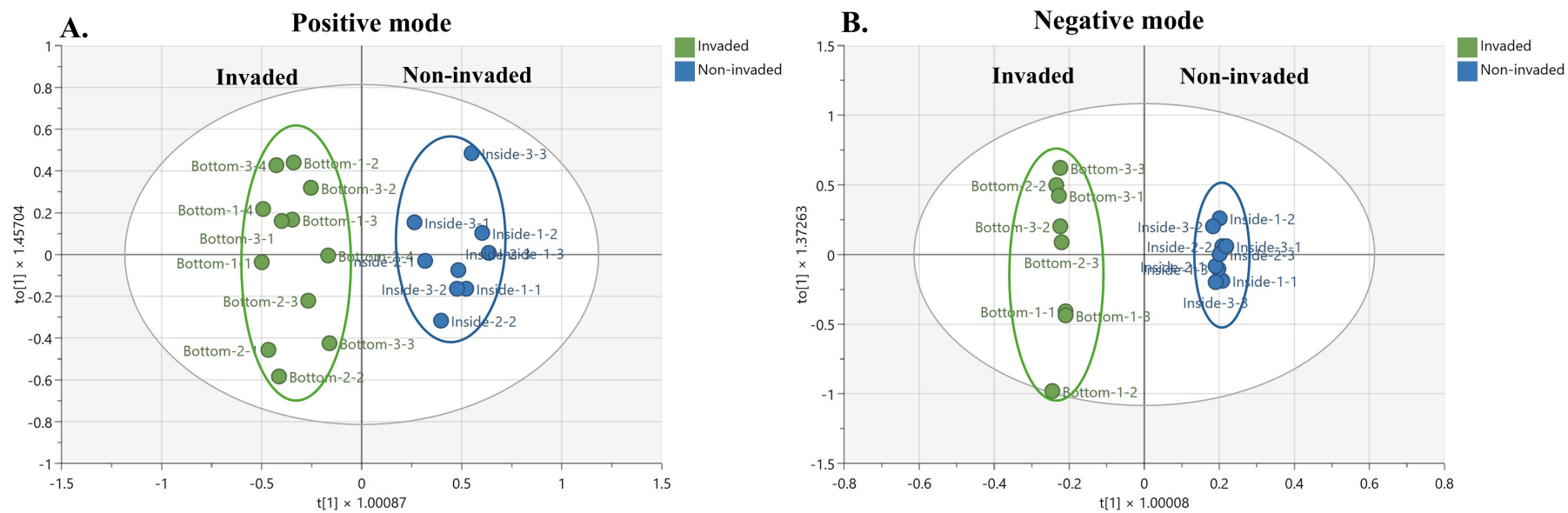


Figure 3.8. OPLS-DA scores plots of invaded vs. non-invaded cells. OPLS-DA scores plot for invaded vs. non-invaded cells in positive ($R^2Y = 0.926$, $Q^2 = 0.798$) and negative ($R^2Y = 0.997$, $Q^2 = 0.924$) ionisation mode.

Table 3.1. Significantly altered positive ions between invaded and non-invaded cells detected by 3D OrbiSIMS (adjusted p value < 0.05 and VIP > 1).

m/z	Assigned formula	Δ ppm	DBE	FC	Adj. p -value	VIP	Putative metabolite
77.0389	C ₆ H ₅	4.20	4.5	0.23	1.99E-04	1.36	1-Methylpyrrolinium
	C ₂ H ₈ NP	0.18	0				
80.0499				0.38	1.95E-02	1.08	
81.0452				0.19	5.13E-03	1.34	
83.0609				0.21	5.65E-03	1.43	
84.0449				0.23	1.13E-02	1.32	
84.0813	C ₅ H ₁₀ N	0.00		0.30	8.31E-03	1.26	
86.0969				0.45	5.53E-03	1.05	
92.0499	C ₆ H ₆ N	4.62	4.5	0.23	2.08E-03	1.34	
	C ₂ H ₉ N ₂ P	1.25	0				
92.0580	C ₂ H ₈ O ₂ N ₂	-0.32	0	0.24	1.13E-03	1.42	2-Hydroxypyridine
93.0452	CH ₈ N ₃ P	1.78	0	0.20	2.39E-03	1.48	
93.0577	C ₆ H ₇ N	4.30	4	0.23	2.39E-03	1.42	
93.0703	C ₇ H ₉	4.55	3.5	0.19	2.91E-03	1.40	
94.0655	C ₆ H ₈ N	3.99	3.5	0.27	2.11E-03	1.33	
95.0496	C ₆ H ₆ O	5.00		0.37	5.06E-06	1.15	
95.0608	C ₅ H ₆ N ₂	4.00		0.18	2.41E-03	1.55	
96.0448	C ₅ H ₅ NO	4.00		0.20	2.18E-03	1.48	
96.0812				0.34	6.17E-03	1.22	
97.0401				0.27	2.32E-03	1.30	
98.9845	CH ₃ ONPNa	0.55	1.5	0.34	1.26E-03	1.29	Phosphate
100.0760				0.34	8.15E-03	1.09	
102.0468	C ₄ H ₉ NP	0.87	1.5	0.33	7.11E-06	1.12	
102.9260				0.28	3.84E-03	1.28	Choline
104.0498	C ₃ H ₉ N ₂ P	0.14	1	0.30	1.36E-03	1.23	
104.1073	C ₅ H ₁₄ NO	2.00		0.33	3.65E-03	1.28	
105.0451	C ₂ H ₈ N ₃ P	0.63	1	0.26	8.01E-05	1.42	
105.0703	C ₈ H ₈	4.00		0.36	9.51E-04	1.20	
106.0654	C ₃ H ₁₁ N ₂ P	-0.33	0	0.25	1.29E-03	1.44	

Table 3.1 (continued)

m/z	Assigned formula	Δ ppm	DBE	FC	Adj. <i>p</i> -value	VIP	Putative metabolite
107.0495	C ₃ H ₁₀ ONP	0.46	0	0.18	2.15E-03	1.39	Histamine
107.0607	C ₂ H ₁₀ N ₃ P	0.15	0	0.25	2.40E-03	1.34	
110.0603				0.20	2.39E-03	1.39	
110.0716				0.14	3.63E-03	1.63	
112.0873	C ₅ H ₉ N ₃	3.00		0.16	2.37E-03	1.47	
116.0499	C ₄ H ₉ N ₂ P	0.99	2	0.20	1.10E-03	1.41	
119.0356				0.22	2.40E-03	1.34	
119.0607	C ₃ H ₁₀ N ₃ P	0.13	1	0.19	2.10E-03	1.42	
120.0447	C ₃ H ₉ ON ₂ P	0.00	1	0.38	2.12E-03	1.12	
120.0811	C ₄ H ₁₃ N ₂ P	0.12	0	0.17	2.09E-03	1.56	
121.0764	C ₃ H ₁₂ N ₃ P	0.54	0	0.33	3.44E-03	1.25	Mandelonitrile
125.0001	C ₃ H ₅ ONPNa	0.04	2.5	0.33	1.20E-03	1.33	
125.0712				0.35	6.89E-03	1.19	
126.0035	CH ₄ O ₆ N	1.47	0.5	0.45	1.01E-02	1.01	
	C ₂ H ₃ O ₃ N ₂ Na	-0.70	2.5				
130.0656	C ₅ H ₁₁ N ₂ P	1.27	2	0.20	1.36E-03	1.35	
131.0607	C ₄ H ₁₀ N ₃ P	0.12	2	0.44	5.94E-03	1.04	
134.0603	C ₈ H ₇ ON	2.00		0.20	1.80E-03	1.39	
136.0508	C ₆ H ₆ ON ₃	1.93	5.5	0.27	2.19E-03	1.31	
136.0621	C ₅ H ₅ N ₅	2.00		0.26	6.15E-03	1.24	Adenine 2-Phenylacetamide Nicotiny methylamide
136.0760	C ₈ H ₉ ON	2.00		0.16	2.33E-03	1.47	
137.0712	C ₇ H ₉ ON ₂	1.90	4.5	0.34	2.02E-03	1.16	
139.0158	C ₃ H ₇ O ₄ P	2.00		0.31	2.05E-03	1.41	
144.0447	C ₅ H ₉ ON ₂ P	0.00	3	0.28	2.24E-03	1.23	
145.0764	C ₅ H ₁₂ N ₃ P	0.45	2	0.44	8.51E-03	1.06	
146.0603	C ₉ H ₈ ON	1.78	6.5	0.19	1.02E-03	1.39	
	C ₅ H ₁₁ ON ₂ P	-0.34	2				
153.0410	C ₅ H ₄ O ₂ N ₄	2.00		0.24	2.24E-03	1.25	
156.0811	C ₇ H ₁₃ N ₂ P	0.10	3	0.18	1.32E-03	1.43	Xanthine
159.0920	C ₆ H ₁₄ N ₃ P	0.10	2	0.19	2.11E-03	1.39	
160.0761	C ₁₀ H ₉ ON	3.00		0.28	2.05E-03	1.28	Indoleacetaldehyde

Table 3.1 (continued)

m/z	Assigned formula	Δ ppm	DBE	FC	Adj. <i>p</i> -value	VIP	Putative metabolite
164.0233	C ₅ H ₉ O ₄ P	0.02	2	0.41	2.32E-03	1.05	Phosphorylcholine
	C ₁₀ H ₅ ONa	0.24	8.5				
	C ₆ H ₈ ONPNa	-1.65	4				
170.0604	C ₇ H ₁₁ ON ₂ P	0.29	4	0.31	2.16E-03	1.18	
174.9857				0.42	9.24E-03	1.07	
184.0742	C ₅ H ₁₅ O ₄ NP	2.00		0.45	2.10E-03	1.09	
190.9632	C ₃ H ₆ O ₄ P ₂ Na	-0.80	2	0.33	2.23E-03	1.20	
212.1146	C ₈ H ₁₀ O ₂ N ₄	2.00		3.37	3.94E-03	1.03	
216.9789	C ₅ H ₃ O ₅ N ₃ PS	0.49	6	0.39	5.89E-03	1.10	
	C ₅ H ₈ O ₄ P ₂ Na	-0.47	3				
229.1018	C ₁₄ H ₁₆ NP	1.37	8	0.30	1.29E-02	1.23	
	C ₁₀ H ₁₉ N ₂ P ₂	0.02	3.5				
	C ₁₃ H ₁₈ SNa	-1.49	5				
252.9192	C ₉ H ₄ ONP ₃ S	0.71	9.5	0.32	2.16E-03	1.24	
	C ₃ H ₅ O ₆ P ₃ Na	0.32	3				
254.9522	C ₃ H ₂ O ₉ N ₃ P	-0.47	5	0.43	1.92E-02	1.05	
	C ₉ H ₆ O ₃ P ₃	-0.90	8.5				
	CH ₅ O ₁₂ NS	-1.97	0				
	C ₁₄ H ₂ P ₂ Na	-0.75	15				
	C ₁₀ H ₅ NP ₃ Na	-1.97	10.5				
	C ₆ H ₆ O ₂ N ₂ P ₂ SNa	-1.80	6				
	C ₂ H ₉ O ₂ N ₃ P ₃ SNa	0.58	11.5				
	C ₃ H ₂ O ₈ N ₃ PS	1.20	5				
270.9298	C ₉ H ₆ O ₂ P ₃ S	0.79	8.5	0.36	1.84E-02	1.06	
	C ₃ H ₇ O ₇ P ₃ Na	0.79	8.5				

m/z is accurate mass. Assigned formula is theoretical formula calculated by MFP software. Δ ppm is mass error. DBE is double bond equivalent. FC represents fold change. Adjusted *p* is generated using Benjamini-Hochberg correction.

Table 3.2. Significantly altered negative ions between invaded and non-invaded cells detected by 3D OrbiSIMS (FC > 1.5 or < 0.5, and VIP > 1).

m/z	Assigned formula	Δ ppm	DBE	FC	Adj. <i>p</i> -value	VIP	Putative metabolite/Other possible formula
176.9963				0.49	0.54	1.15	
201.9763	C ₉ H ₃ N ₂ PS	1.47	10	2.29	0.44	1.07	C ₅ H ₆ N ₃ P ₂ S
206.9552	C ₈ H ₂ NO ₂ PS	1.28	9	5.28	0.39	1.54	C ₄ H ₅ N ₂ O ₂ P ₂ S
220.8813				0.50	0.53	1.48	
240.9151				0.38	0.08	1.41	
253.9936	C ₉ H ₉ ON ₂ P ₃	1.10	8	3.58	0.42	1.38	CH ₈ O ₁₀ N ₃ S, C ₇ H ₁₂ O ₄ P ₂ S, C ₃ H ₁₅ O ₄ NP ₃ S
254.8757				3.65	0.46	1.22	
282.8558				2.79	0.41	1.57	
284.8589				3.06	0.37	1.50	
300.8365				2.04	0.35	1.15	
300.8664				2.32	0.46	1.13	
302.8513				3.92	0.37	1.74	
356.8053				0.38	0.38	1.23	
366.0222	C ₂₈ H ₂ N ₂	-0.40	29	2.07	0.38	1.04	C ₁₃ H ₈ O ₁₀ N ₃ , C ₁₅ H ₁₀ O ₁₁ , C ₂₄ H ₅ N ₃ P C ₉ H ₁₂ O ₄ P ₂ , C ₁₅ H ₁₅ O ₄ N ₁ P ₃ , C ₇ H ₁₄ O ₁₃ N ₂ S C ₃ H ₁₇ O ₁₃ N ₃ PS, C ₉ H ₂₁ O ₇ P ₃ S
390.8707				0.40	0.33	1.40	
402.8023				2.80	0.39	1.58	
418.7793				2.02	0.39	1.25	
420.8003				2.55	0.54	1.73	
432.8064				0.42	0.26	1.50	
448.7842				0.46	0.23	1.09	
512.7728				0.34	0.43	1.29	
861.5495	C ₄₅ H ₈₂ O ₁₃ P	-0.41	5.5	0.50	0.37	1.00	PI 36:2/PI O-36:3;O

m/z is accurate mass. Assigned formula is theoretical formula calculated by MFP software. Δ ppm is mass error. DBE is double bond equivalent. FC represents fold change. Adjusted *p* is generated using Benjamini-Hochberg correction.

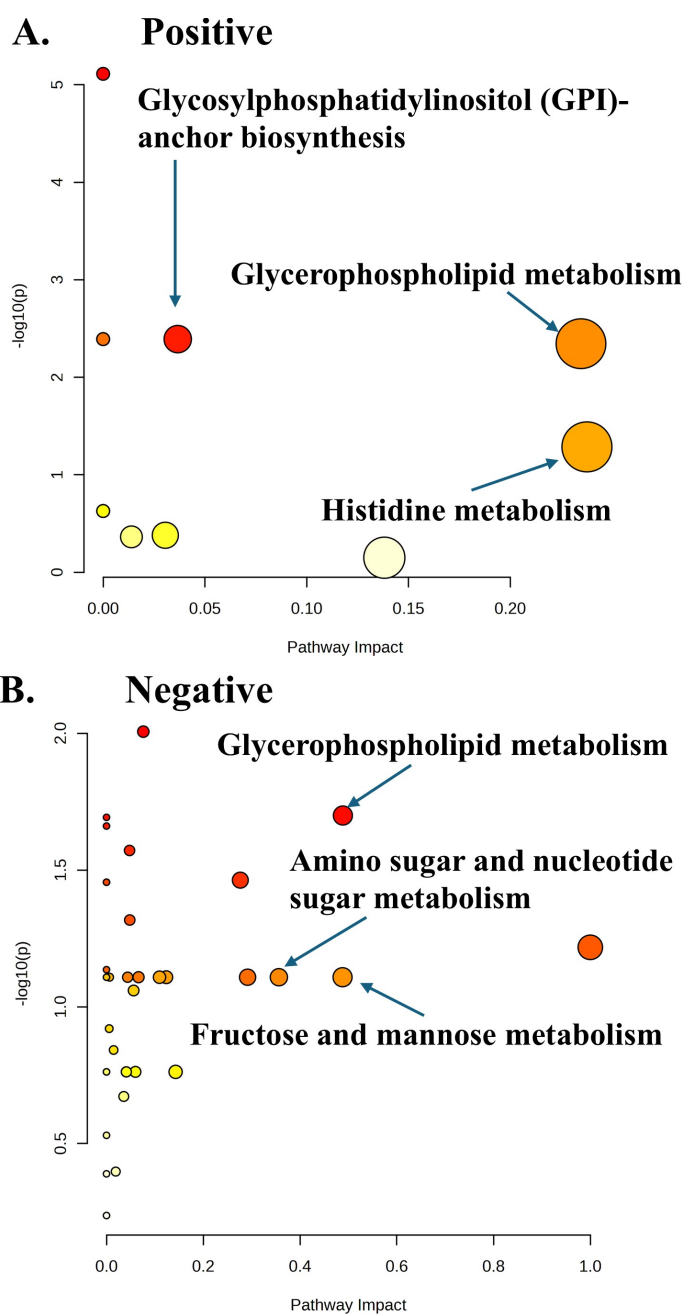


Figure 3.9. Pathway analysis of invaded vs. non-invaded cells analysed by 3D OrbiSIMS. The size of bubble represents the number of compounds mapped on the pathway. The colour represents the degree of significance: white (non-significant) to red (statistically significant). The impact factor represents the importance of an identified pathway within the whole metabolic network.

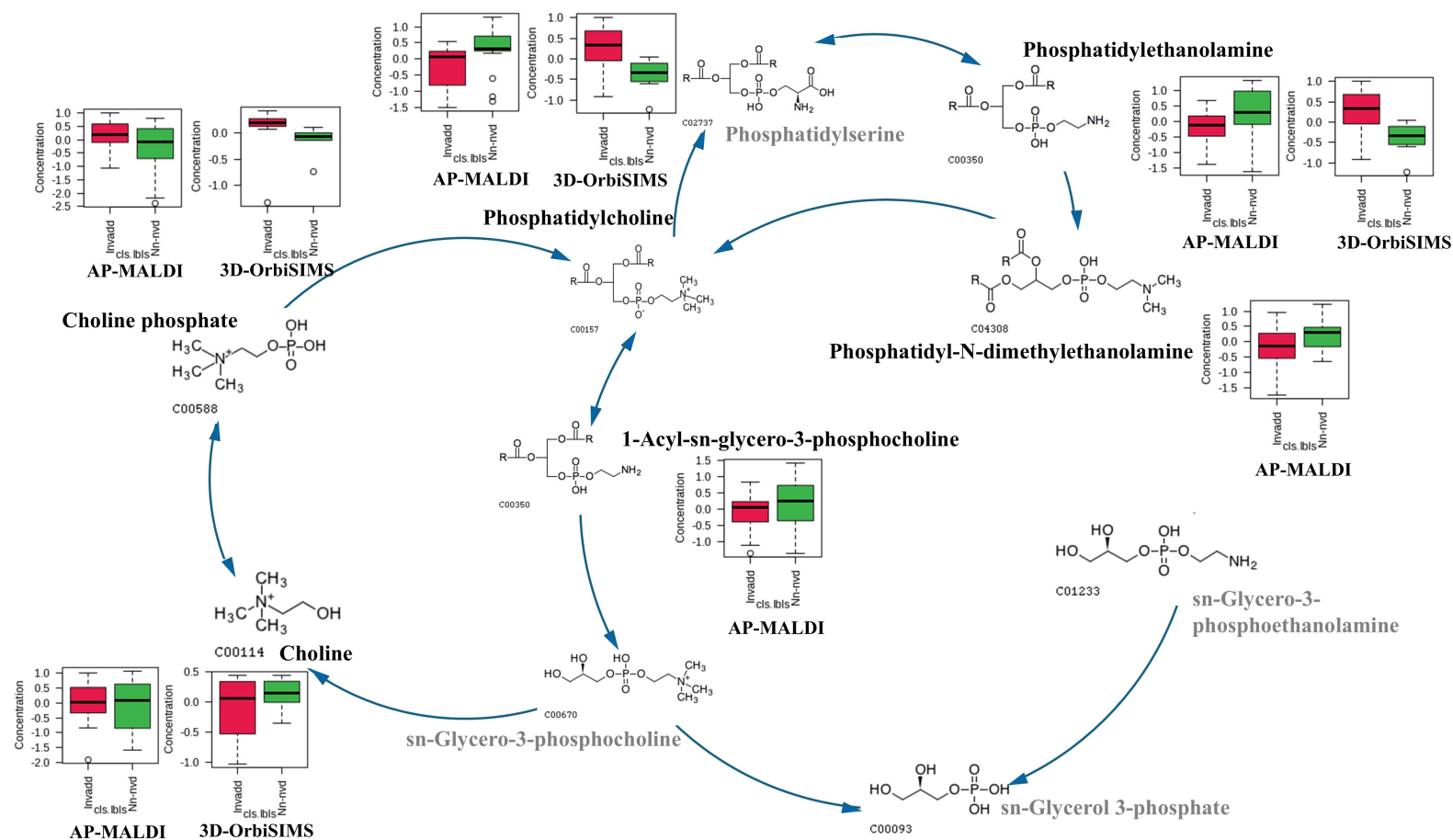


Figure 3.10. Metabolites identified in glycerophospholipid metabolism and their abundance between invaded and non-invaded cells. The normalised abundance of metabolites are shown with invaded cells in red and non-invaded cells in green. The abundance of metabolites was exported from MetaboAnalyst v6.0, and reflects transformed and scaled data for visualisation.

Table 3.3. Significant metabolic pathways between invaded and non-invaded cells analysed by 3D OrbiSIMS in positive mode (adjusted p value < 0.05 and impact factor > 0.1).

Pathway name	Total compounds	Hits	Raw p	Adjusted p	Impact factor	Overlap score
Glycerophospholipid metabolism	36	4	4.53E-03	9.81E-03	0.23	0.75

"Total compounds" refers to the number of compounds involved in a specific pathway. "Hits" represents the number of ions mapped to the pathway. Adjusted p is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network. Overlap score is the ratio of the number of unique m/z values to the number of ions mapped to the pathway.

3.3.5 Metabolic differences between invaded and non-invaded cells detected by AP-MALDI-MS

Based on the results of the 3D OrbiSIMS analysis, staining cells with neutral red may have obscured signals from certain metabolites. Additionally, during the process of spraying matrix, the dye dispersed across the plastic membrane of the insert, further complicating the localisation of cells. Hence, AP-MALDI-MS analysis was operated in label-free mode.

The ions of histidinol (m/z 159.1244) and malic acid (m/z 152.0558), previously detected in GIN31 cells under identical conditions using AP-MALDI-MS, were used for image visualisation. As shown in Figure 3.11, both invaded and non-invaded samples exhibited distinct spatial patterns compared to their blank controls, reflecting the presence of GIN31 cells on the insert membrane.

Images based on the first-two principal components (PC) exhibited a pattern similar to the images constructed using the previous two targeted ions, presented in Figure 3.12. The ions contributing to the positive loading of PC2 are primarily from cells, while those contributing to the negative loading of PC2 are associated with the membrane of insert. These results indicated that cells and the membrane of inserts can be effectively distinguished.

Next, spatial-aware shrunken centroids clustering, a spatially constrained clustering method, was applied to distinguish single cells, shown in Figure 3.13. This method is an unsupervised statistical approach that groups nearby spatial locations with similar

molecular signals while reducing noise, allowing single cells to be distinguished from the insert membrane based on their spatial and molecular characteristics²²⁷. The segmentation pattern closely resembles that produced by PCA, further supporting the conclusion that the identified regions correspond to cells.

The invaded and non-invaded cells were clearly separated on the well-constructed OPLS-DA scores plot (Figures 3.14 A and B.10), suggesting the presence of metabolic differences. The significant altered ions are summarised in Table 3.5. Finally, four metabolic pathways were identified as significant, shown in Figure 3.14 B and Table 3.4.

Tyrosine metabolism contributes to the progression of GBM. In our study, tyrosine itself was not detected, but its downstream metabolites, such as 3-(4-hydroxyphenyl)pyruvate, 3,4-dihydroxy-L-phenylalanine, and dopaquinone, were found to be more abundant in non-invaded cells. Supporting this observation, Yamashita *et al.* found that tyrosine metabolism is upregulated in the GBM tumour core, compared to the edge²²⁸. This suggests that altered tyrosine metabolism may be associated with differences in invasive potential.

Nicotinamide and aspartate, key metabolites in nicotinate and nicotinamide metabolism, were found at lower levels in invaded cells. It has been reported that invasive GBM cells exhibit elevated reactive oxygen species and energy consumption, increasing the demand for NAD⁺ to maintain redox balance²²⁹. Precursors like nicotinamide and aspartate may be rapidly consumed in invaded cells to sustain NAD⁺ production, reflecting increased NAD⁺ turnover required for GBM invasion.

The one-carbon metabolism pathway utilises folate to transfer one-carbon units essential for the synthesis of nucleotides and amino acids. Within this context, adenosine plays a crucial role in purine salvage and cellular energy metabolism. Adenosine depletion has been proven to inhibit glioblastoma stem cell invasion²³⁰. In our study, the abundance of adenosine was lower in invaded cells, possibly reflecting increased purine turnover and energy demand, as these cells rapidly fuel adenosine for nucleotide recycling and energy production to support their invasive behaviour.

The glycerophospholipid metabolism was identified in both 3D OrbiSIMS and

AP-MALDI-MS results, as presented in Figure 3.10. The abundances of choline phosphate and choline in invaded and non-invaded cells were consistent across both methods.

Choline phosphate is the predominant choline compound in GBM and a precursor for PC, which is the most abundant phospholipid in cell membranes²³¹. Elevated choline phosphate supports rapid membrane turnover for proliferation and invasion, by enhancing membrane fluidity and plasticity²³². We found that the level of choline phosphate was higher in the invaded cells, suggesting its involvement in membrane remodelling processes that promote GBM invasion.

Choline is converted to choline phosphate through a phosphorylation reaction catalysed by choline kinase (CK). Cells with high CK activity show increased migration. It has been reported that CK activity supports epithelial–mesenchymal transition-like process in GBM, facilitating cell migration and extracellular matrix degradation, thereby enhancing invasiveness²³³. Inhibition of choline kinase significantly suppresses GBM invasion^{234,235}.

Collectively, these findings proved the role of glycerophospholipid metabolism in promoting GBM invasion, and presented a potential therapeutic target for GBM invasion.

Table 3.4. Significant metabolic pathways between invaded and non-invaded cells analysed by AP-MALDI-MS (adjusted p value < 0.05 and impact factor > 0.1).

Pathway name	Total compounds	Hits	Raw p	Adjusted p	Impact factor	Overlap score
Tyrosine metabolism	42	5	5.51E-05	1.88E-03	0.21	1.00
One carbon pool by folate	26	5	1.63E-03	1.24E-02	0.18	1.00
Glycerophospholipid metabolism	36	6	2.19E-03	1.24E-02	0.26	1.00
Nicotinate and nicotinamide metabolism	15	2	1.12E-02	3.46E-02	0.19	1.00

"Total compounds" refers to the number of compounds involved in a specific pathway. "Hits" represents the number of ions mapped to the pathway. Adjusted p is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network. Overlap score is the ratio of the number of unique m/z values to the number of ions mapped to the pathway.

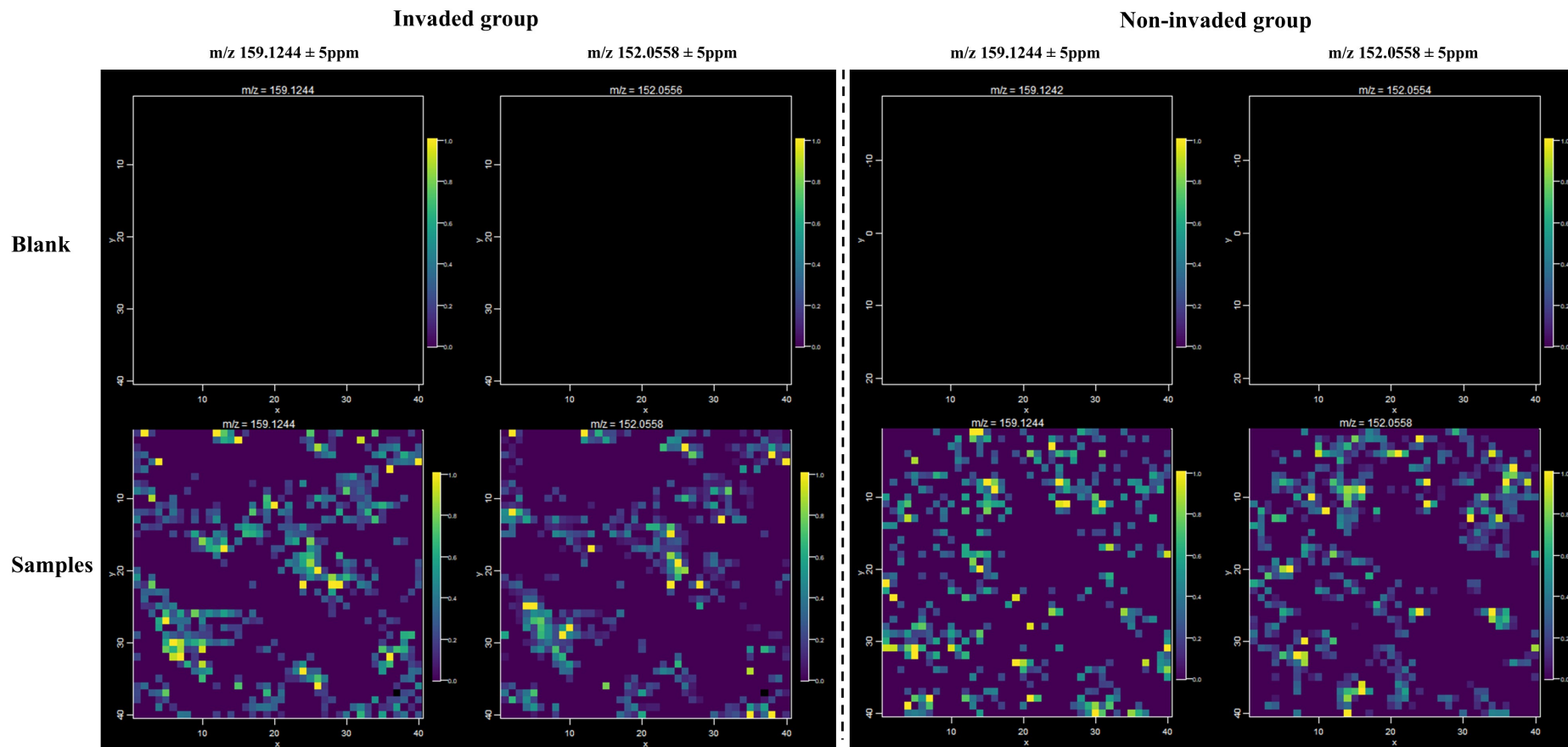


Figure 3.11. The presence of GIN31 cells on the insert membrane. Images of invaded group (left) and non-invaded group (right) were constructed by the exact mass of histidinol (m/z 159.1244) and malic acid (m/z 152.0558), with mass error 5 ppm. The figure compares the signals of two ions between a blank membrane (top) and an experimental sample which contains cells on the insert membrane (bottom). Histidinol and malic acid are detected only in the experimental sample, with no signal observed in the blank membrane.

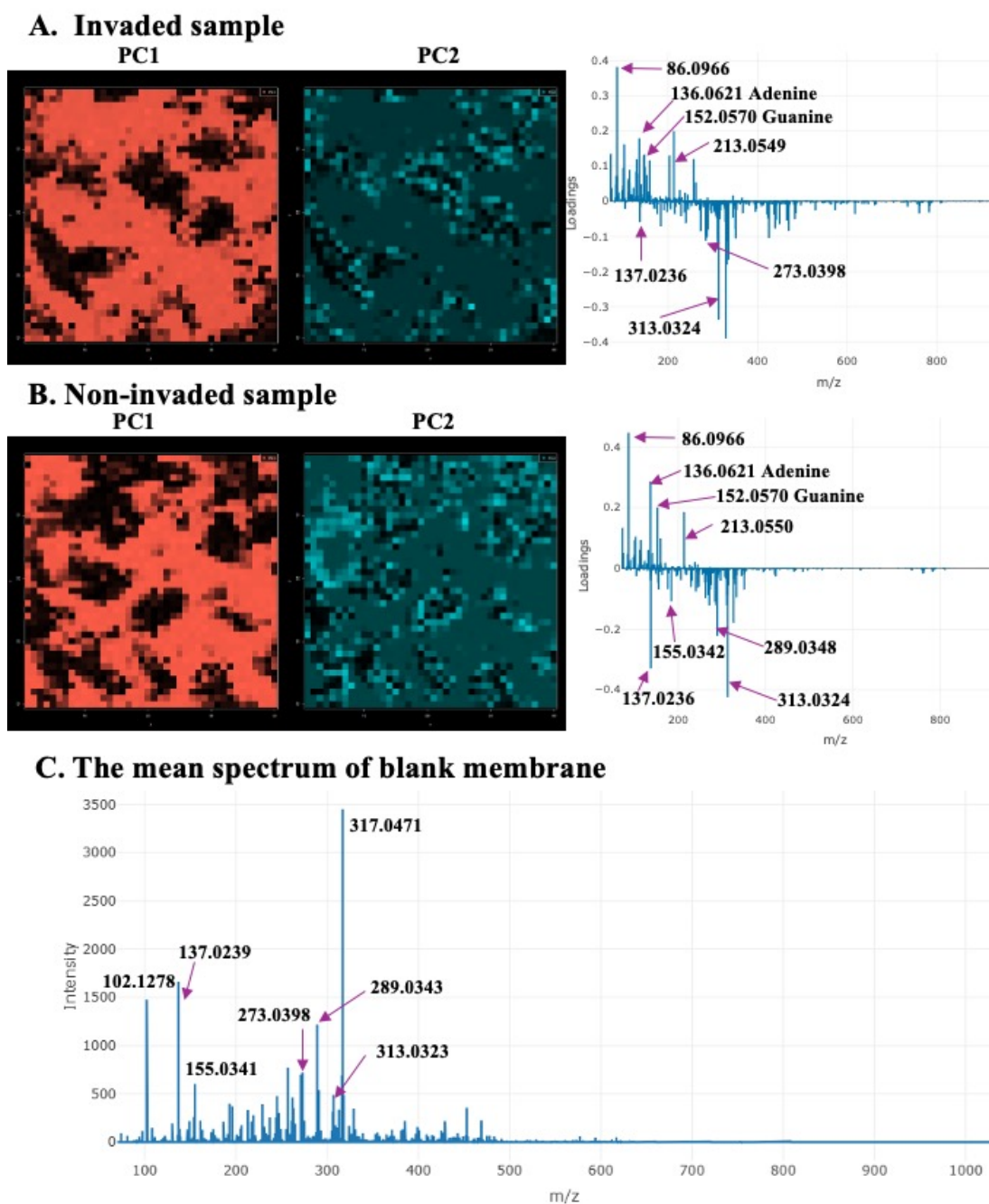


Figure 3.12. PCA-assisted segmentation. (A) and (B) show images generated by first-two principal components. Ions contributing to the positive loading of PC2 are primarily from cells, while those contributing to the negative loading of PC2 are associated with the blank membrane. (C) is the mean spectrum of the membrane.

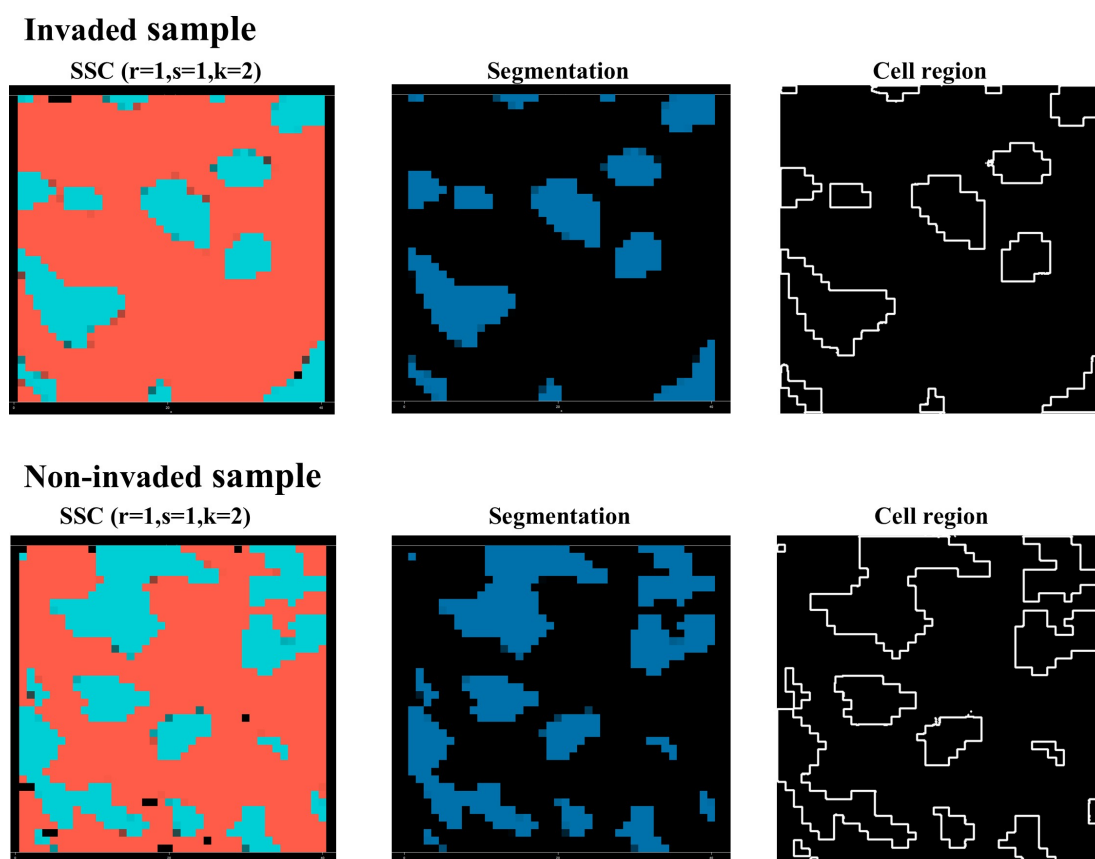


Figure 3.13. Spatial segmentation by Spatial-aware Shrunk Centroids Clustering. Spatial data were segmented using an unsupervised clustering approach that incorporates both molecular similarity and spatial proximity, enabling the identification of cells on the insert membrane. In this method, "r" defines the size of the spatial neighbourhood around each pixel, "s" controls the relative importance of spatial information compared with spectral data, and "k" represents the number of segmented clusters.

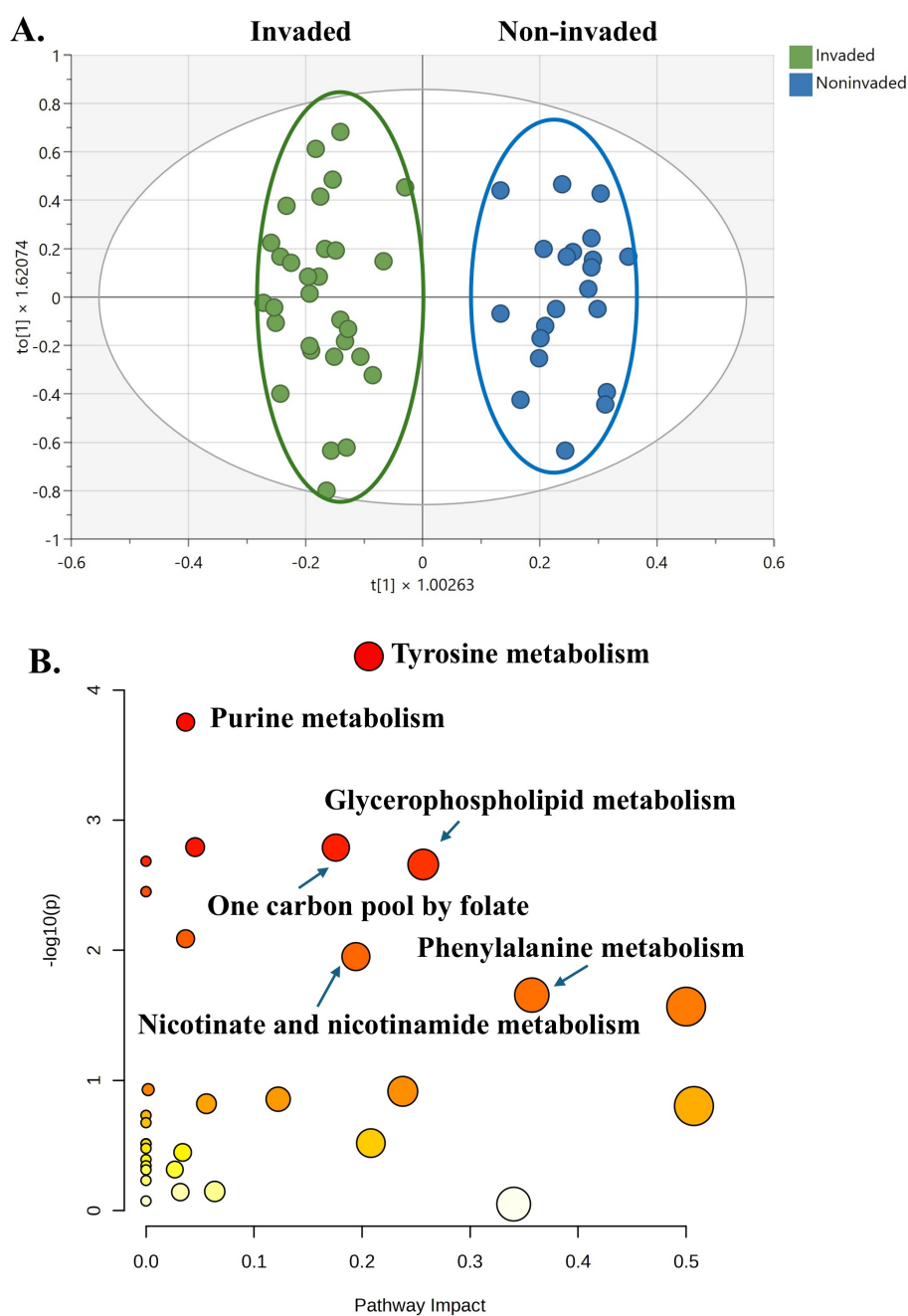


Figure 3.14. OPLS-DA model and pathway analysis of invaded vs. non-invaded cells analysed by AP-MALDI-MS. (A): OPLS-DA model of invaded vs. non-invaded cells ($R^2Y=0.926$, $Q^2=0.716$). Four invaded samples and three non-invaded samples were analysed. (B): The size of bubble represents the number of compounds mapped on a particular pathway. The colour represents the degree of significance: white (non-significant) to red (statistically significant). The impact factor represents the importance of an identified pathway within the whole metabolic network.

Table 3.5. Significantly altered metabolites between invaded and non-invaded cells detected by AP-MALDI-MS (adjusted *p* value < 0.05 and VIP > 1).

m/z	Assigned formula	Δ ppm	DBE	FC	Adj. <i>p</i> -value	VIP	Putative metabolites
114.0665	C ₄ H ₇ ON ₃	3.0		0.91	2.14E-03	1.01	Creatinine
186.2219				0.46	2.37E-04	1.83	
196.0608	C ₉ H ₉ O ₄ N	2.0		0.39	3.44E-02	1.96	
247.0605	C ₁₃ H ₁₁ O ₅	1.6	8.5	0.70	2.70E-02	1.41	Dopaquinone Isopimpinellin
257.0449	C ₁₄ H ₉ O ₅	1.7	10.5	0.70	9.00E-03	1.20	
	C ₁₀ H ₁₂ O ₅ NP	0.5	6.0				
	C ₆ H ₁₅ O ₅ N ₂ P ₂	-0.7	1.5				Adenosine Oleamide
268.1046	C ₁₀ H ₁₃ O ₄ N ₅	2.0		0.14	4.58E-03	1.77	
282.2797	C ₁₈ H ₃₅ ON			0.40	1.76E-02	1.68	
289.0348	C ₁₄ H ₉ O ₇	1.8	10.5	0.72	1.80E-02	1.34	
	C ₁₀ H ₁₂ O ₇ NP	0.7	6.0				
	C ₆ H ₁₅ O ₇ N ₂ P ₂	-0.3	1.5				
291.0503	C ₁₄ H ₁₁ O ₇	1.3	9.5	0.69	2.37E-02	1.37	
	C ₁₀ H ₁₄ O ₇ NP	0.2	5.0				
	C ₆ H ₁₇ O ₇ N ₂ P ₂	-0.9	0.5				
312.0241	C ₉ H ₁₃ O ₁₀ P	0.0	4.0	0.95	3.44E-02	1.01	
326.3788				0.08	4.58E-03	2.37	

m/z is exact mass. Assigned formula is theoretical formula calculated by MFP software. Δ ppm is mass error. DBE is short for double bond equivalent. FC is short for fold change. Adjusted *p* is generated using Benjamini-Hochberg correction.

3.4 Conclusion

This study successfully applied single-cell metabolomics to investigate the metabolic mechanisms underlying GBM invasion using 3D OrbiSIMS and AP-MALDI-MS. Distinct metabolic profiles were observed between invaded and non-invaded cells, with the involvement of tyrosine metabolism, nicotinate and nicotinamide metabolism, one-carbon pool by folate, and glycerophospholipid metabolism, providing new insights into GBM invasion from a metabolomics perspective.

Although our findings highlight changes in metabolite abundance, they offer limited evidence of pathway regulation. Incorporating enzyme activity and gene expression would provide comprehensive interpretation of the observed metabolic changes. High intra-group variation was observed in the negative mode of 3D-OrbiSIMS analysis, which could be attributed to cellular heterogeneity leading to various metabolic profiles. However, the potential influence of neutral red staining cannot be excluded.

Our study was also limited by the use of a single cell line. Including additional GBM cell lines or *in vivo* models would be important to validate these findings. Moreover, no drug treatment was applied in the current study; therefore, our results cannot confirm whether repurposed Afuresertib and Caledonine alter glycerophospholipid metabolism to inhibit GBM invasion.

Chapter 4

Method development of capillary-flow LC-MS-based metabolite profiling

4.1 Introduction

As described in Chapter 3, although the application of 3D OrbiSIMS and AP-MALDI enables the identification of distinct metabolic profiles at the single-cell level, several challenges remain, including a limited number of identified metabolites and low confidence in metabolite identification. These limitations hinder precise metabolic characterisation during glioblastoma (GBM) invasion, thereby necessitating the development of a more robust analytical method. Liquid chromatography–mass spectrometry (LC–MS)-based metabolite profiling has been widely applied to investigate metabolic signatures associated with GBM. Researchers are continually striving to increase the sensitivity of LC–MS method by improving LC and ionisation techniques, particularly the application of low-flow LC–MS¹²⁶.

As illustrated in Figure 4.1, low-flow LC-MS can be classified into three types: nano-flow, capillary-flow, and micro-flow LC-MS. Compared with the analytical flow rate, low-flow LC-MS improves the detection of metabolites with superior sensitivity. Chetwynd *et al.* demonstrated that nano-flow LC-MS provided reproducibility comparable to the conventional LC-MS, with 2-2,000 fold improved in detection

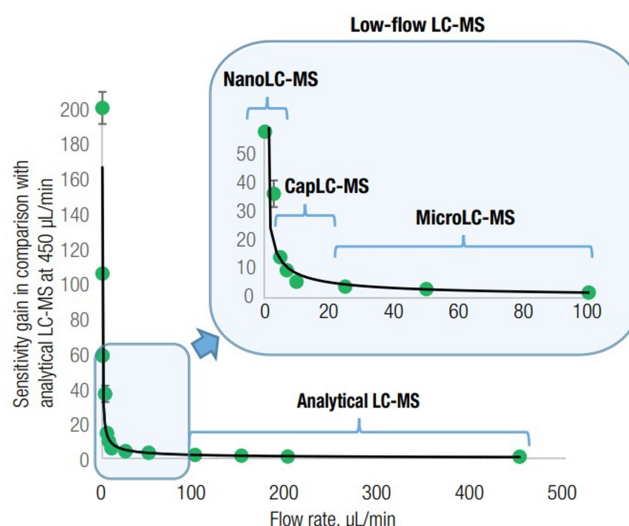


Figure 4.1. Increased sensitivity with low-flow LC-MS. Sensitivity gain = $38.168 \times [\text{Flow rate, } \mu\text{L/min}]^{-0.638}$. The figure is copied from the official guide "Capillary-flow LC-MS: Combining High Sensitivity, Robustness, and Throughput" by Thermo Fisher Scientific.

limits for metabolites²³⁶. Further, the increased sensitivity allows low-flow LC-MS particularly desirable in handling samples of limited amounts. Deng *et al.* reported a nano-LC-MS approach to analyse intracellular metabolites in IDH1 R132H-mutant GBM (1×10^5 cells/run)¹²³. Luo *et al.* developed a nano-flow metabolomics workflow capable of profiling down to 100 human breast cancer cells¹²².

Given its enhanced sensitivity and the ability to accommodate scarce samples, we hypothesised that low flow LC-MS could be applied to analyse samples (around 1×10^4 cells) obtained from Transwell inserts, enabling the detection of metabolic differences between invaded and non-invaded cells.

In this study, a capillary-flow LC-MS-based metabolite profiling method (1 $\mu\text{L/min}$) was developed, with the reliable detection limit of 5,000 GIN31 cells.

4.2 Materials and methods

4.2.1 Materials and instruments

The materials related to cell culture have been described in Chapter 2.2.1.

LC-MS grade ammonium formate (Lot No.: NX749402), methanol (Lot No.: 221115C006) and acetonitrile (Lot No.: 24B084010) were supplied by VWR Chemicals,

LLC (Lutterworth, UK). Glutamine (Lot No.: SLBD9625V) and LC-MS grade ammonium acetate (Lot No.: BCBV8695) were ordered from Sigma-Aldrich Co. LLC (Merck KGaA, Darmstadt, Germany). LC-MS grade formic acid (Lot No.: 193272) was ordered from Thermo Fisher Scientific Inc. (Hemel Hempstead, UK).

Nano-capillary ZIC[®]-HILIC column (5 μm , 0.075×150 mm, Lot No.: K95724865) was manufactured by Merck Sequant[®] (Darmstadt, Germany). ZIC[®]-HILIC capillary HPLC column (5 μm , 0.3×150 mm, Lot No.: TA2039177) was manufactured by Merck Supelco[®] (Darmstadt, Germany). EASY-Spray[™] nano-flow emitter (I.D. 10 μm , Lot No.: 2968883), EASY-Spray[™] micro-flow emitter (I.D. 20 μm , Lot No.: 01201158), EASY-Spray[™] PepMap[™] Neo column (2 μm , 0.075×500 mm, Lot No.: 2732462) and EASY-Spray[™] PepMap[™] RSLC column (3 μm , 0.075×150 mm, serial No.: 10759519) were supplied by Thermo Fisher Scientific Inc. (Hemel Hempstead, UK).

TriVersa NanoMate[®] LESA[®] nanoelectrospray ionisation system, ESI chip (Lot No.: G8P488TF) and LC coupler (I.D. 15 μm , Lot No.: C2-092421-10) were supplied by Advion Interchim Scientific Inc. (New York, USA). Vanquish[™] Neo UHPLC system and Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer were manufactured by Thermo Fisher Scientific Inc. (Hemel Hempstead, UK).

4.2.2 Sample preparation

4.2.2.1 Preparation of authentic standards

Five mixtures of authentic standard solutions (stdA-stdE, total 266 standards, 200 μM) were previously prepared and stored at -80°C , details in Chapter 2.2.6.2. They were diluted to 100 μM , 20 μM , 1 μM , 500 nM, 200 nM, 100 nM, 50 nM, and 10 nM with 95% aqueous acetonitrile. After centrifugation (14,000 g/ 5 min), the supernatant was transferred to LC-MS vials for analysis.

4.2.2.2 Extraction of intracellular metabolites

GIN31 cells were seeded at densities ranging from 625 to 2×10^4 cells/well. After 24 h, cells were washed with pre-warmed PBS before being quenched by pre-cooled

(−40 °C) methanol. Cell suspensions were vortexed for 1 h at 4 °C. Subsequently, the supernatant was collected and dried under a nitrogen stream by intermittently flushing the inside of the tube with methanol, before being reconstituted by LC-MS grade methanol. The samples were centrifuged again (14,000 g/ 5 min), and the supernatant was transferred to LC-MS vials for analysis.

4.2.3 Data analysis

Statistical analysis was performed on GraphPad Prism v10.3.0 (GraphPad Software, San Diego, CA), with p value < 0.05 considered statistically significant. Venn diagrams were generated on TBtools-II²⁰¹.

The total ion chromatogram (TIC) and extracted ion chromatogram (EIC) were displayed on Freestyle™ v1.4 (Thermo Fisher Scientific, Waltham, MA, USA). Peak screening and integration were performed on TraceFinder™ v4.1 (Thermo Fisher Scientific, Waltham, MA, USA). Metabolite identification was performed on Compound Discoverer 3.3 SP3 (Thermo Fisher Scientific, Waltham, MA, USA).

4.3 Results and discussion

4.3.1 Ionisation source

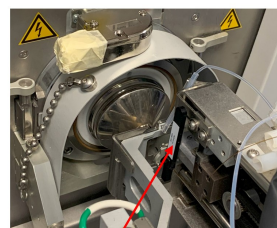
Conventional ionisation sources have difficulty maintaining a stable spray at low-flow rates. Two low-flow ionisation sources (Figure 4.2) were evaluated: TriVersa NanoMate® chip-based ESI and EASY-Spray™ emitter. The separation and detection conditions are presented in Table A.7 and A.8.

As shown in Figure 4.3, compared to chip-based ESI, the EASY-Spray™ emitter provided a more stable spray throughout the analysis and generated a higher intensity of glutamine with narrower peak width, demonstrating superior spray stability and ionisation efficiency.

TriVersa NanoMate® robot does not support polarity switching mode, limiting its ability to acquire positive and negative ions simultaneously. Additionally, the

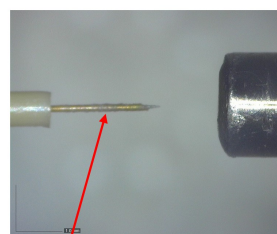
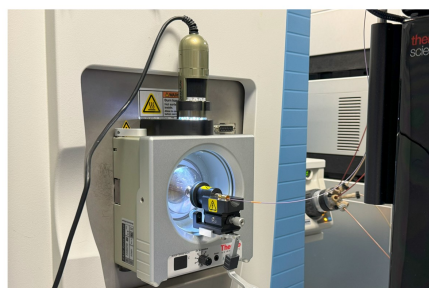
instrument setup, maintenance, and troubleshooting of the TriVersa NanoMate[®] robot were more complex, making the EASY-Spray[™] emitter a more suitable option for low-flow applications.

NanoMate[®] chip-based ESI



LC coupler

EASY-Spray[™]



The end of emitter

Figure 4.2. Instrument setup of ionisation sources. The LC coupler was aligned to nozzles (size 2.5 μm) on the chip before spraying. The emitter was centred and 2–3 mm from the ion transfer tube.

4.3.2 Washing solution

Strong and weak wash solutions were used to clean the auto-sampler in LC system. They were optimised based on the information presented in Table 4.1, with detection conditions displayed in Table A.9 and A.10.

Table 4.1. Optimisation of wash solutions.

Solution	PepMap [™] RSLC C ₁₈ column		Nano-capillary ZIC [®] -HILIC column	
	Typical	Adjusted	Typical	Adjusted
Strong wash	90% acetonitrile	90% acetonitrile	10% acetonitrile	90% acetonitrile
Weak wash	10% acetonitrile	90% acetonitrile	90% acetonitrile	90% acetonitrile

All the solutions were added with 0.1% formic acid.

For HILIC columns, a strong wash typically consists of 90–100% water to remove residuals inside the injection needle. However, as shown in Figure 4.4, when the

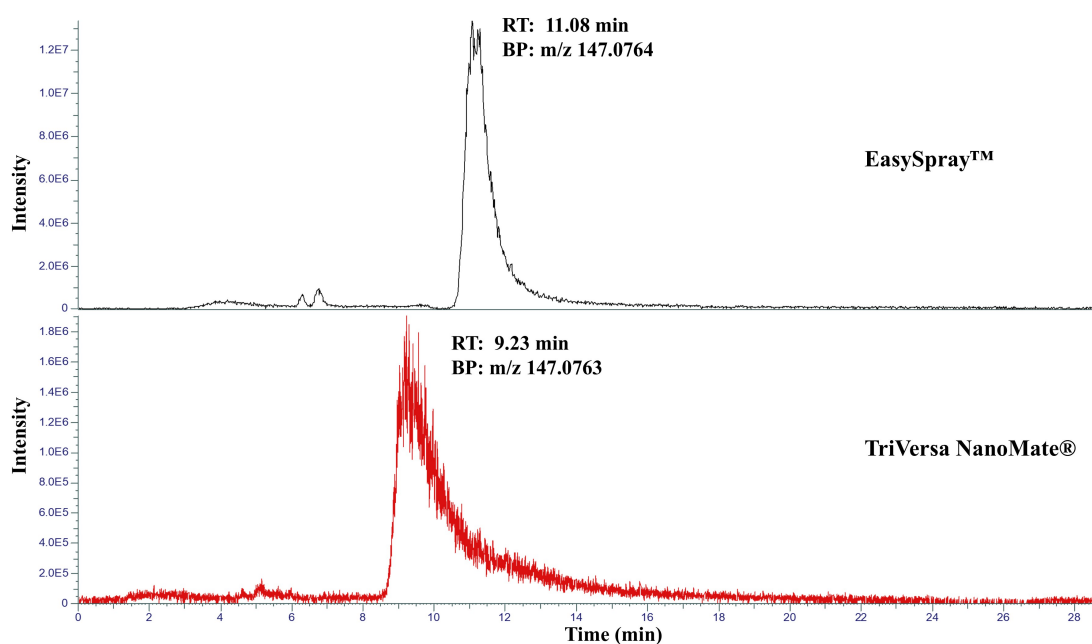


Figure 4.3. Comparison of chip-based ESI and EASY-Spray™ emitter. The EICs of glutamine (m/z 147.0764 positive) analysed using two ionisation sources. RT represents retention time. BP (basic peak) refers to the most intense ion on the spectrum.

strong wash solution contained 10% acetonitrile (a highly aqueous composition), the analytes eluted too fast or did not retain properly in the column. After changing to 90% acetonitrile, the retention of analytes was improved and higher intensity was observed, although retention and separation remained poor. This suggested that excess aqueous solvent in the autosampler can hinder proper loading of analytes (polar metabolites) onto the column or disrupt the hydrophilic interaction retention mechanism. It was supported by the results on the RSLC C_{18} column. After the acetonitrile content in the weak wash solution was increased, analyte peaks eluted with higher intensity.

Hence, since the standards tested in the study were polar metabolites, 90% acetonitrile with 0.1% formic acid was selected as the wash solution.

4.3.3 Analytical column

In low-flow LC-MS metabolomics applications, C_{18} columns are widely used, whereas HILIC columns have been employed in a smaller fraction of studies. These two types of column were evaluated, with their specifications shown in Table 4.2. The separation and detection conditions are presented in Table A.9 and A.10.

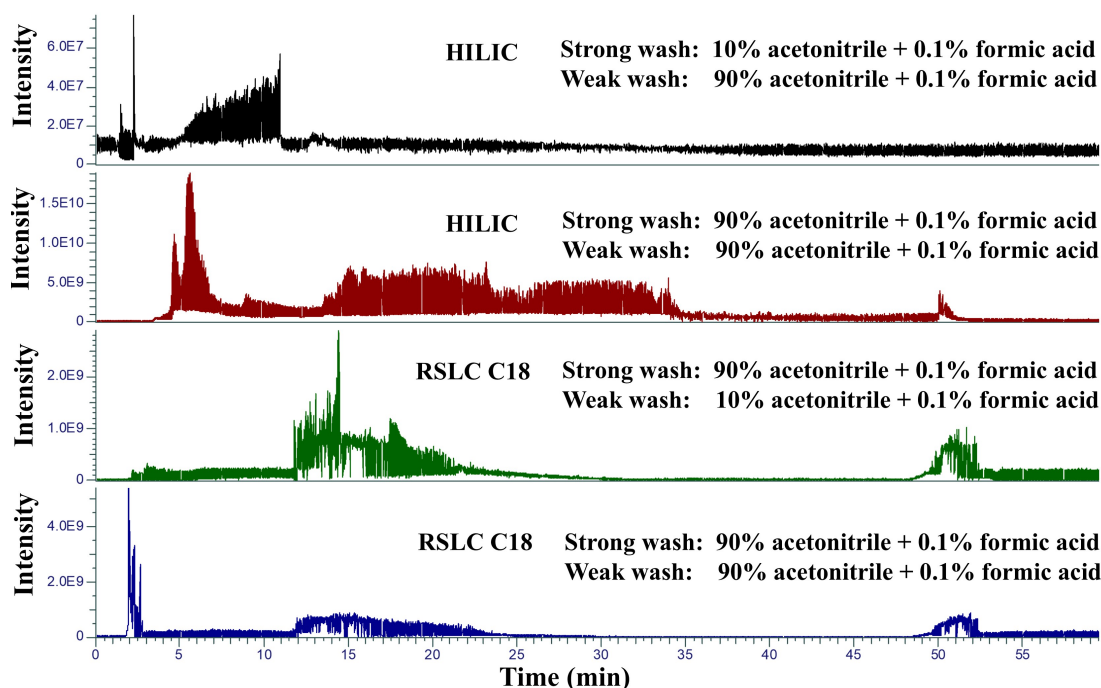


Figure 4.4. Optimisation of wash solutions. The TICs of StdA solution were acquired from the RSLC C₁₈ and ZIC-HILIC columns, with different strong and weak wash solutions.

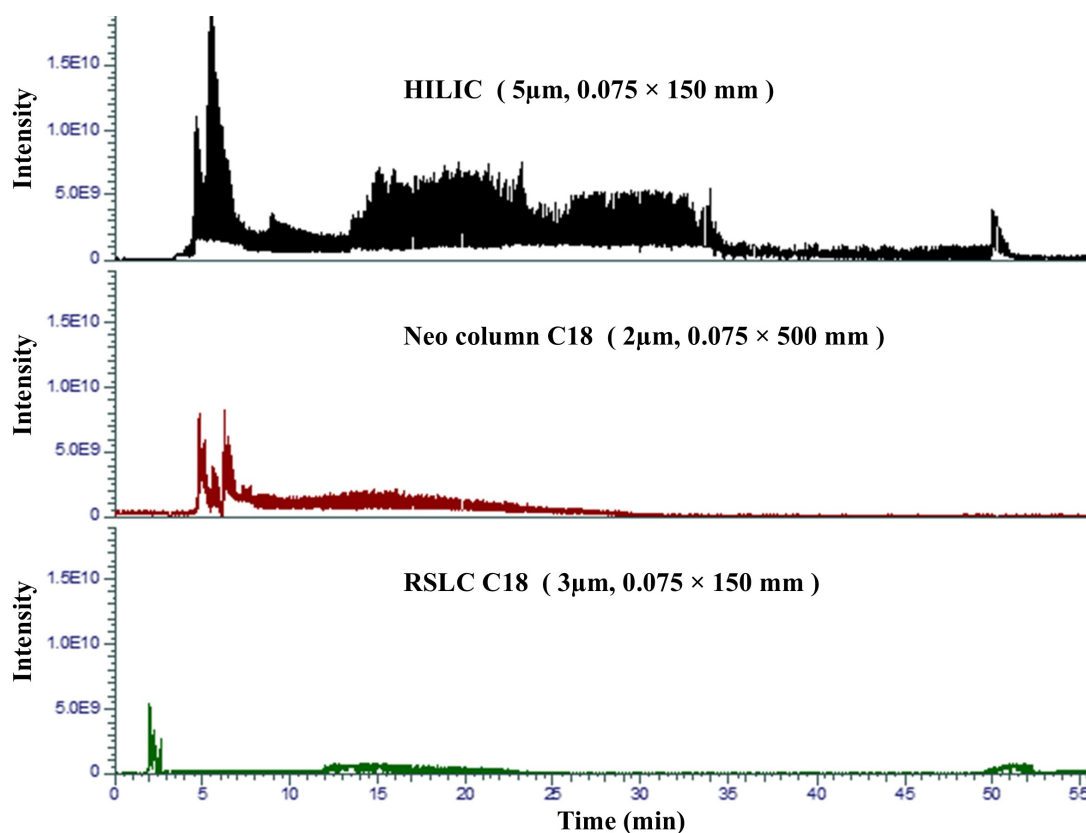


Figure 4.5. Optimisation of analytical columns. The TICs of StdA solution were obtained from the C₁₈ and ZIC-HILIC columns at nano-flow rate (300 nL/min).

Table 4.2. Specifications of analytical columns.

Type	Name	Particle size	Dimensions
C ₁₈	EASY-Spray™ PepMap™ Neo column	2 µm	0.075 × 500 mm
	EASY-Spray™ PepMap™ RSLC column	3 µm	0.075 × 150 mm
HILIC	Nano-capillary ZIC®-HILIC column	5 µm	0.075 × 150 mm

As shown in Figure 4.5, analytes were better retained on the ZIC-HILIC column, with retention times exceeding 5 min compared to the estimated dead time of 1.66 min, whereas on the C₁₈ columns, analytes eluted near the dead time, indicating poor retention.

A total of 29 standards were detected on the ZIC-HILIC column, more than on either of the C₁₈ columns, with 18 of these exclusively detected on the ZIC-HILIC column (Figure 4.6). Moreover, while some metabolites were detected on both Neo C₁₈ and ZIC-HILIC columns, they exhibited better peak shape and higher intensity on the ZIC-HILIC column, including glycine, arginine, leucine, lysine, methionine, histidine and threonine.

As discussed in the section 1.4.3.1, the reversed-phase chromatography (C₁₈) and the HILIC chromatography operate under different retention mechanisms. Compared to the C₁₈ column, HILIC column presents superior capability in capturing a wider range of polar compounds, including amino acids, nucleotides, sugars, and other hydrophilic metabolites^{237,238}. In ZIC-HILIC columns, highly polar analytes are better retained because they preferentially partition from the organic-rich mobile phase into the water-enriched layer associated with the stationary phase, where additional electrostatic interactions can further stabilise retention. This results in improved retention, selectivity, and peak shape for polar metabolites²³⁹. Ladd *et al.* found that ZIC-*p*HILIC column significantly improved separation and peak shape for polar compounds at nano-flow rate, compared to C₁₈ column²⁴⁰.

Considering the number of detected ions, peak shape, and signal intensity, the results showed that ZIC-HILIC column was more suitable for profiling polar metabolites under nano-flow conditions, compared to C₁₈ columns. However, poor separation highlighted the importance of further optimisation of chromatographic conditions.

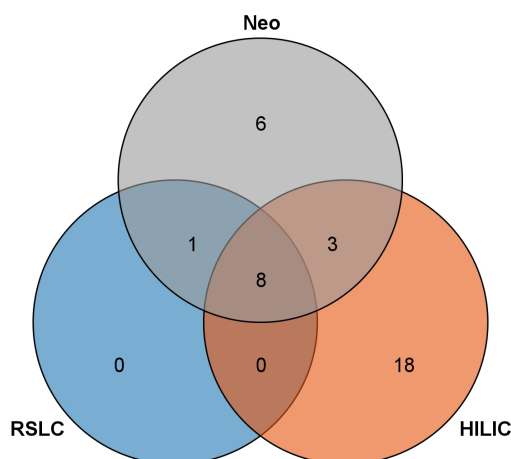


Figure 4.6. The number of detected standards on three columns at nano-flow rate (300 nL/min).

4.3.4 Separation on ZIC-HILIC column at nano-flow rate

Solvent selection, pH and gradient optimisation are crucial for achieving good separation. Five compositions were evaluated, presented in Table 4.3. The separation and detection conditions are summarised in Table A.11 and A.12.

Table 4.3. Compositions of mobile phase evaluated at nano-flow rate.

Composition	A (aqueous phase)	B (organic phase)
1	0.1% formic acid in water (pH 2.61)	0.08% formic acid in acetonitrile
2	5mM NH ₄ Ac in water (pH 7.08)	
3	15mM NH ₄ Ac in water (pH 7.0)	
4	5mM NH ₄ HCOO in water (pH 5.72)	
5	5mM NH ₄ Ac in water	acetonitrile

NH₄Ac refers to ammonium acetate. NH₄HCOO refers to ammonium formate.

First, the performance of formic acid in the organic phase was assessed. As presented in Figure 4.7, 0.08% formic acid in acetonitrile provided better sample separation than pure acetonitrile. The addition of formic acid improved the peak shape and narrowed the peak widths for analytes, such as 3-methoxyramine, histidine, and threonine (Figure B.11). Therefore, 0.08% formic acid in acetonitrile was selected as the organic phase.

Next, the performance of aqueous phases were compared. As shown in Figures 4.8 and 4.9, although ammonium acetate and ammonium formate provided

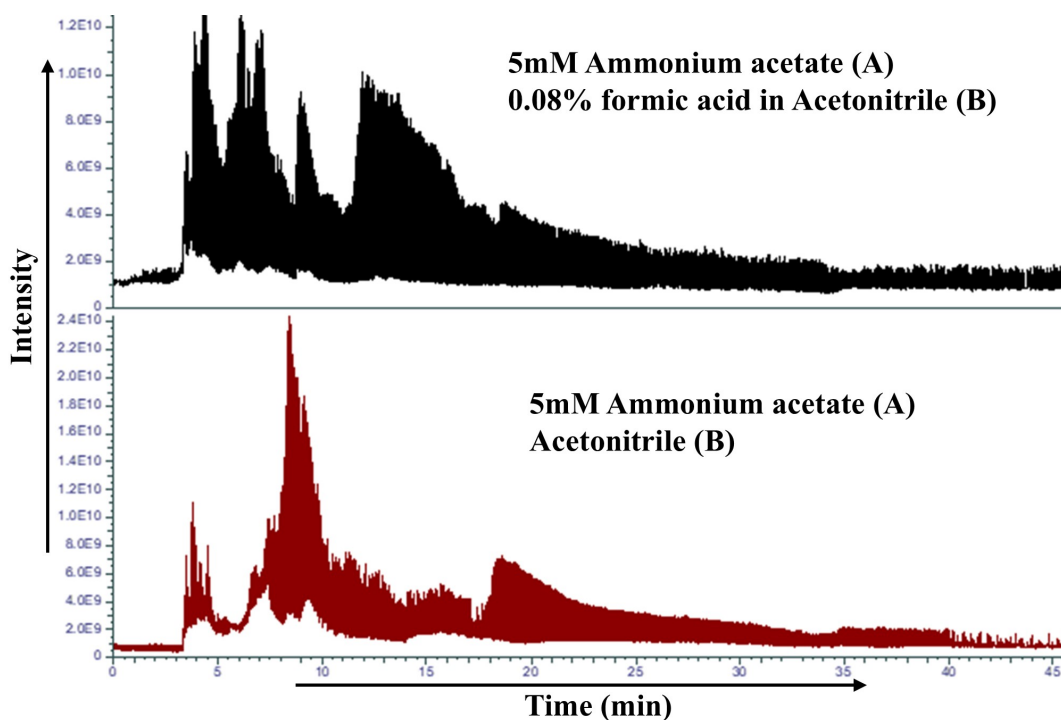


Figure 4.7. Optimisation of organic phase at nano-flow rate (300 nL/min). The TICs of acetonitrile with formic acid is shown in black, while without formic acid is shown in red.

better chromatographic separation compared to formic acid, and all four aqueous phases showed similar performance in the number of detected standards (22 detected across all), 0.1% formic acid in water produced sharper peaks and higher intensity (Figures B.12 and B.13), particularly for amino acids. Current results indicated that 0.1% formic acid in water was the optimal aqueous phase at nano-flow rate.

The gradient profile was further optimised to improve chromatographic separation. The optimal result was a gradient from 80% B (0.08% formic acid in acetonitrile) to 20% B over 30 min, though the improvement remained limited. Moreover, reducing the flow rate (to 200 and 150 nL/min) did not improve the separation.

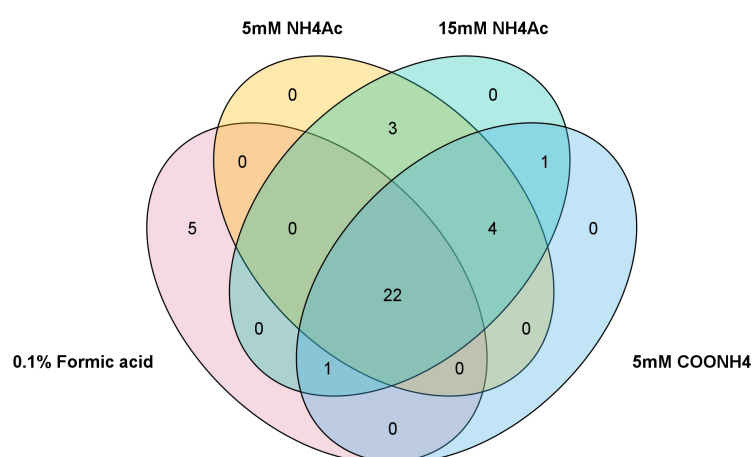


Figure 4.8. The number of detected standards separated by different aqueous phases at nano-flow rate (300 nL/min). NH₄Ac refers to ammonium acetate. NH₄HCOO refers to ammonium formate.

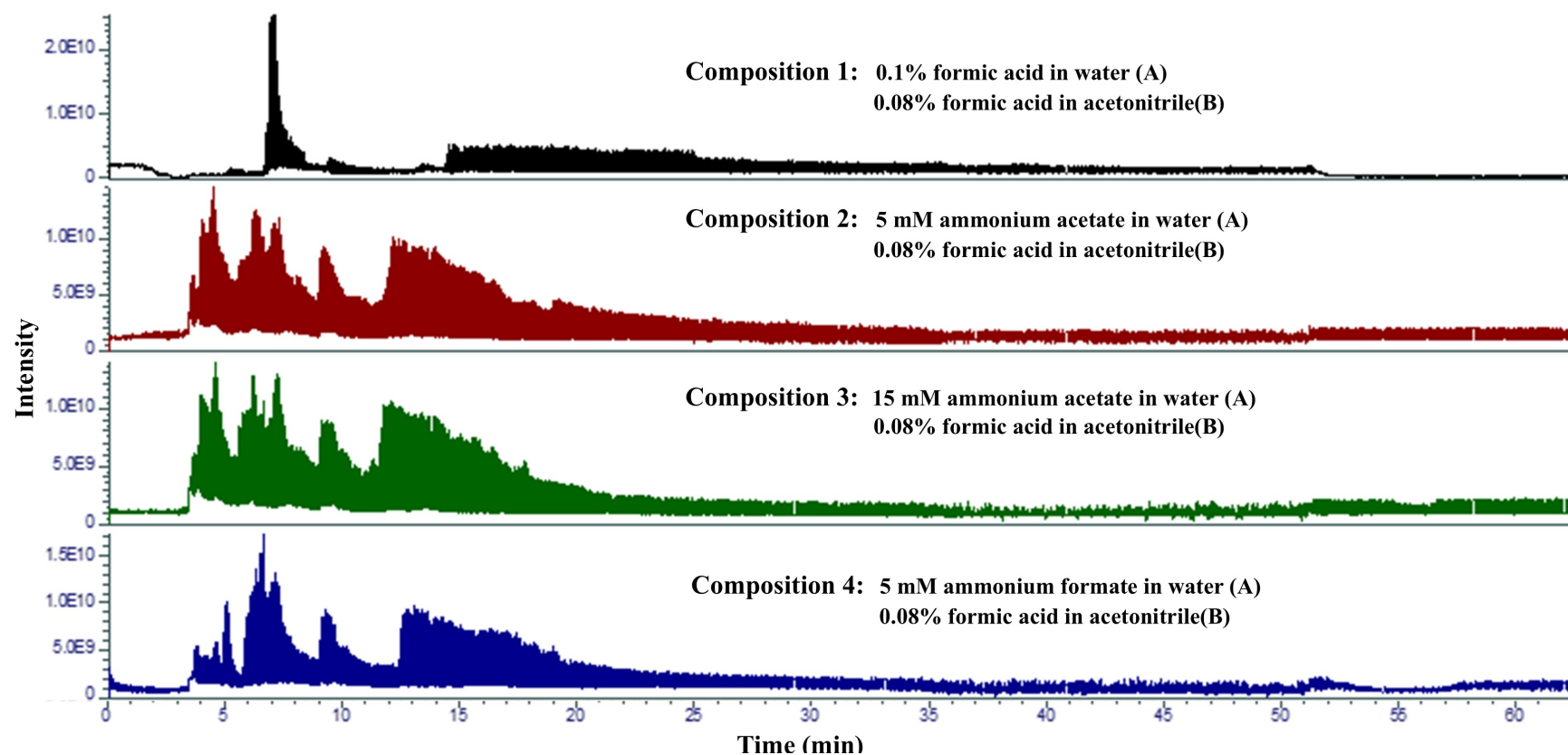


Figure 4.9. Optimisation of aqueous phases at nano-flow rate (300 nL/min). The TICs of StdA were separated by different aqueous phases.

4.3.5 Nano-flow, capillary-flow, and micro-flow performance

Micro-flow, capillary-flow and nano-flow experiments were conducted according to the information in Table 4.4. Standards were analysed by gradient in Table A.11 and MS parameters in Table A.12.

Table 4.4. Nano-flow, capillary-flow, and micro-flow separation.

Flow mode	Micro-flow	Capillary-flow	Nano-flow
Analytes	StdA-StdE, 20 μ M		
Mobile phases	0.1% formic acid in water (A)		
	0.08% formic acid in acetonitrile (B)		
Column	Capillary column (5 μ m, 0.3 x150 mm)		Nano-capillary column (5 μ m, 0.075 x150 mm)
Flow rate (μ L/min)	5, 10	1	0.3
Emitter I.D. (μ m)	20	20	10

UHPLC system was operated in capillary/nano-flow mode (I.D. 20 μ m, flow rate < 5 μ L/min) for capillary/nano-flow separation, and micro-flow mode (I.D. 50 μ m, flow rate > 5 μ L/min) for micro-flow separation.

Results indicated that micro-flow rates offered superior analyte detection and chromatographic separation relative to nano-flow rate. As shown in Figure B.14, the TICs of StdA at micro-flow rates differed notably from that at nano-flow rate (300 nL/min), with better separation and peak shapes observed at 5 μ L/min and 10 μ L/min. Moreover, micro-flow rates detected more standards than nano-flow rate, with 80 standards undetectable at nano-flow rate (Figure 4.10).

Further, the flow rate was decreased to the capillary-flow rate (1 μ L/min). Similar to the results of micro-flow rates, the TICs of five standard solutions (StdA-StdE) separated at 1 μ L/min exhibited better separation than that at 300 nL/min (Figure B.15 to B.17). Regarding the number of detected standards, capillary-flow rate showed similar performance with micro-flow rates, detecting a greater number of standards compared to nano-flow rate (Figure 4.11). Furthermore, cell extracts analysed at nano-flow and capillary-flow (1 μ L/min) also exhibited markedly different TICs, with the nano-flow rate producing no proper peaks on the TICs (data not shown).

Current results suggested that capillary-flow rate (1 μ L/min) was worth for further method development, offering superior separation and detection compared to the nano-flow rate, while comparable to micro-flow rates.

Our findings showed that the nano-flow method failed to achieve good separation and reasonable peak shape of standards compared to the micro-flow and capillary-flow methods. Gika *et al.* reported similar results that they did not obtain reasonable peak shapes from nano-ZIC-HILIC column. Some peaks became symmetrical when a diluted sample (10 fold) was injected, indicating that the nano-ZIC-HILIC column had a very low loading capacity²⁴¹. Moreover, the number of detected standards at the nano-flow rate was distinct from that gained at the micro-flow and capillary-flow rates. A possible reason could be solubility limitations. In nano-flow application, the extremely low volume of organic solvents can raise challenges for dissolving certain analytes. This limit may result in inadequate analyte dissolution, adversely affecting separation and detection^{123,242}.

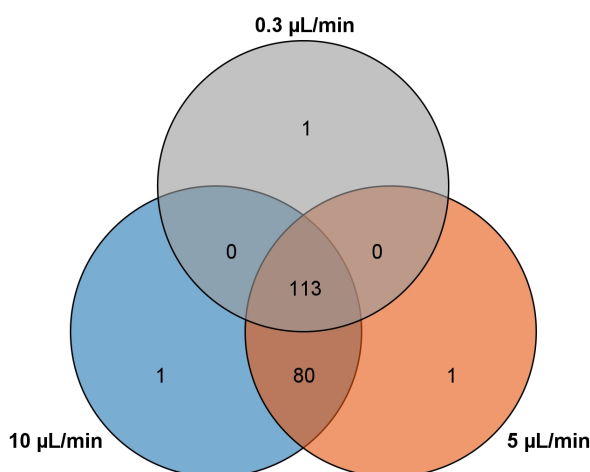


Figure 4.10. The number of detected standards at nano-flow (300 nL/min) and micro-flow rate (5 and 10 µL/min).

4.3.6 Separation on ZIC-HILIC column at capillary-flow rate

To enhance separation at capillary-flow rate (1 µL/min), mobile phases were optimised as listed in Table 4.5, with separation and detection conditions summarised in Table A.12 and A.13.

Figure B.18 shows that buffer salts (ammonium acetate and ammonium formate) improved chromatographic separation and enhanced spray stability (signal dropped at 35 min) compared to 0.1% formic acid. As shown in Figure 4.12 A, composition 2

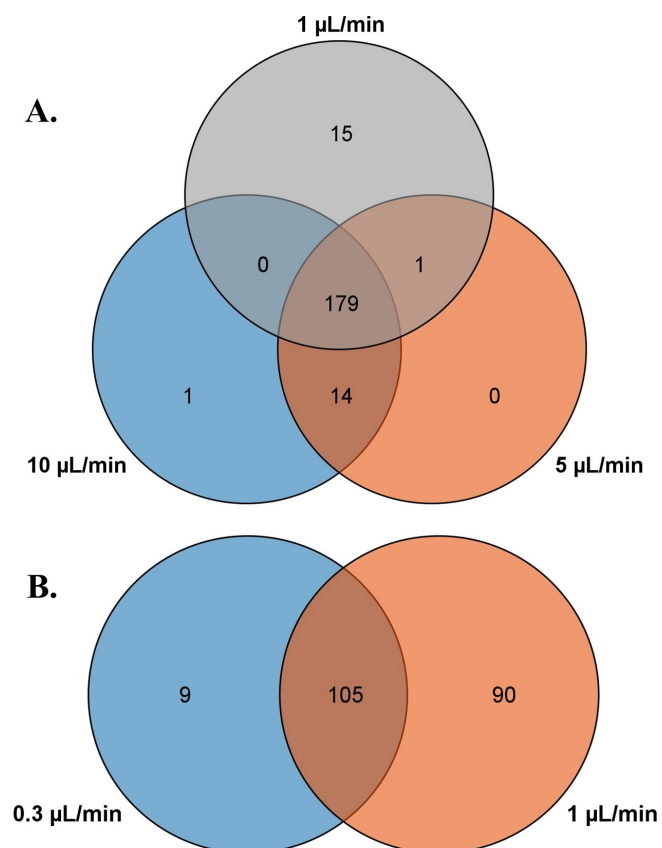


Figure 4.11. The number of detected standards at the capillary-flow rate (1 µL/min). (A) shows the comparison between capillary-flow and micro-flow methods, while (B) compares capillary-flow with nano-flow methods.

Table 4.5. Mobile phases evaluated at capillary-flow rate.

Composition	A (aqueous phase)	B (organic phase)
1	0.1% formic acid in water	0.08% formic acid in acetonitrile
2	5mM ammonium acetate in water	acetonitrile
3	15mM ammonium acetate in water	acetonitrile
4	5mM ammonium formate in water	acetonitrile
5	5mM ammonium acetate in water	0.08% formic acid in acetonitrile

(ammonium acetate) detected the highest number (203) of standards, slightly exceeding composition 1 (192), composition 3 (199), and composition 4 (199). Moreover, ammonium acetate had higher intensities for 65% detected standards compared to other additives.

5 mM ammonium acetate exhibited similar TIC and the comparable number of detected standards to 15 mM ammonium acetate, suggesting that enhanced ionic strength did not produce significant improvements. In negative ion mode, buffer salts enabled the detection of more standards than 0.1% formic acid (Figure 4.15).

Based on the results of separation, the number of detected standards and their intensities, 5 mM ammonium acetate as the aqueous mobile phase presented the best overall performance. However, compared to 0.1% formic acid, the peak shapes obtained with ammonium acetate and ammonium formate were suboptimal, particularly for amino acids (Figures 4.13 to 4.15).

To address the issue of poor peak shape with ammonium acetate, formic acid was added to the organic phase (acetonitrile). As shown in Figure B.18, Figure 4.12 B, Figure 4.13 to 4.15, 0.08% formic acid in acetonitrile (composition 5) performed similarly in TIC separation and the number of detected standards to pure acetonitrile (composition 2), but produced sharper and more symmetric peaks for amino acids, though at the expense of signal intensity. These findings suggested that 0.08% formic acid in acetonitrile was the optimal organic mobile phase.

Using 5 mM ammonium acetate (A) and 0.08% formic acid in acetonitrile (B) as mobile phases, the gradient profile was further optimised, shown in Figures B.19 and B.20. Increasing the initial organic phase to above 90% B caused unstable spray, with droplet formation at the emitter tip. Reducing it to 70% B improved peak sharpness but worsened TIC separation compared to the 80%–20% B gradient over 30 minutes. Moreover, extending or shortening the gradient time resulted in poorer TIC separation and peak shapes, as demonstrated using leucine, uridine, and adenine.

To enhance separation, two gradients were set in the same duration of separation: one targeting analytes eluting between 0–10 min and the other for those eluting between

10–30 min. However, varying the proportion of the mobile phases and the duration of the gradient did not significantly improve the overall separation (data not shown).

Hence, optimisation of mobile phases and gradient profile at a capillary flow rate (1 $\mu\text{L}/\text{min}$) demonstrated that 5 mM ammonium acetate (aqueous) and 0.08% formic acid in acetonitrile (organic), with a gradient from 80% to 20% B over 30 min, provided optimal balance.

The salts in mobile phase are used to control ionic interactions between the analytes and the stationary phase. Ammonium formate and ammonium acetate were chosen for their relatively high solubility in the mobile phase containing high levels of acetonitrile. Our results demonstrated that 5 mM ammonium acetate as the aqueous mobile phase presented better performance than 0.1% formic acid, likely due to its greater buffering capacity and ionic strength. The limited buffering ability of formic acid results in less stable pH control, leading to poor equilibria in the analyte distribution between the stationary and mobile phases, which adversely affect retention. Ammonium acetate could also stabilise electrostatic interactions, thereby enhancing chromatographic separation²⁴³.

Despite its optimisation, the separation at capillary-flow rate (1 $\mu\text{L}/\text{min}$) still had several limitations:

First, it had an overall analysis time of more than 90 min/run, restricting its ability for high-throughput applications. This prolonged duration was not only due to the inherently low flow rate for sample loading and separation, but also because the HILIC column required extensive conditioning and re-equilibration^{243,244}. We have observed that re-equilibration of 8 to 10 column volumes between sample injections is sufficient to achieve adequate analytical reproducibility.

Moreover, some metabolites exhibited poor peak shapes on the ZIC[®]-HILIC capillary HPLC column. The ZIC-*p*HILIC column is a polymer-based HILIC column with a zwitterionic stationary phase, and tolerates a wide pH range¹¹⁸. It has been reported that ZIC-*p*HILIC column could improve retention compared to the HILIC column in conventional metabolomics studies²⁴⁵. In addition, Zhang *et al.* demonstrated

that operating the ZIC-*p*HILIC column at high pH significantly improved the peak shapes of organic acids and sugars, which is particularly beneficial for extending the coverage of polar metabolites in human urine²⁴⁶. Taken together, these results suggest that the ZIC-*p*HILIC column has strong potential to improve chromatographic performance under low-flow applications.

Last but not least, chromatographic separation remains suboptimal. To some extent, it could be attributed to the use of 1 μ L/min flow rate, which lies at the lower boundary of the recommended operating range of the ZIC[®]-HILIC capillary HPLC column. At such a low flow rate, longitudinal diffusion becomes more significant, leading to increased peak broadening and reduced peak symmetry²⁴⁷. Beyond increasing the flow rate on the same column, it may be beneficial to evaluate this flow rate using columns with a smaller internal diameter (100 or 150 μ m) and a smaller particle size (3.5 μ m) to enhance separation.

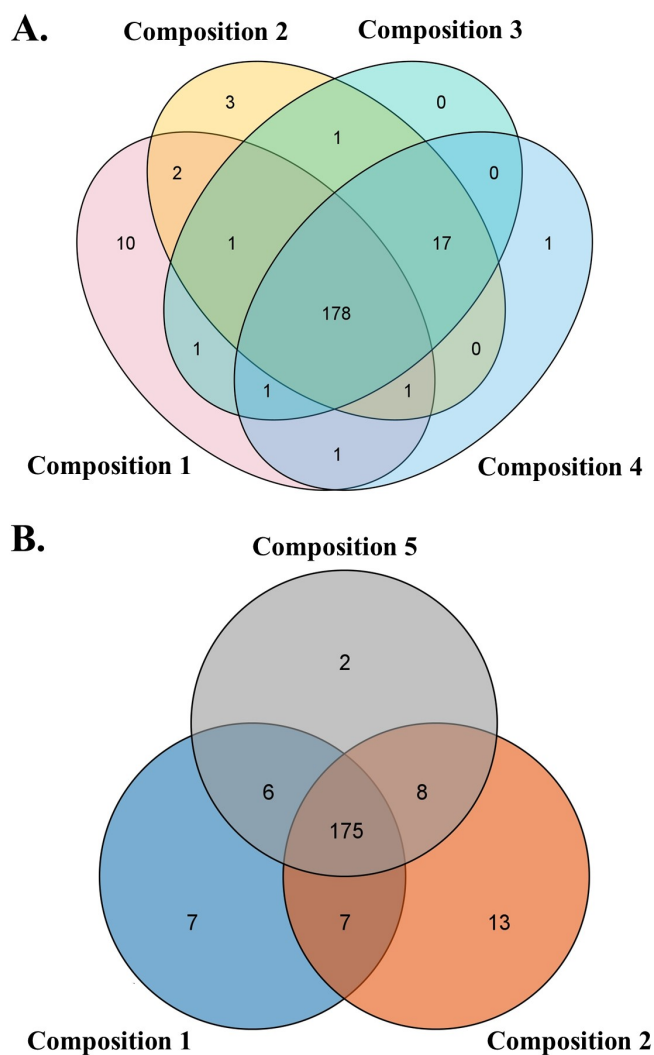


Figure 4.12. The number of detected standards by different mobile phases at capillary-flow rate (1 $\mu\text{L}/\text{min}$). (A): The number of standards was detected by the aqueous mobile phases with 0.1% formic acid (composition 1), 5mM ammonium acetate (composition 2), 15mM ammonium acetate (composition 3) and 5mM ammonium formate (composition 4). (B): The number of standards was detected by the organic mobile phases with (composition 2) and without formic acid (composition 5).

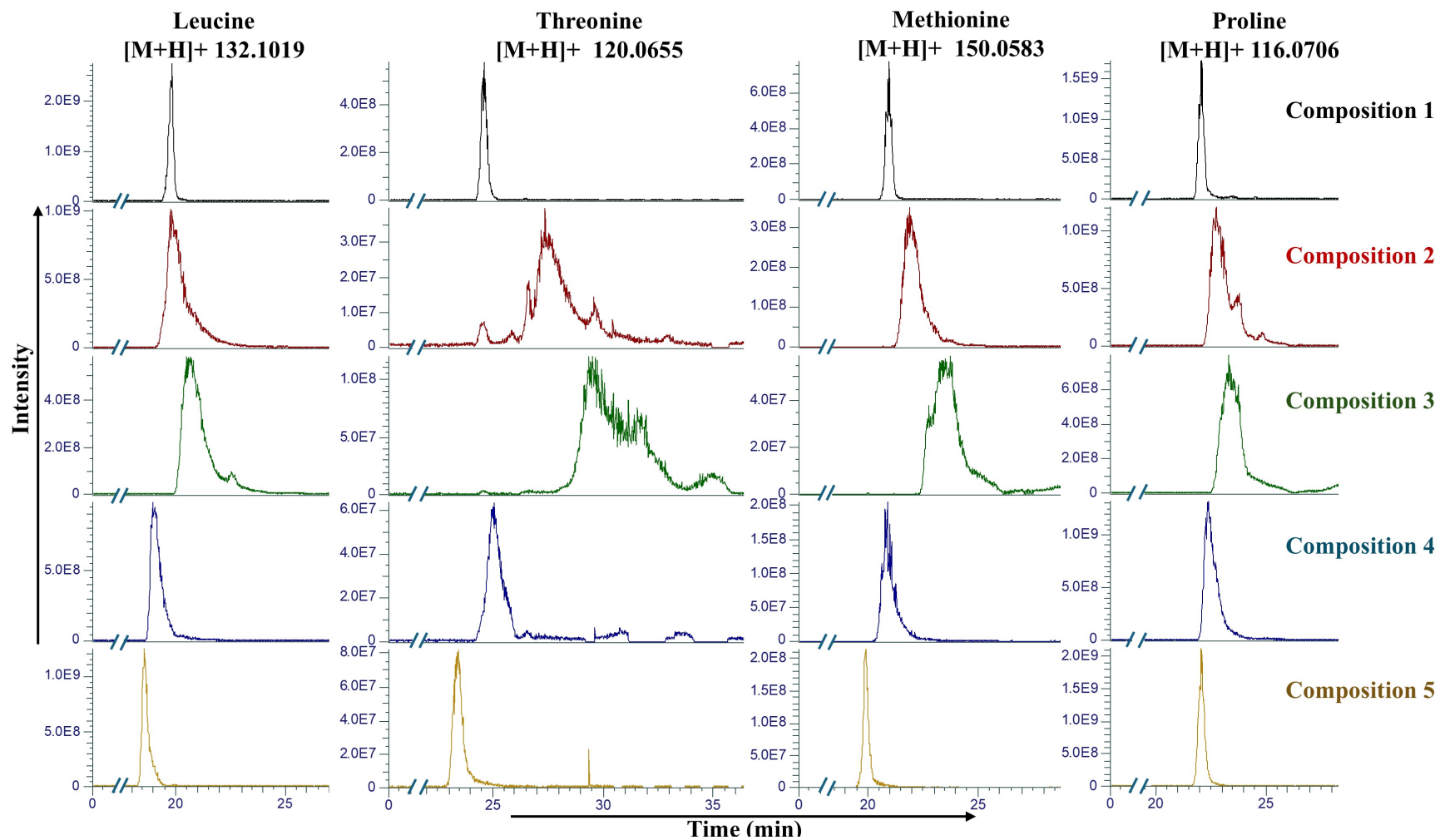


Figure 4.13. Performance of different mobile phases at capillary flow rate (1 $\mu\text{L}/\text{min}$). The EICs of leucine, threonine, methionine and proline were separated by the mobile phase with different additives.

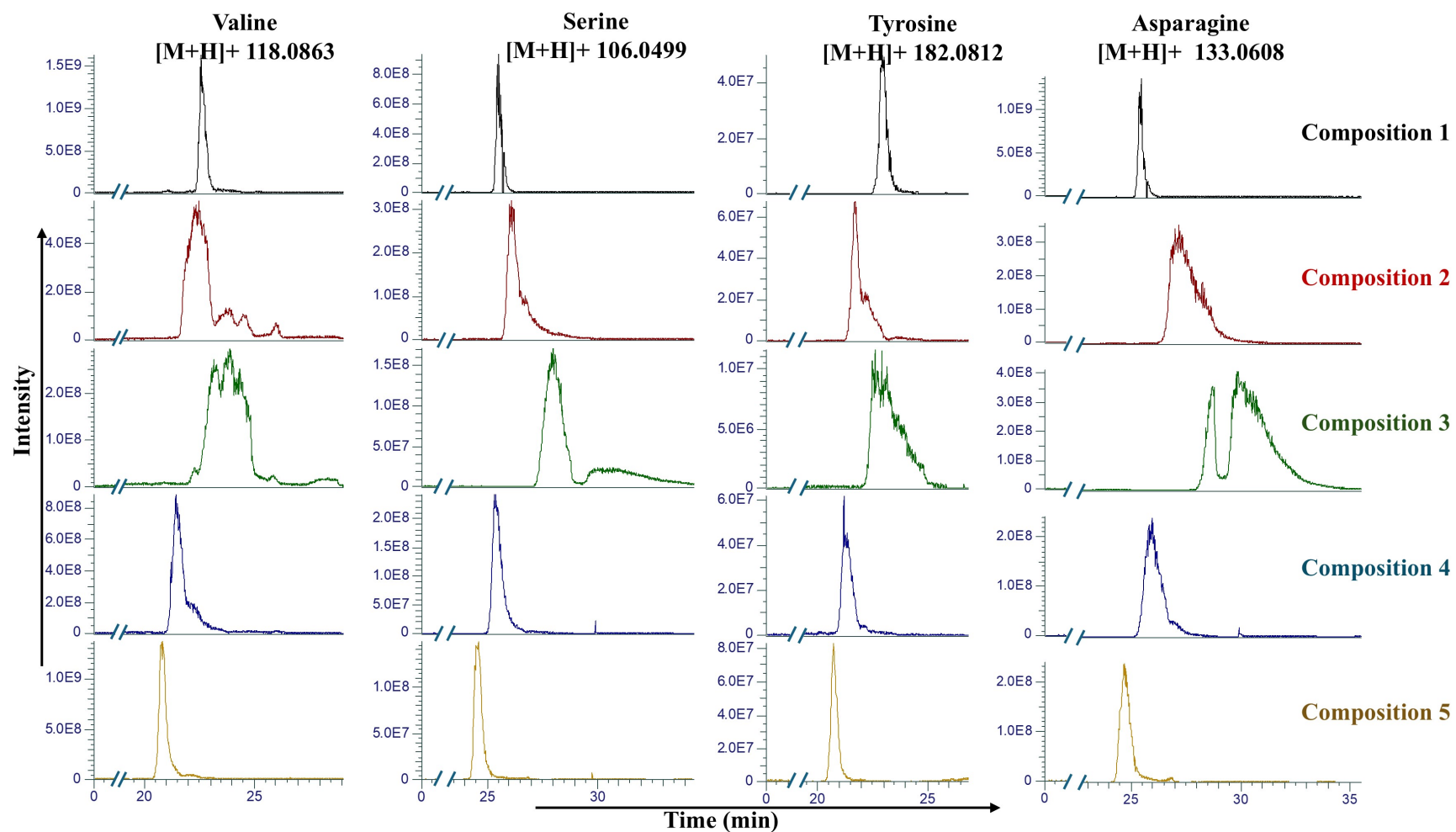


Figure 4.14. Performance of different mobile phases at capillary flow rate (continued-1). The EICs of valine, serine, tyrosine and asparagine were separated by the mobile phase with different additives.

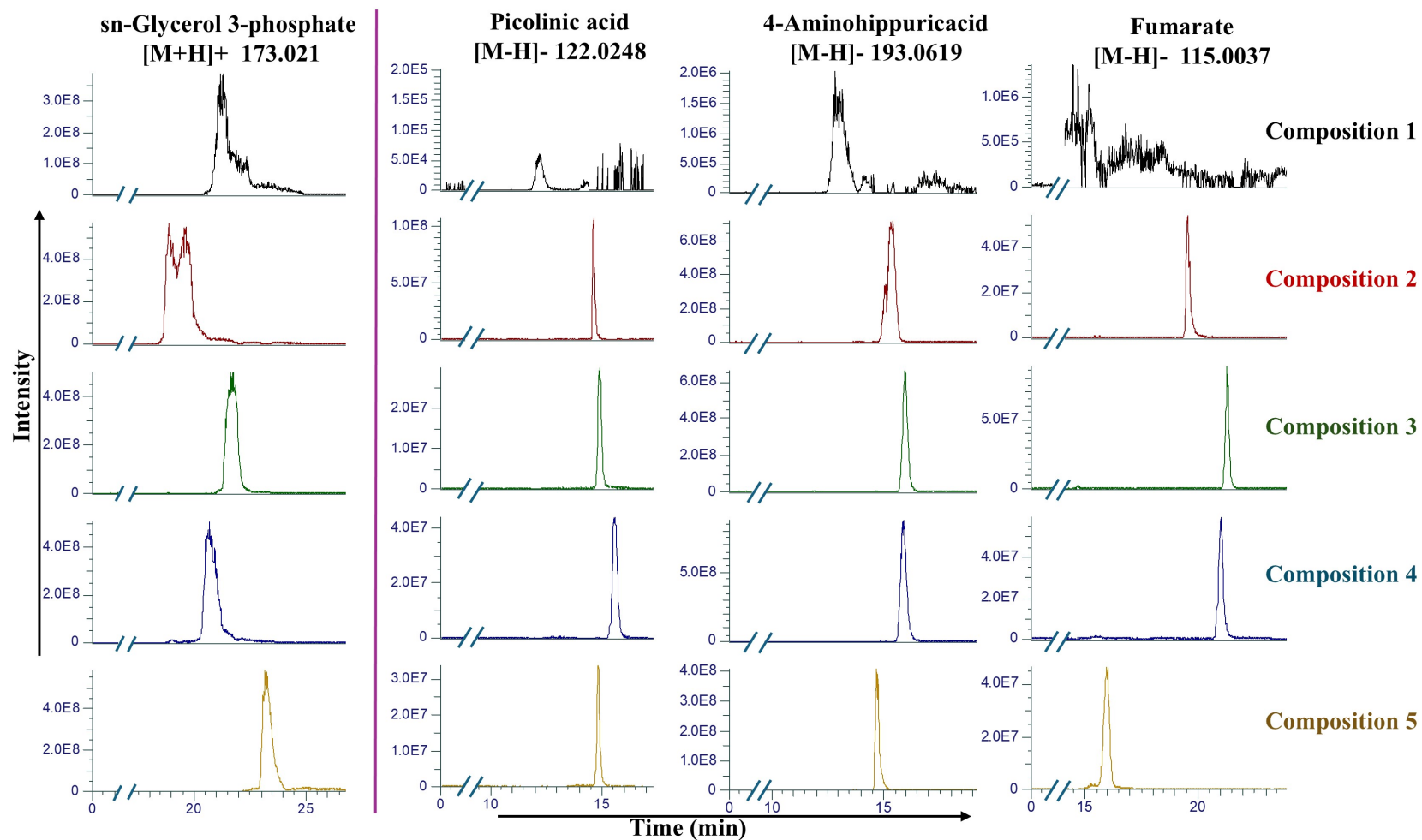


Figure 4.15. Performance of different mobile phases at capillary flow rate (continued-2). The EICs of sn-glycero-3-phosphate, picolinic acid, 4-aminohippuric acid and fumarate were separated by the mobile phase with different additives.

4.3.7 EASY-Spray™ emitters

Using the optimal mobile phases and the gradient profile, two emitters were optimised (Figure 4.16). The separation and detection conditions are summarised in Table A.12 and A.14.

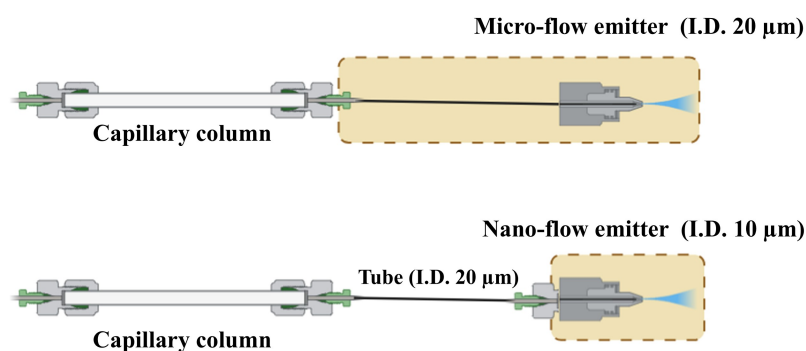


Figure 4.16. Instrumental setup of micro-flow and nano-flow emitter. The internal diameter is 20 µm for micro-flow emitter and 10 µm for nano-flow emitter. The micro-flow emitter has a silica-fused transfer line, whereas the nano-flow emitter does not. The figure was created with BioRender.com.

As shown in Figure 4.17, the micro-flow emitter showed a gradual pressure increase from 0 to 30 min due to the rising aqueous phase. In contrast, the nano-flow emitter had a sharp pressure drop at 6 min and recovery by 13 min. Initial pressure was higher for the nano-flow emitter (51 bar) than the micro-flow emitter (28 bar).

As displayed in Figure 4.18, the nano-flow emitter showed missing signals from 8–13 min, likely due to peak compression from high pressure, and poor separation from 15–27 min, unlike those observed with the micro-flow emitter. Both emitters showed similar spray stability, with signal drop at 45 min.

Moreover, EASY-Spray™ transfer line with I.D. 20 µm silica emitter is recommended for capillary-flow application according to the manufacturer's instructions. Hence, the micro-flow emitter (I.D. 20 µm) was decided for further optimisation.

However, this emitter may be suboptimal at a flow rate of 1 µL/min, as spraying worsened with higher aqueous content. A capillary-flow emitter (I.D. 15 µm) should be considered in future optimisation.

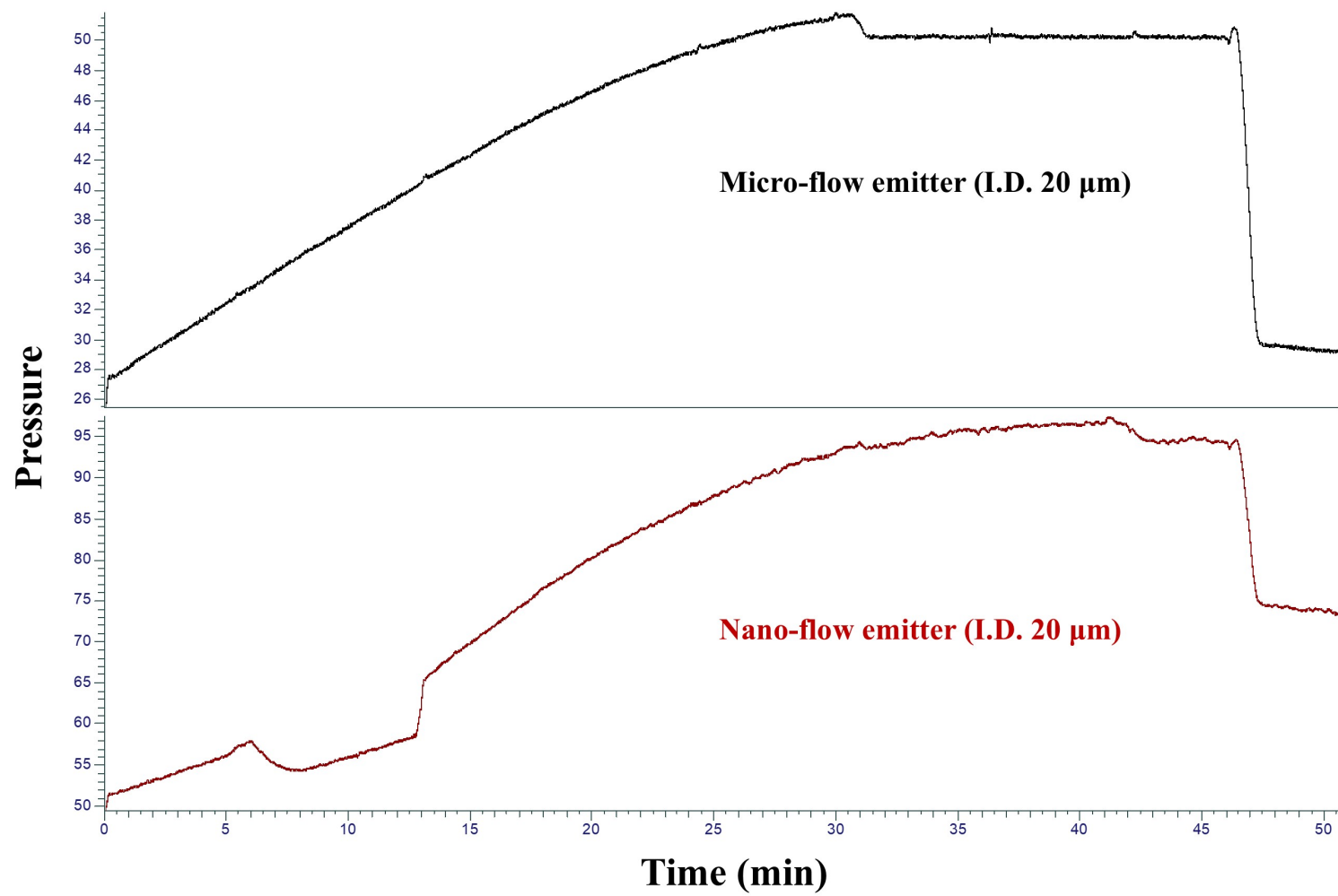


Figure 4.17. System pressure of UHPLC coupled with nano-flow emitter and micro-flow emitter.

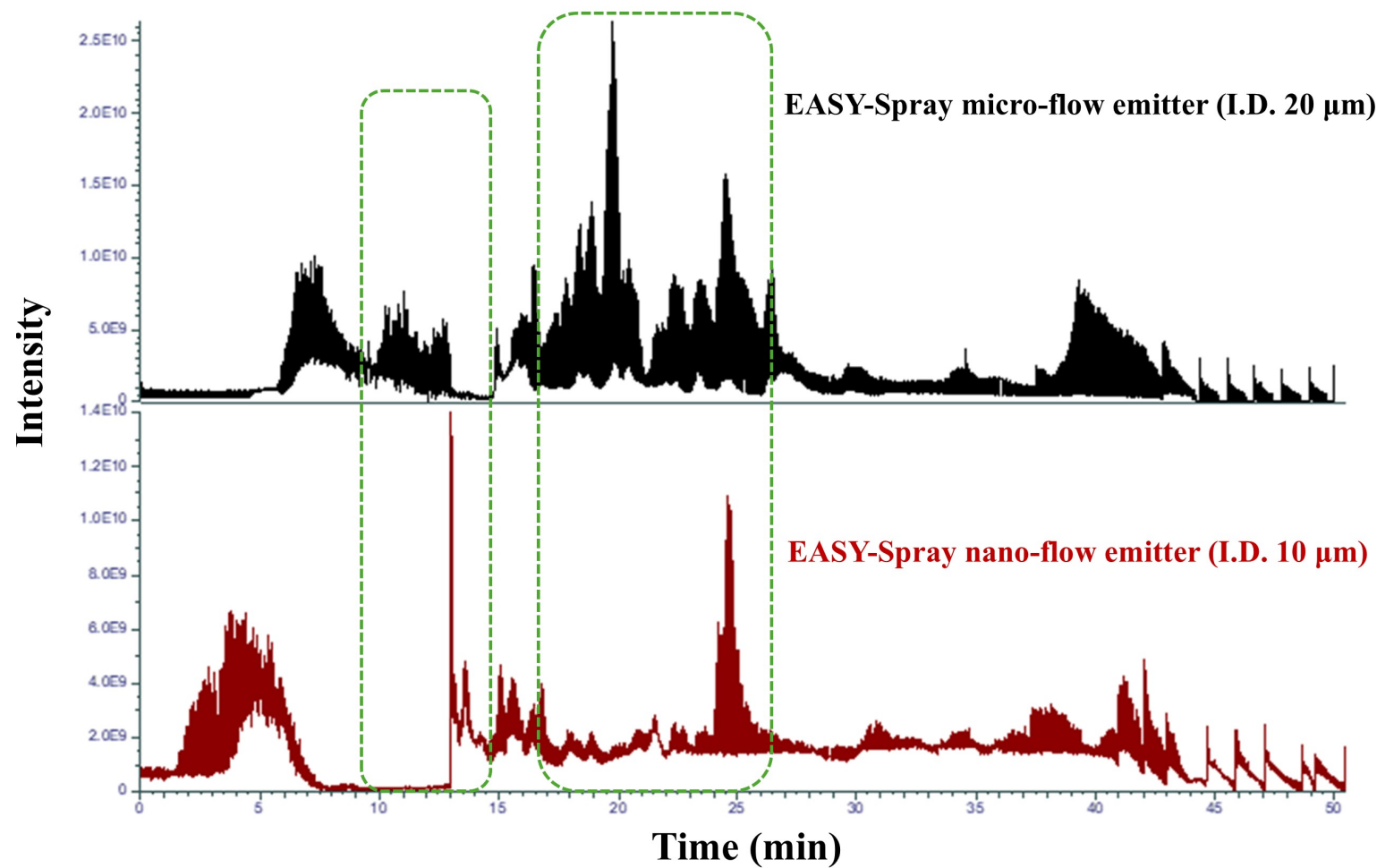


Figure 4.18. Comparison of nano-flow emitter and micro-flow emitter. The TICs of StdA were separated and detected on UHPLC system coupled with nano-flow emitter (red) and micro-flow emitter (black).

4.3.8 Column temperature

Given that the upper temperature limit for the ZIC[®]-HILIC capillary HPLC column is 60 °C, three temperatures were evaluated: 30 °C, 45 °C, and 60 °C. The separation and detection conditions are summarised in Table A.12 and A.14, with micro-flow emitter.

Among three temperatures, 45 °C provided the best balance in separation quality. As shown in Figure 4.19, at 30 °C, peaks eluting around 14–16 min were likely missing, compared to 45 °C. At 60 °C, peaks eluting at 18–20 min showed poorer separation than those at 45 °C.

On the EICs (Figure 4.20), deoxyuridine, leucine, decanoyl-carnitine, creatinine, and sn-glycero-3-phosphocholine were used as examples. Peak shapes were similar across the three temperatures, and retention time remained largely unaffected, except for creatinine. The peak shape of creatinine at 45 °C was better than other two temperatures. However, it was difficult to draw a definitive conclusion about intensity.

Hence, 45 °C was determined to be the optimal column temperature.

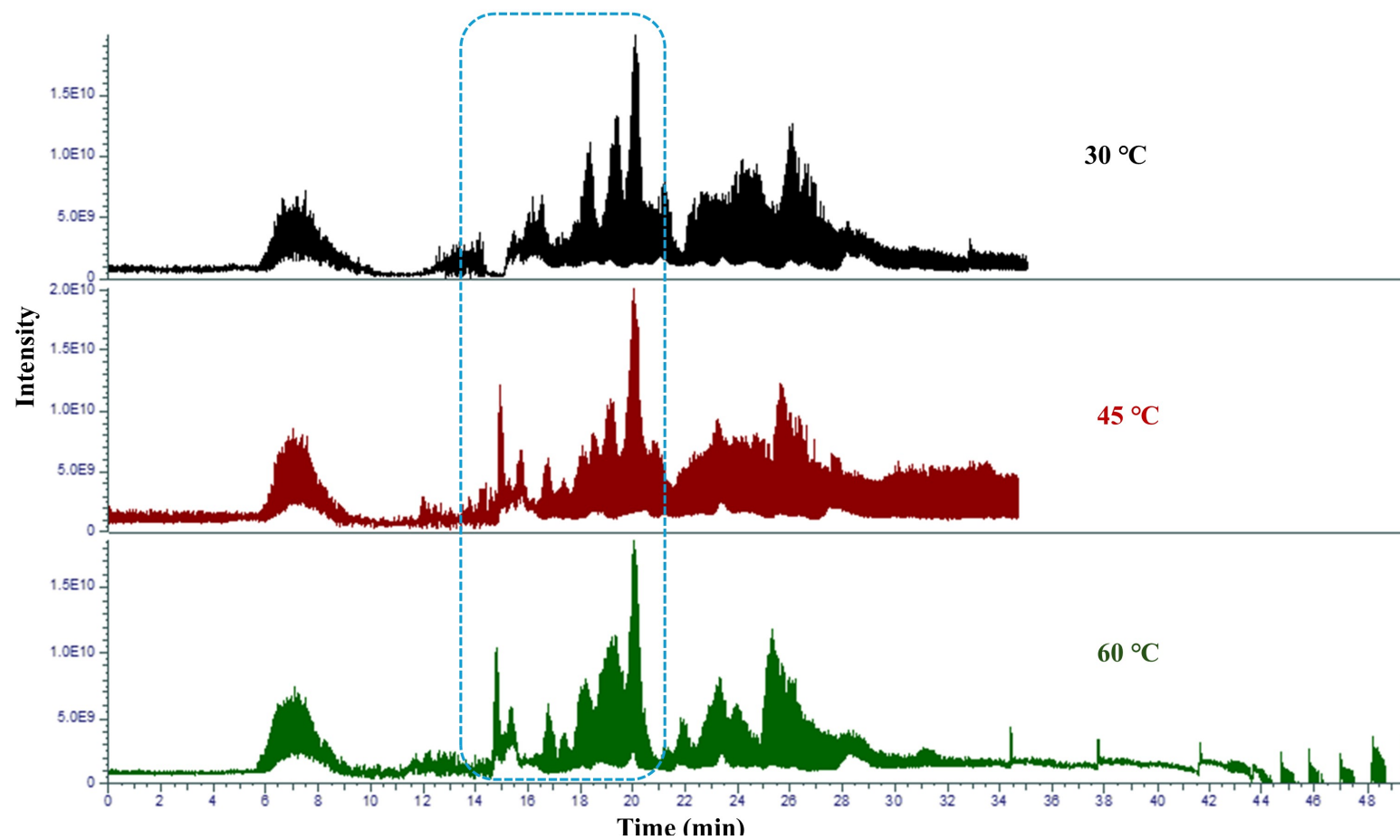


Figure 4.19. Optimal column temperature (TICs). The TICs of StdA were obtained under different column temperatures: 30 °C (black), 45 °C (red) and 60 °C (green).

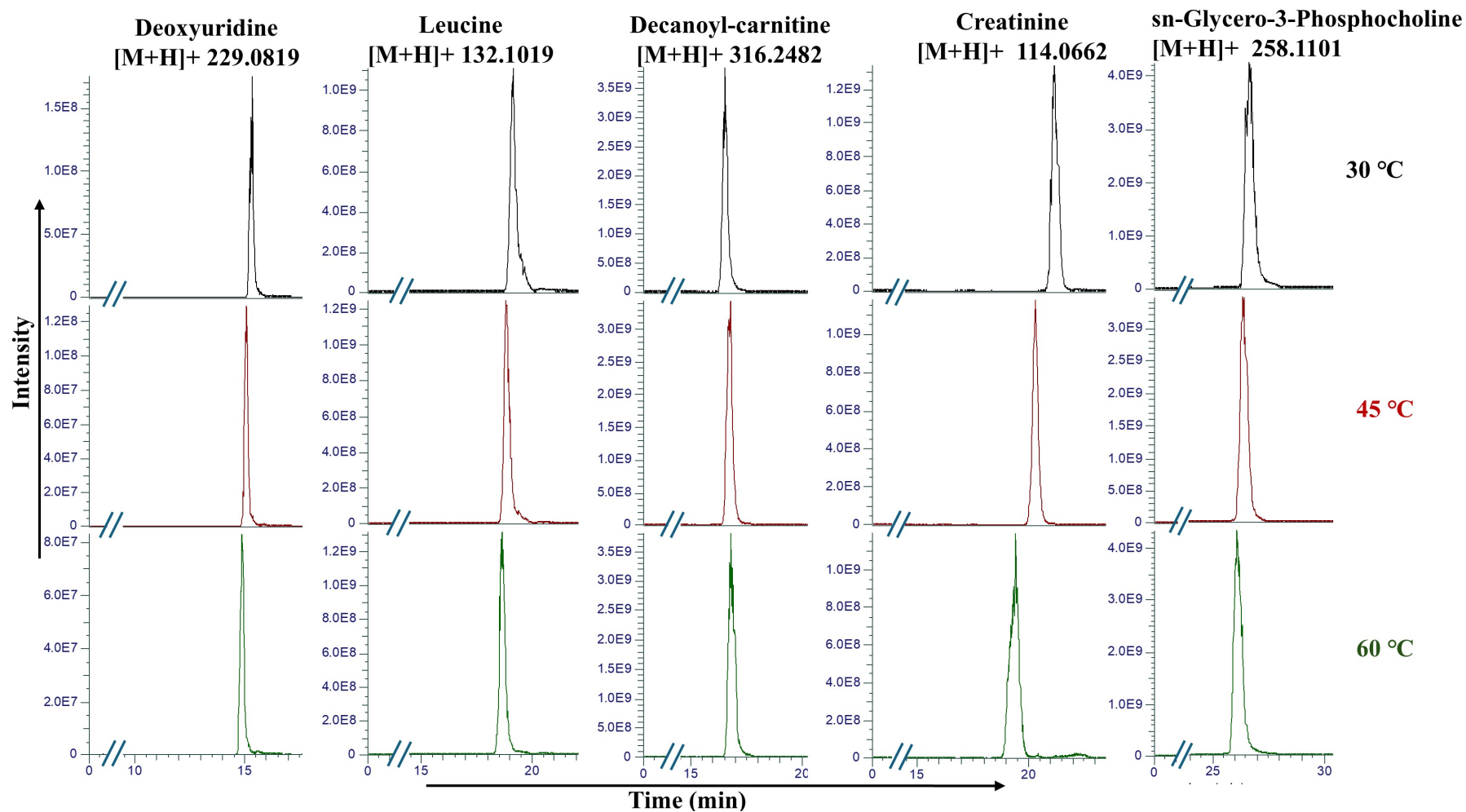


Figure 4.20. Optimal column temperature (EICs). The EICs of deoxyuridine, leucine, decanoyl-carnitine, creatinine and sn-glycero-3-phosphocholine were obtained under 30 °C (black), 45 °C (red) and 60 °C (green).

4.3.9 Injection mode and volume

Regarding the injection mode, direct injection was utilised, allowing samples to be loaded without extensive preprocessing, reducing the risk of sample loss²⁴⁸. Nevertheless, trap-elute mode is a common approach in low-flow studies, allowing for larger sample volumes to be loaded at higher flow rates, thus reducing the sample loading time and the peak broadening caused by diffusion during direct injection²⁴⁹. Considering these advantages, pre-concentration onto a trap cartridge is worth being evaluated in further studies.

It was a 25 μL loop used in our study. For low injection volume (20-200 nL), shown in Figures 4.21 and 4.22, minimal impact on TIC separation and peak shape was observed when injection volume decreased (examples: inosine, O-Butanoylcarnitine, adenine, orotate, 4-aminohippuric acid, 5-hydroxyindoleacetate). Although peak area decreased with reducing injection volume, the change was non-linear, suggesting injection inaccuracy (Figure 4.23). Moreover, injecting 20 μM StdA at 1000 nL had higher peak area than injecting 100 μM StdA at 200 nL, further supporting possible injection inaccuracy at low volumes, since both deliver 20 pmol of analyte.

The manufacturer confirmed accurate 1000 nL injection using a 25 μL loop ($\text{RSD} \leq 0.5\%$ for 500–2000 nL). The sharp and symmetric peak shapes were observed at 1000 nL injection volume, suggesting that column overloading is unlikely. Given these findings, 1000 nL injection volume was selected.

The possible injection inaccuracy at low injection volumes could be due to sample loss, as the extremely small volume could be retained or dispersed within the large sample loop (25 μL). Moreover, the large sample loop resulted in a longer loading time. In the future, smaller sample loops (1 or 10 μL) would be better for achieving accurate low-volume injections.

Given that only one injection was made at a single concentration, future experiments should include multiple replicate injections at each concentration to properly evaluate the accuracy and precision of the injection.

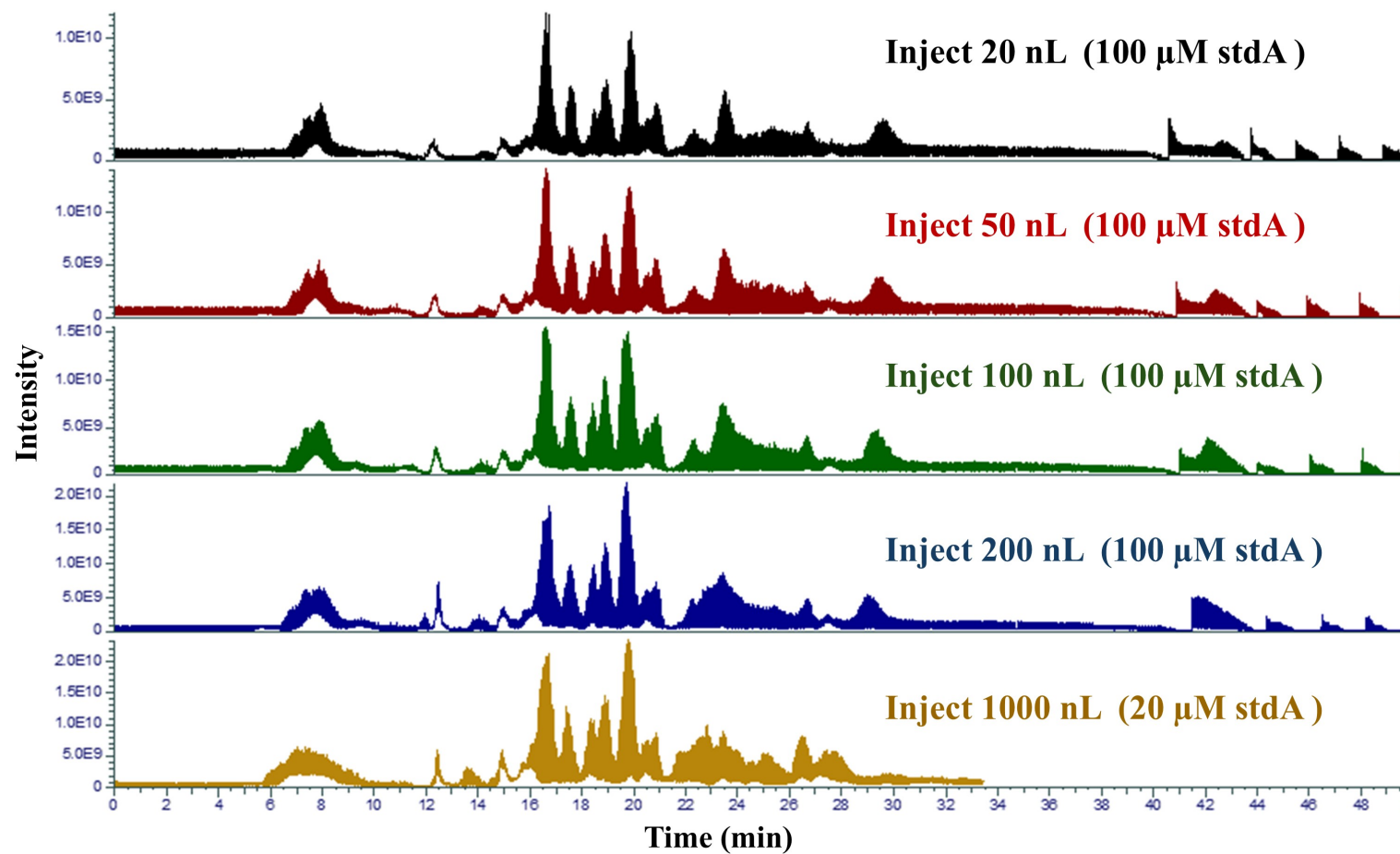


Figure 4.21. Effect of injection volume on separation. The total ion chromatograms of StdA were obtained under different injection volumes: 20 nL (black), 50 nL (red), 100 nL (green), 200 nL (blue), and 1000 nL (yellow).

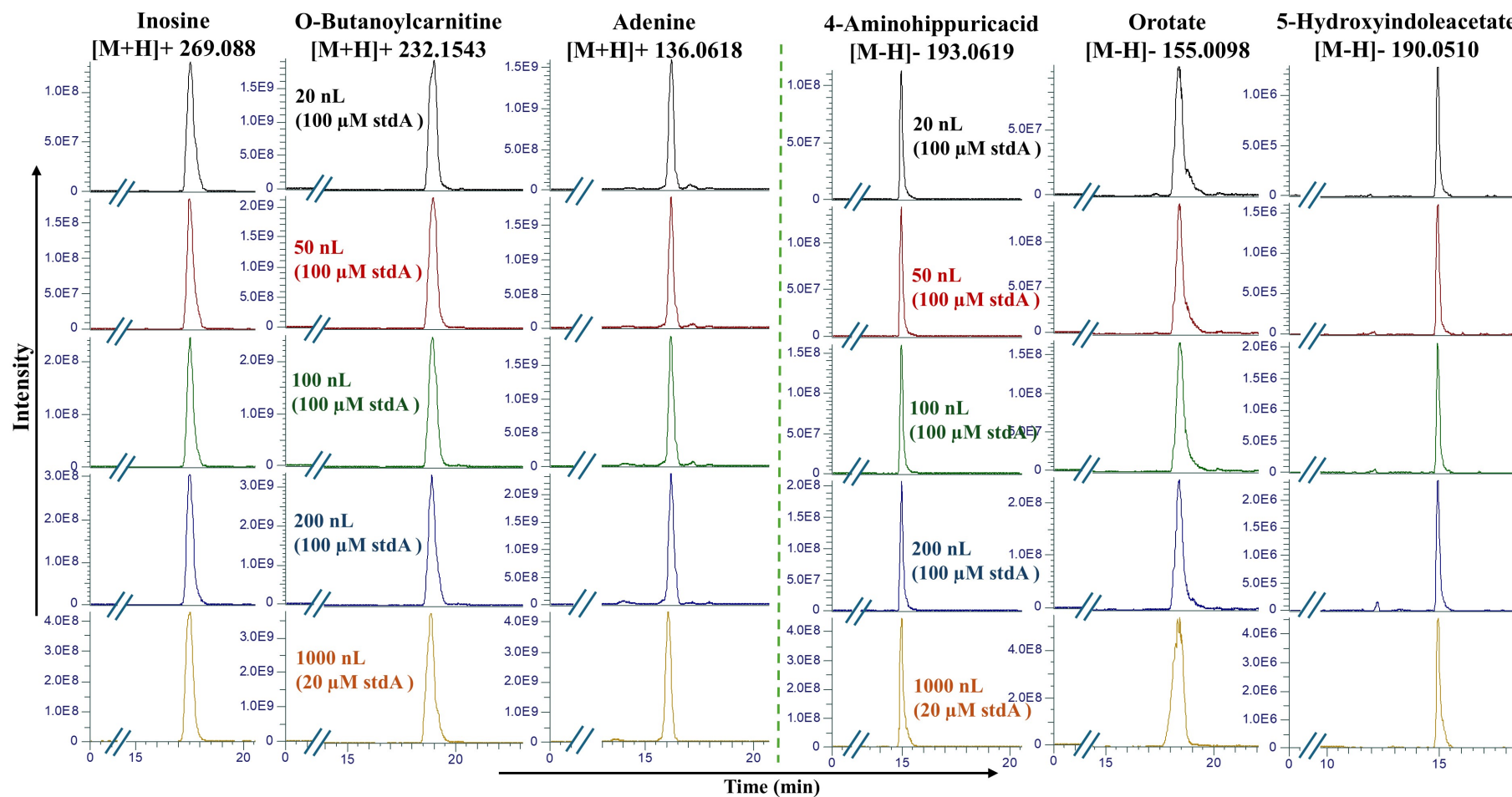


Figure 4.22. Impact of injection volume in positive and negative mode. The EICs of inosine, O-butanoylcarnitine, and adenine were detected in positive mode, while orotate, 4-aminohippuric acid, 5-hydroxyindoleacetate were detected in negative mode.

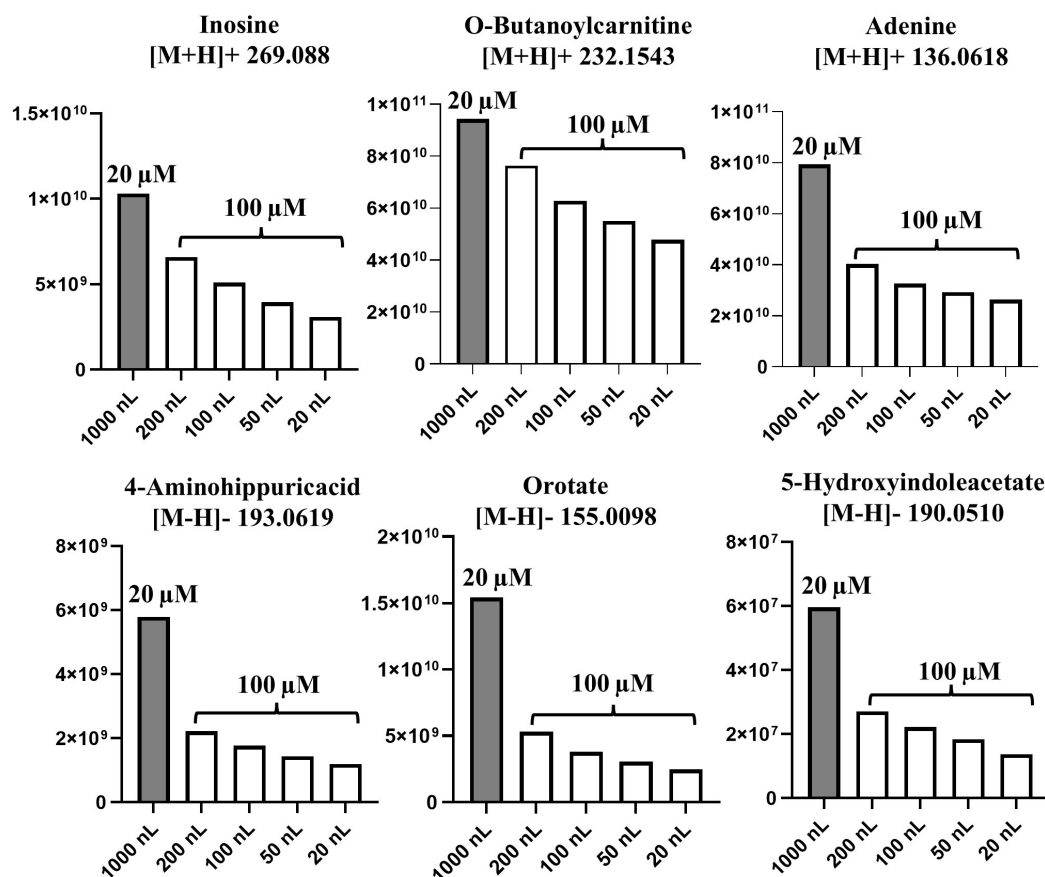


Figure 4.23. Comparison of injection volumes in positive and negative mode.

4.3.10 MS parameters

Given that no additional gas was applied to the Easy-SprayTM ionisation source, capillary temperature and spray voltage became the main adjustable parameters and were optimised as details in Table 4.6. The LC conditions are presented in Table 4.7 and other detection parameters are shown in Table A.12.

Table 4.6. MS parameters optimised at capillary flow rate.

MS parameters	Settings
Capillary temperature	250 °C, 275 °C, 300 °C
Spray voltage	± 1.5 kV, ± 1.7 kV, ± 1.9 kV, ± 2.1 kV, ± 2.3 kV

As presented in Figure 4.24, the sum of intensities from all detected ions in StdA was highest at 275 °C, suggesting improved ionisation efficiency and ion transmission.

For spray voltage, the maximum voltage applicable to the micro-flow emitter is

Table 4.7. Optimal separation conditions for capillary-flow LC-MS method.

Column	ZIC®-HILIC capillary HPLC column (5 µm, 0.3×150 mm)
Analyte	StdA
Column temperature	45 °C
Injection volume	1 µL
Emitter	EASY-Spray™ micro-flow emitter (I.D. 20 µm)

Gradient table		
Time (min)	Flow rate (µL/min)	B%
0	1	80
30	1	20
31	1	5
41	1	5
42	1	80
50	1	80
Re-equilibration (> 8 column volumes)		

± 2.3 kV, with an incremental increase of 0.2 kV recommended. Figure 4.24 shows that the sum of intensities from all detected ions in StdA was highest at ± 2.1 kV. Moreover, for the single standards, most signals showed the highest intensity at ± 2.1 kV compared to other spray voltages.

Based on these findings, a capillary temperature of 275 °C and a spray voltage ± 2.1 kV were found to be optimal. Nevertheless, scan settings, such as resolution, automatic gain control, maximum injection time, isolation window, and normalised collision energy, still require further optimisation.

To summarise, the optimal separation and detected conditions at capillary-flow rate (1 µL/min) are presented in Table 4.7 and Table 4.8.

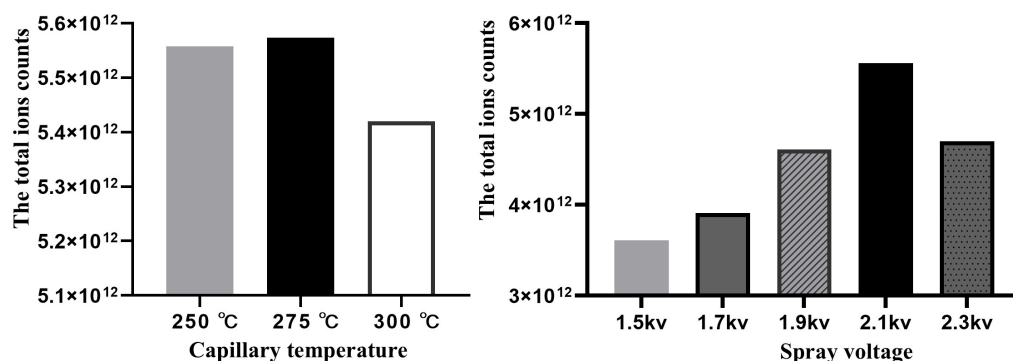


Figure 4.24. Optimal capillary temperature and spray voltage. The sum of intensities from all detected ions in StdA was measured across different capillary temperatures and spray voltages.

Table 4.8. Optimal detection parameters for capillary-flow LC-MS method.

MS parameters	EASY-Spray TM
Resolution	70000 at m/z 200
Scan range	m/z 70-1050
Scan mode	Full MS-SIM
Capillary temperature	275 °C
Sheath gas	0
Aux gas	0
Sweep gas	0
Spray voltage (+) kV	2.1
Spray voltage (-) kV	2.1

4.3.11 Standards concentration

Serial concentrations of StdA solution (1 μ M, 500 nM, 200 nM, 100 nM, 50 nM and 10 nM) were injected to UHPLC system, with solvent blanks intermittently injected between samples.

As shown in Figure 4.25, the highest number of analytes was detected in 20 μ M StdA, but it caused residuals in the sampler. The residual was undetectable after injecting 200 nM StdA. Both 200 nM and 100 nM StdA had similar results, with 30 analytes detected. In balance of detection and minimisation of residuals, 100 nM StdA was identified as the optimal standard concentration.

Double-gradient analysis (Figure B.21) showed that not all analytes in StdA solution (20 μ M) had residual in the sampler. Moreover, compared to higher concentrations, some standards were not detected at 100 nM due to dilution effects. These suggested that a uniform concentration of 100 nM may not be suitable for all standards, and concentration optimisation should ideally be performed individually for each compound, based on its specific properties.

After analysing all standard solutions (StdA to StdE, 100 nM), total 149 standards could be detected by the developed capillary-flow method, details in <https://github.com/Rui-eebebeb/Standards-database-Capillary-flow>.

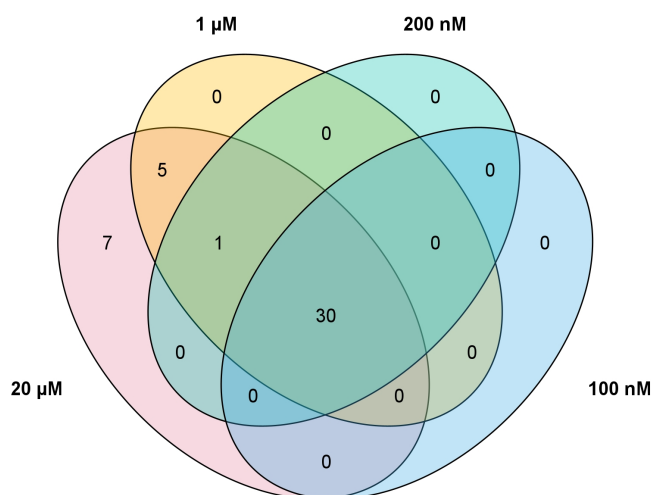


Figure 4.25. The number of detected standards in serial concentrations of StdA solutions. Serial concentrations of StdA solutions (20 µM, 1 µM, 200 nM and 100 nM) were analysed by the optimal capillary-flow method.

4.3.12 Extraction solvent and reconstitution volume

As shown in Figure 4.26 A and B, methanol extracted significantly more ions than acetonitrile ($p = 0.0062$) and had a higher total ion intensities ($p = 0.0209$), indicating that methanol was the optimal extraction solvent.

To further determine the ideal extraction volume, different volumes of methanol were evaluated (Figure 4.26 C and D). The sum of total ion intensities and the number of detected ions were highest with 200 µL methanol, comparable to 600 µL, suggesting that 200 µL of methanol was identified as the optimal extraction solvent. In the practical procedure, a total of 600 µL of methanol was used by adding 200 µL three times.

For reconstitution volume, shown in Figure 4.27, the intensities of some metabolites detected in cell extracts reconstituted with 25 µL of methanol were significantly lower than those reconstituted with 50 µL, indicating incomplete dissolution of dried extracts. A further decrease in intensity was observed when the volume increased from 50 µL to 100 µL, suggesting that higher volumes diluted analytes. Finally, 50 µL of methanol was selected as the optimal reconstitution volume.

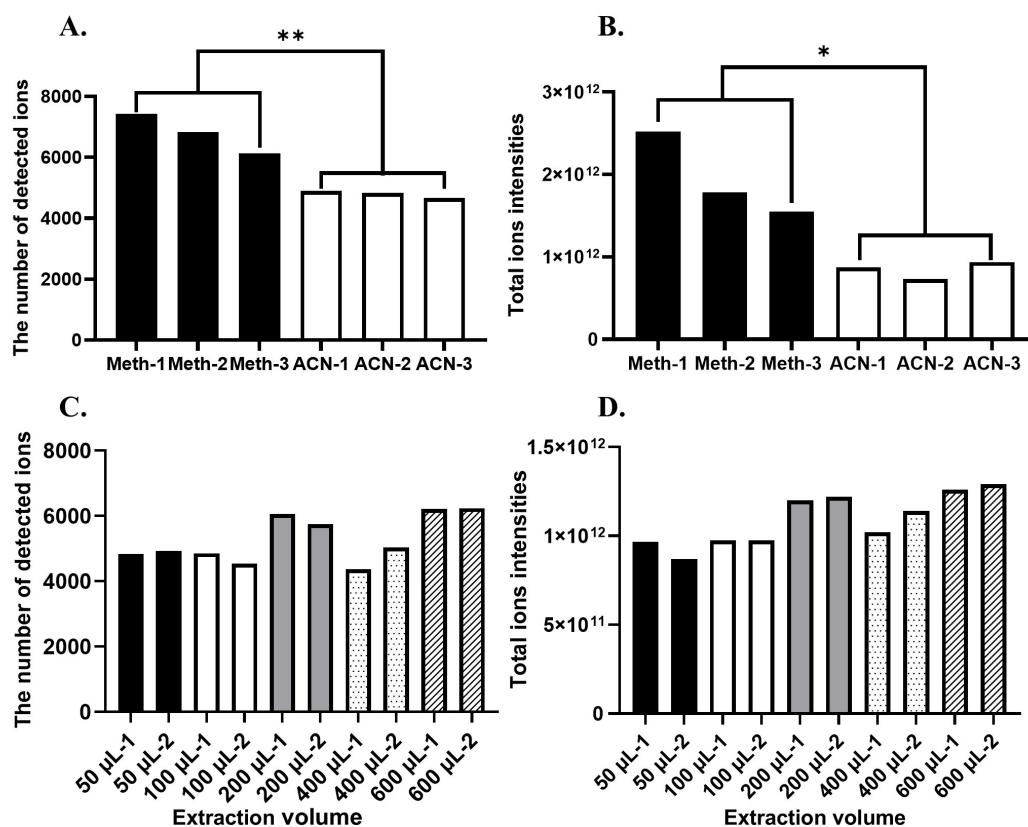


Figure 4.26. Optimal extraction solvent and volume. (A): The number of ions detected from cells extracted by acetonitrile (ACN) and methanol (Meth). (B): The sum of intensities from all ions detected in cells extracted by acetonitrile (ACN) and methanol (Meth). (C): The number of ions detected from cells extracted by different volumes of methanol. (D): The sum of intensities from all ions detected in cells extracted by different volumes of methanol. Samples were extracted from 2×10^4 GIN31 cells. The symbol of * and ** means $p < 0.05$.

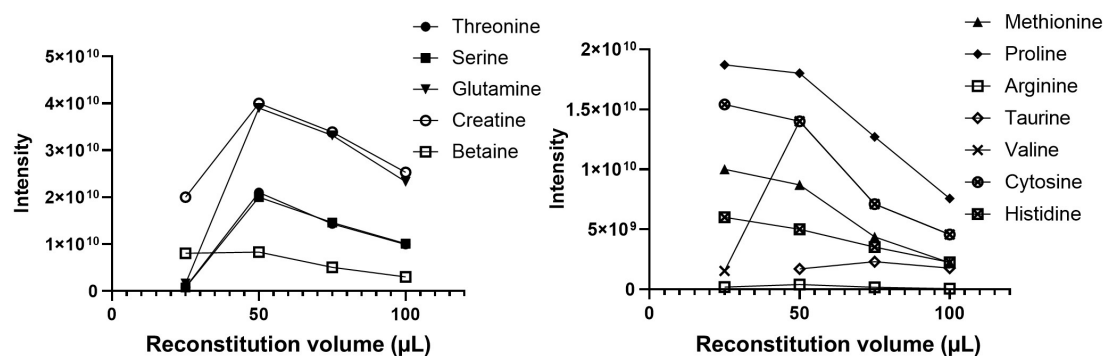


Figure 4.27. Optimal reconstitution volume. Samples were extracted from 2×10^4 GIN31 cells.

4.3.13 Feature detection limit

Cell extracts from 625 to 20,000 cells were reconstituted in 50 μ L methanol and analysed using the developed capillary-flow method.

The relationship between cell input and feature detection indicated a threshold-dependent sampling efficiency. As presented in Figure 4.28 A, the number of detected features exhibit a sigmoidal dependence on cell number ($R^2 = 0.99$), with half-maximal detection observed at 5,240 cells, indicating that a threshold where a minimum number of cells is required to overcome technical noise. Also, it suggested that loading more than 10,000 cells provides minimal contribution to feature detection, indicating a saturation point under the given analytical conditions.

In parallel, cumulative abundance analysis (Figure 4.28 B) shows that higher cell inputs yield broader range coverage, as evidenced by the increasing number of features required to account for 95% of total abundance, from 188 features at 1,250 cells to 502 features at 10,000 cells, suggesting improved detection of features with greater input.

Collectively, these results highlight the importance of sufficient cell input to ensure comprehensive molecular coverage. Based on the detection threshold and abundance plateau, a minimum of 5,000 cells is necessary to capture molecular features, while inputs of 10,000 cells provide near-saturation coverage with minimal gains beyond that point.

To assess whether this plateau in features was due to column overloading, we examined the relationship between sample input and signal intensity. The peak area was linearly correlated with the number of cells loaded (average $R^2 = 0.9594$) for a representative set of features (Figure 4.29). This confirmed that column saturation was not occurring. The observed limit is more likely due to the separation capacity of the LC system and/or the dynamic range of the mass spectrometer.

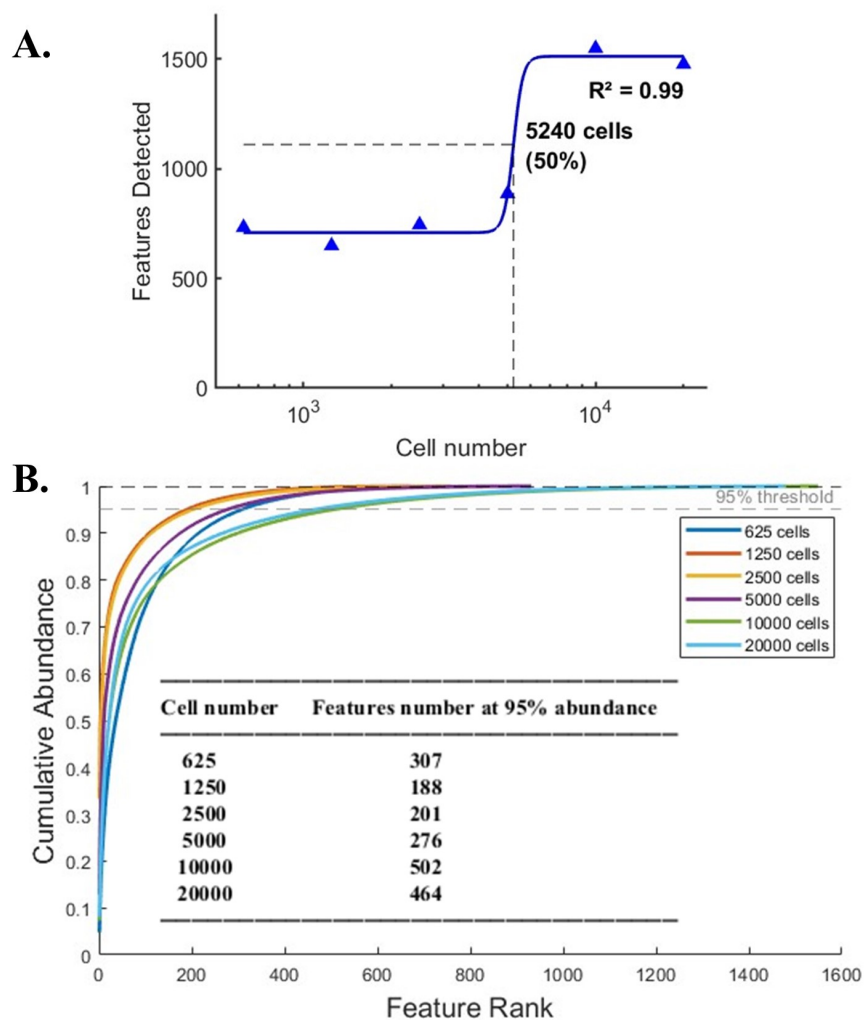


Figure 4.28. Feature detection limit of capillary-flow LC-MS method. (A): The feature detection curve of different cell inputs. (B): Cumulative abundance curves of different cell inputs ranked by descending feature intensity. The dashed horizontal line marks the 95% cumulative abundance threshold. The accompanying table summarises the number of features required to reach 95% cumulative abundance at each cell number.

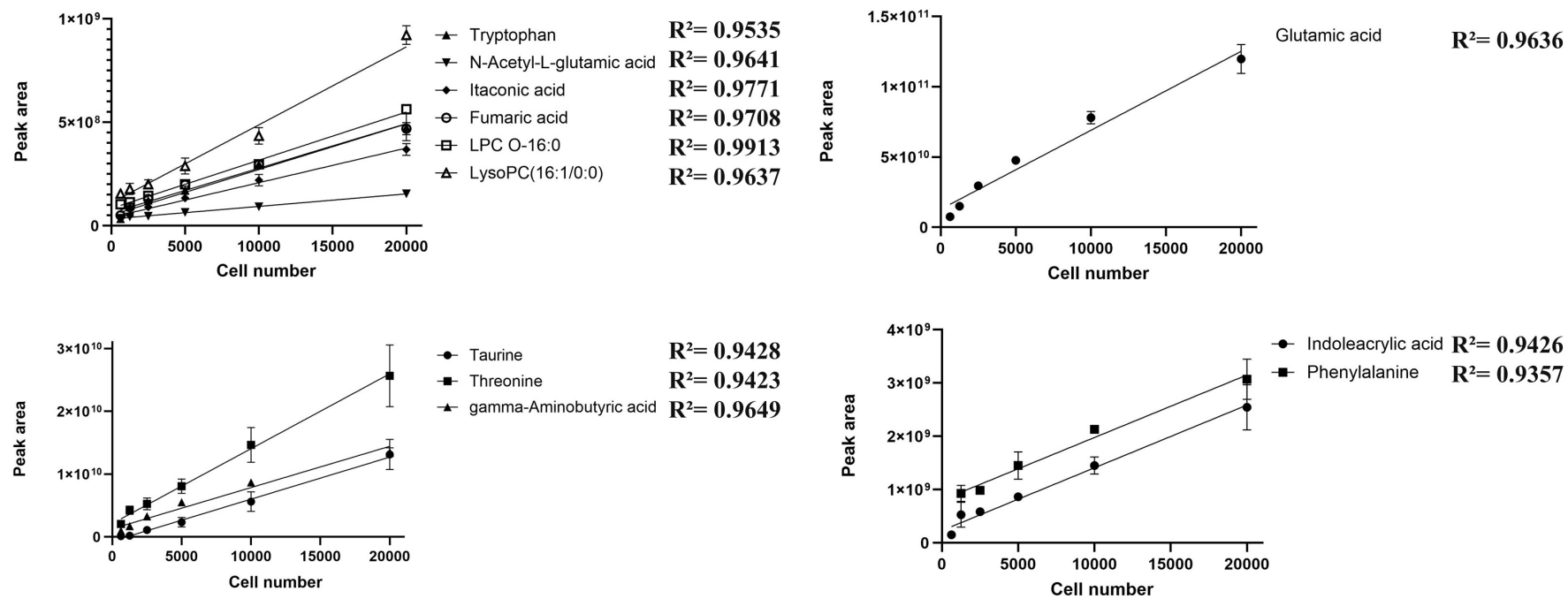


Figure 4.29. Linear correlation between peak area and cell input.

4.4 Conclusion

This study presented an optimised capillary-flow LC-MS method for metabolomics application, capable of detecting metabolites from as few as 5,000 GIN31 cells. These findings highlight key technical considerations for low-flow LC-MS analysis, paving the way for its future application in GBM research.

Nevertheless, it is acknowledged that this method is not yet fully optimised and still requires further refinement, as the current results are limited by the consumables currently available. It can not be considered as a general protocol for low-flow metabolomics studies, as it was optimised specifically for GIN31 cells. To establish broader utility, future studies should apply this method to diverse biological samples and assess its robustness and reproducibility. Additionally, the differences observed between nano-flow and capillary-/micro-flow methods require further investigation to elucidate the underlying mechanisms.

Chapter 5

Metabolic characterisation of glioblastoma invasion using capillary-flow LC-MS-based metabolite profiling

5.1 Introduction

Glioblastoma (GBM) can invade cross tissue barriers and the extracellular matrix (ECM) by remodelling their cytoskeleton, facilitating the colonisation of distant areas from the primary tumour site²⁰³. Understanding the metabolic drivers of GBM invasion is key to uncovering its biology and finding new therapeutic targets.

As described in Chapter 3, single-cell metabolomics was applied to identify metabolic signatures associated with GBM invasion, utilising 3D OrbiSIMS and AP-MALDI-MS. However, several challenges remain for these techniques, including the limited number of identified metabolites, low confidence in metabolite identification, redundant annotation of individual ions, and inconsistencies in abundance of some metabolites between two methods. These limitations may affect the consistency of interpreting metabolomics results.

To address these challenges, capillary-flow liquid chromatography-mass spectrometry (capillary-flow LC-MS)–based metabolite profiling has been developed. Inheriting the principles of conventional LC-MS, capillary-flow LC-MS enables chromatographic separation before MS detection, simplifying the signal landscape by spreading analytes over time, rather than simultaneously detecting everything²⁵⁰. It also provides additional identification vectors, including retention time and MS/MS spectra, thereby improving the reliability of metabolite identification²⁵¹. Furthermore, as discussed in Chapter 4, the lower flow rate enhances sensitivity for low abundance analytes, enabling the analysis of smaller biomass samples. The developed capillary-flow method could detect metabolites from as few as 5,000 cells, making it capable of analysing samples (1×10^4 cells) obtained from the invasion assay.

As illustrated in Figure 5.1, capillary-flow LC-MS–based metabolite profiling was applied as a complementary method to analyse invasion samples, characterising metabolic signatures of GBM invasion. Moreover, the developed method was evaluated for carryover effect, reproducibility, and statistical reliability.

5.2 Materials and methods

5.2.1 Materials

The materials related to cell culture and invasion assay have been described in Chapter 2.2.1. The materials related to the capillary-flow LC-MS method have been described in Chapter 4.2.1. GIN31 cells were cultured under the conditions described in Chapter 2.2.2.

5.2.2 Sample preparation

The invasion assay followed the steps described in Chapter 2.2.5.

GIN31 (2×10^4 cells/insert) suspended in DMEM were seeded in pre-soaked inserts. The plate was further incubated for 12 h.

When incubation ended, for non-invaded samples, the medium inside the inserts

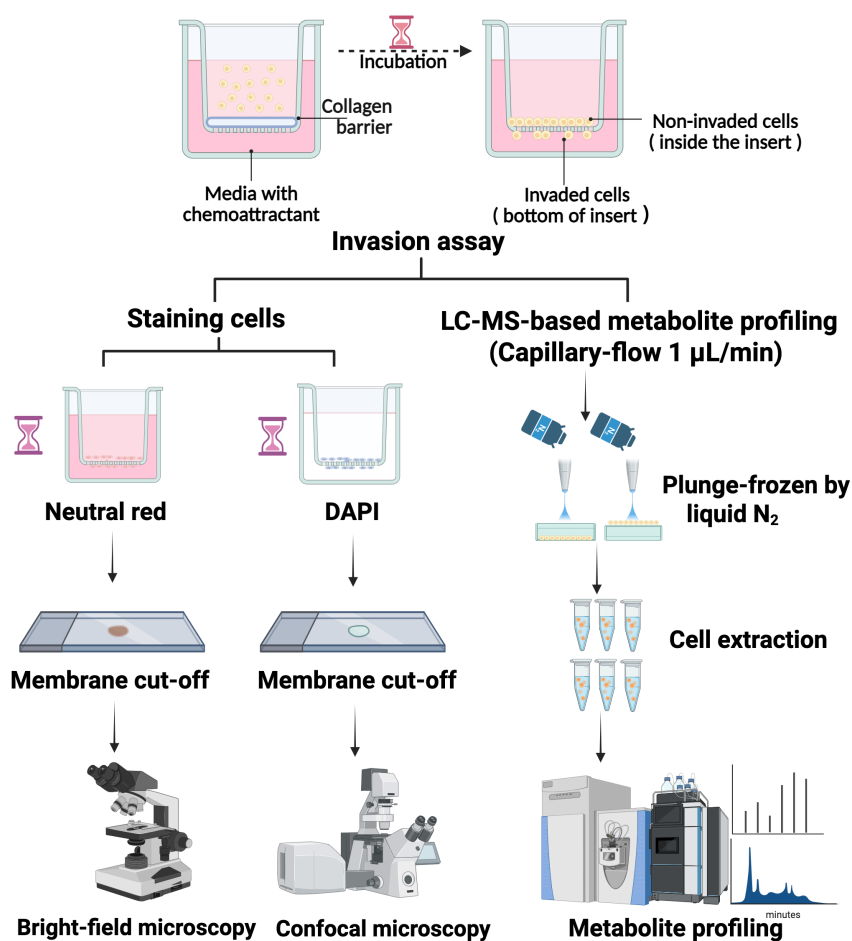


Figure 5.1. Workflow for capillary-flow LC-MS-based metabolite profiling. The figure was created with BioRender.com.

was removed, and the cells were gently washed with pre-warmed PBS. The cells at the bottom of the inserts were removed. The inserts were then plunge-frozen with liquid nitrogen.

For invaded samples, the inserts were gently washed with pre-warmed PBS before removing non-invaded cells. Then, the inserts were placed upside-down and plunge-frozen by liquid nitrogen.

After inserts being transferred onto the ice, the membrane was carefully cut off and placed in a tube containing 200 μL of methanol before being vortexed for 1 h at 4 $^{\circ}\text{C}$. Subsequently, the supernatant was transferred to a new tube after centrifugation (14,000 g / 5 min) at 4 $^{\circ}\text{C}$. The membrane was washed twice with 200 μL methanol, and the supernatant was combined with the previous.

Cell extracts were dried under a nitrogen stream by intermittently flushing the inside of the tube with methanol. The dried samples were reconstituted in 50 μ L LC-MS grade methanol and transferred to LC-MS vials for analysis.

Blank samples were inserts without cells and underwent the same steps of invasion assay and extraction. A QC sample was prepared by taking 5 μ L aliquots from each sample and mixed into a vial (excluding blank samples).

5.2.3 Staining and imaging

Neutral red staining was followed the approach in 3D OrbiSIMS analysis (Chapter 3.2.2.2). However, 10 μ L neutral red solution was added to the Transwell chamber for 1 h. Dried membranes were cut off and imaged at four different fields of view using a multi-viewing microscope (Brunel, N-510) equipped with a 10 $\times$ magnification len.

For DAPI staining, cells were fixed with ice-cold methanol for 1 h, followed by staining with 1 μ g/mL DAPI for 15 min. Dried membranes were imaged under a confocal laser scanning microscope (Leica, DMI4000B) using a 10 $\times$ magnification len.

5.2.4 Capillary-flow LC-MS-based metabolite profiling

5.2.4.1 Separation and detection

Reagent-blank samples were first analysed, followed by randomly sequenced experimental samples with intermittent QC samples. Five mixtures of authentic standards solutions (100 nM, StdA-StdE) were injected under identical conditions.

Samples were separated on a ZIC[®]-HILIC capillary HPLC column (5 μ m, 0.3 $\times$ 150 mm) from Merck Supelco[®] (Darmstadt, Germany), using a Vanquish[™] Neo UHPLC system operated in nano/capillary-flow mode (tube I.D 20 μ m, Thermo Fisher Scientific, Hemel Hempstead, UK). The mobile phases were 5 mM ammonium acetate (A) and 0.08% formic acid in acetonitrile (B), using a linear gradient (0-30 min: 20% A to 80% A, 30-31 min: 80% A to 95% A, 31-41 min: 95% A, 41-42 min: 95% A to 20 % A,

42-50 min: 20% A). The injection volume was 1 μ L and the flow rate was 1 μ L/min. The autosampler and column compartment temperature was maintained at 4 °C and 45 °C respectively.

Detection was performed on a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) fitted with an EASY-SprayTM ionisation source. The UHPLC system was connected via an EASY-SprayTM micro-flow emitter (I.D. 20 μ m). Data acquisition was performed in polarity-switching mode with spray voltages of $\pm$ 2.1 kV. The capillary temperature was 275 °C. Spectra were recorded at a mass resolution of 70,000 (m/z 200), across an m/z range of 70-1,050, with an automatic gain control (AGC) of 3×10^6 . Data-dependent MS/MS (top 10) was performed on QC sample at a mass resolution of 17,500 with an AGC target 1×10^5 and stepped normalised collision energies of 20, 30 and 40.

5.2.4.2 Metabolite identification and pathway analysis

Data were processed in Compound Discoverer 3.3 SP3 (Thermo Fisher Scientific, Waltham, MA, USA) using an untargeted metabolomic workflow for peak picking, alignment, metabolite identification, and gap filling. Annotation, identification confidence and pathway analysis were performed as described in sections 2.2.6.3 and 2.2.6.4.

5.2.5 Statistics

Images were processed with Fiji (ImageJ v1.54p) and statistical data analysis was performed on GraphPad Prism v10.3.0 (GraphPad Software, San Diego, CA), with p value < 0.05 considered statistically significant. Univariate analysis, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed as described in Chapter 2.2.7.

5.3 Results and discussion

5.3.1 Carryover

PCA scores plot (Figure 5.2) shows tight clustering of blank samples, clearly separated from pooled QC and other study samples, indicating that blank samples contained minimal biological signal.

To assess any carryover effect, a blank was injected immediately after a QC sample. Of 1470 ions detected in pooled QC, only 91 ions (6.19%) were also found in the blank, suggesting low risk of carryover. Among shared ions, 52.75% (48 ions) had carryover below 5% (Figure 5.3). The remaining 43 ions (except for m/z 423.3568, 885.5508, and 428.3721) were considered either as non-informative features (intensity RSD > 30% in QC technical replicates), or less biologically relevant (low RSD but lacked both annotation and statistical significance between invaded and non-invaded groups).

Furthermore, we checked the 37 confidently annotated metabolites (levels 1 and 2), only N-acetyl-L-aspartic acid showed carryover (3.96%). Collectively, these results proved that carryover is unlikely to affect data quality.

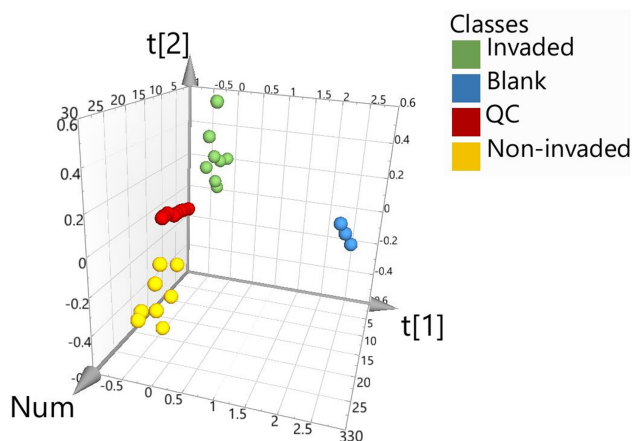


Figure 5.2. PCA scores plot of experimental samples.

5.3.2 Reproducibility, mass accuracy and statistical reliability

Reproducibility was evaluated using the coefficient of variation (CV) across technical replicates of pooled QC. As shown in Figure 5.4 A and B, no meaningful

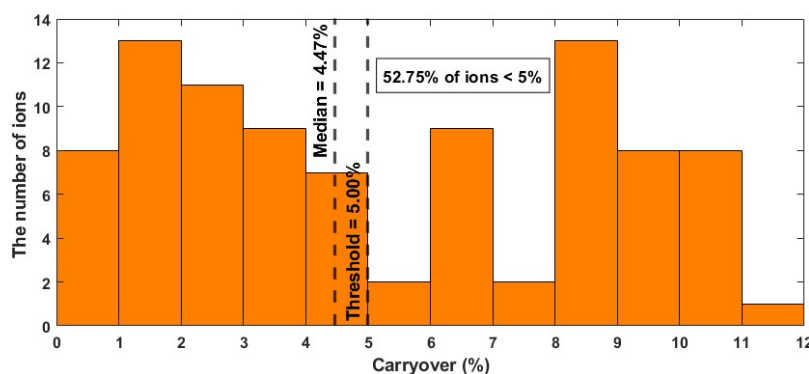


Figure 5.3. Carryover effect.

correlation was observed between peak area and CV ($R^2 = 0.009$), nor between retention time and CV ($R^2 = 0.060$), indicating that feature reproducibility was largely independent of both signal intensity and chromatographic elution time. Half of the features had a CV below 14.28%, reflecting good reproducibility (Figure 5.4 C).

For mass accuracy, mass errors were centred around 0 ppm, with 97.3% of features falling within ± 5 ppm, indicating high mass accuracy and reliable instrument calibration across detected features (Figure 5.4 D).

To assess statistical reliability, we performed a mock-group analysis by randomly splitting the invaded samples into two groups. Shown in Figure 5.5, the p values showed no bias with respect to intensity, and the low false discovery rate (FDR, 3.4%) indicated minimal false positives. This confirmed the robustness of our workflow and suggested that observed differences are unlikely to arise from random variation. These results were consistent in the analysis using non-invaded samples (FDR 4.0%).

Furthermore, QC samples clustered tightly on the PCA scores plot (Figure 5.2), indicating that the instrument performance was stable, and the separation and detection were both reproducible.

Overall, these results collectively demonstrated that the analytical workflow was robust, reproducible, and statistically reliable.

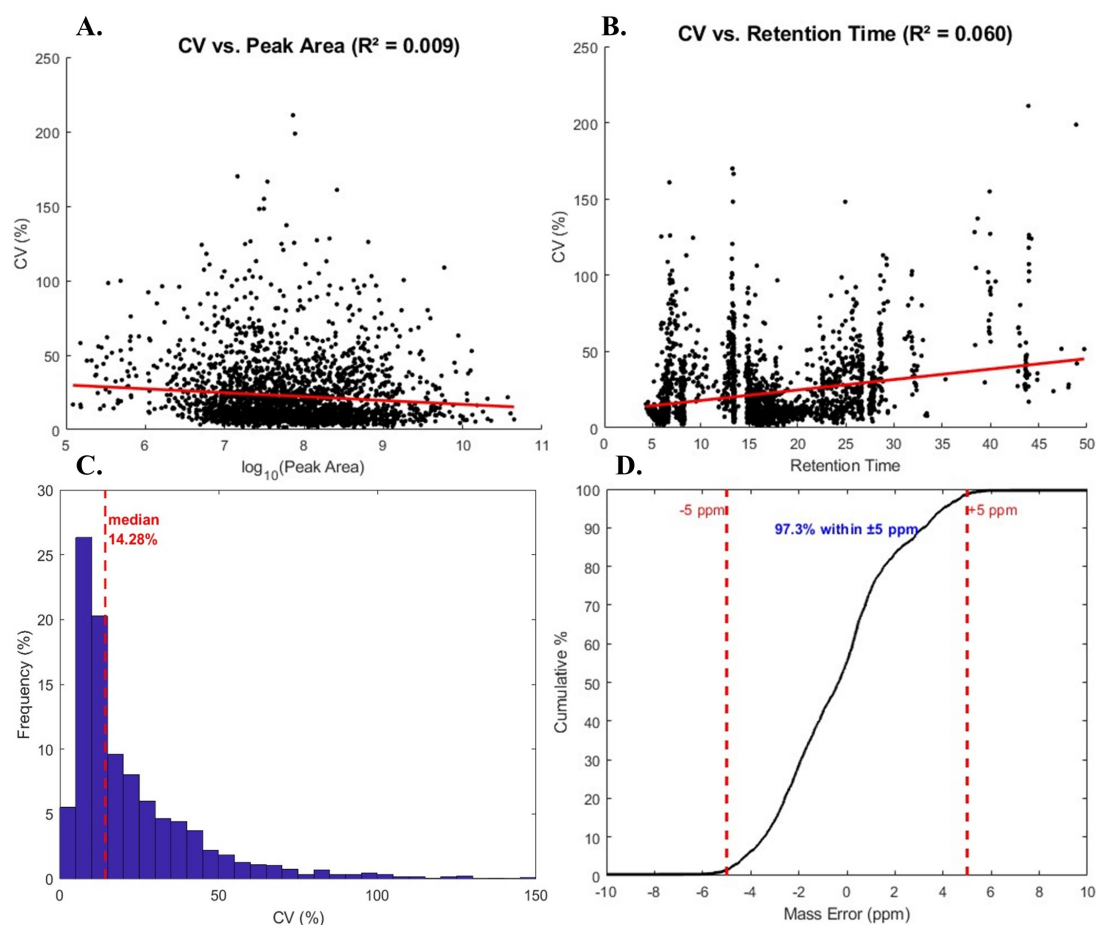


Figure 5.4. Technical reproducibility and mass accuracy. (A) Scatter plot of CV vs. peak area. (B) Scatter plot of CV vs. retention time. (C) Histogram of CV distribution across pooled QC samples. (D) Cumulative distribution of mass error.

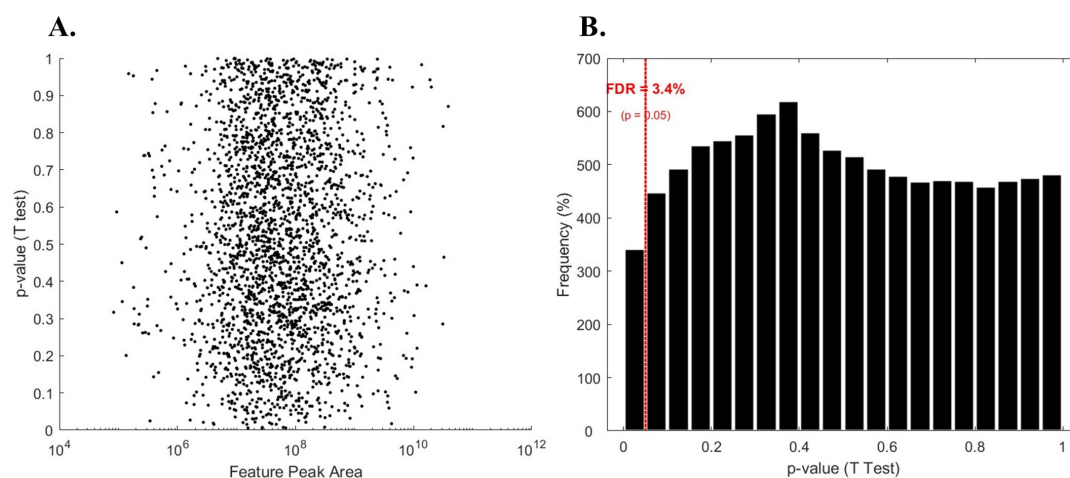


Figure 5.5. Mock-group analysis for estimating false discovery rate. (A) Scatter plot of p values vs. feature peak area. (B) Histogram of p value distribution from the mock comparison.

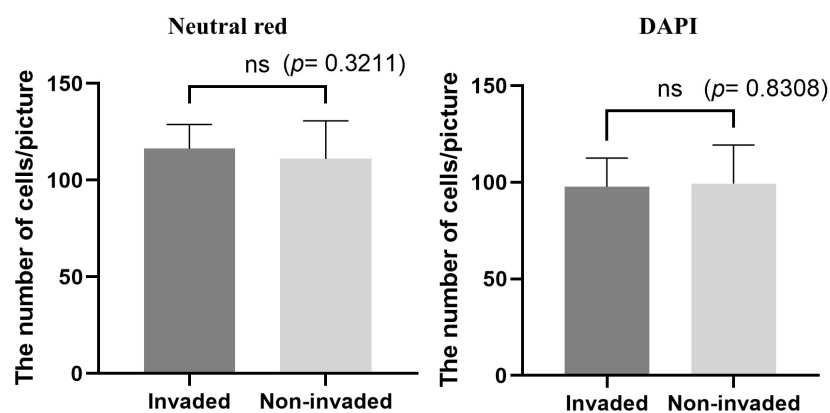


Figure 5.6. Cell counting results for samples stained by neutral red and DAPI.

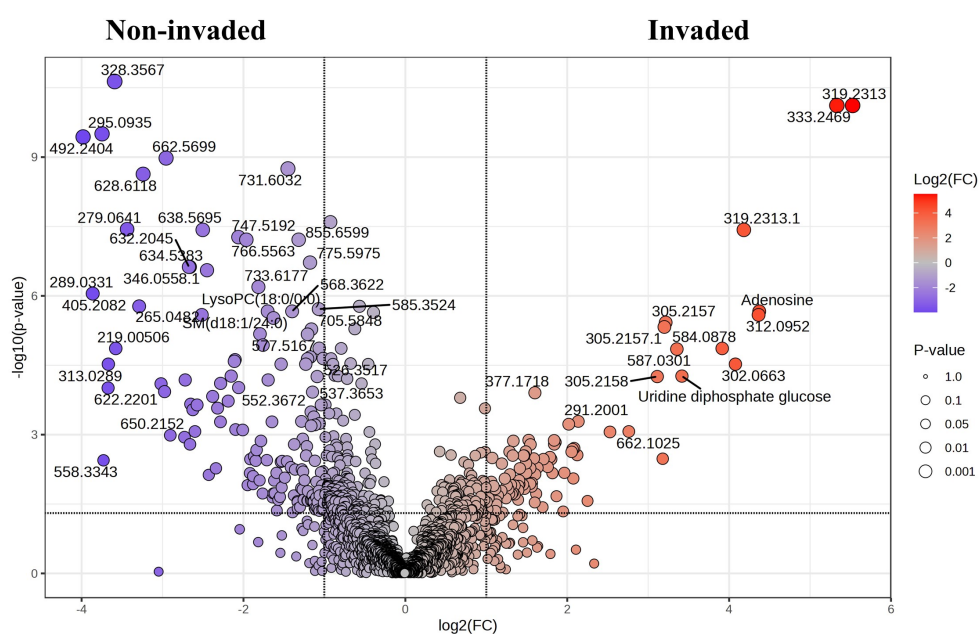


Figure 5.7. Metabolic variation between invaded and non-invaded cells. The ions were highlighted with fold change > 2 and adjusted p value < 0.05.

5.3.3 Metabolic differences between invaded and non-invaded cells

To reduce variation arising from sample preparation, the cell numbers between invaded and non-invaded groups were balanced by increasing the seeding density. As shown in Figure 5.6, no statistical differences were observed between the invaded and non-invaded groups after neutral red and DAPI staining. Therefore, subsequent analyses were not normalised to cell number. Images are presented in Figure B.22.

Volcano plot presents significant differences in metabolite abundance between invaded and non-invaded cells (Figure 5.7). In addition, two groups were separated on a well-constructed OPLS-DA scores plot (Figure 5.8 A and Figure B.23), indicating distinct metabolic profiles. Metabolites with variable importance in projection (VIP) scores > 1 and adjusted p value < 0.05 were considered differentially abundant, summarised in Table A.15. Several metabolic pathways were significantly enriched by identified metabolites (Figure 5.8 B, Figure 5.9 and Table 5.1).

Table 5.1. Significantly altered metabolic pathways between invaded cells and non-invaded cells (adjusted p value < 0.05 and impact factor > 0.1).

Pathway name	Total compounds	Hits	Raw p	Adjusted p	Impact factor
Starch and sucrose metabolism	18	3	4.51E-06	2.71E-05	0.57
Amino sugar and nucleotide sugar metabolism	42	3	7.47E-06	3.84E-05	0.13
Glycerophospholipid metabolism	36	3	1.16E-05	5.22E-05	0.19
Pentose and glucuronate interconversions	19	3	1.32E-05	5.26E-05	0.13
beta-Alanine metabolism	21	3	9.89E-04	2.28E-02	0.10
Histidine metabolism	16	3	4.98E-03	1.05E-02	0.22
Glycine, serine and threonine metabolism	33	3	2.50E-02	4.29E-02	0.21

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted p is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

Uridine diphosphate glucose (UDP-glucose) and N-acetyl-glucosamine 1-phosphate (GlcNAc-1P) are the central metabolites that bridge starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and pentose and glucuronate interconversions. UDP-glucose supplies glucose-6-phosphate to glycolysis or the pentose phosphate pathway to support nucleotide biosynthesis and redox balance^{252,253}. Moreover, it could be oxidised into UDP-glucuronic acid, a precursor for glycosaminoglycans and proteoglycans, thereby modifying the ECM and promoting cell migration²⁵⁴. GlcNAc-1P supports glycoprotein and glycolipid production, cell

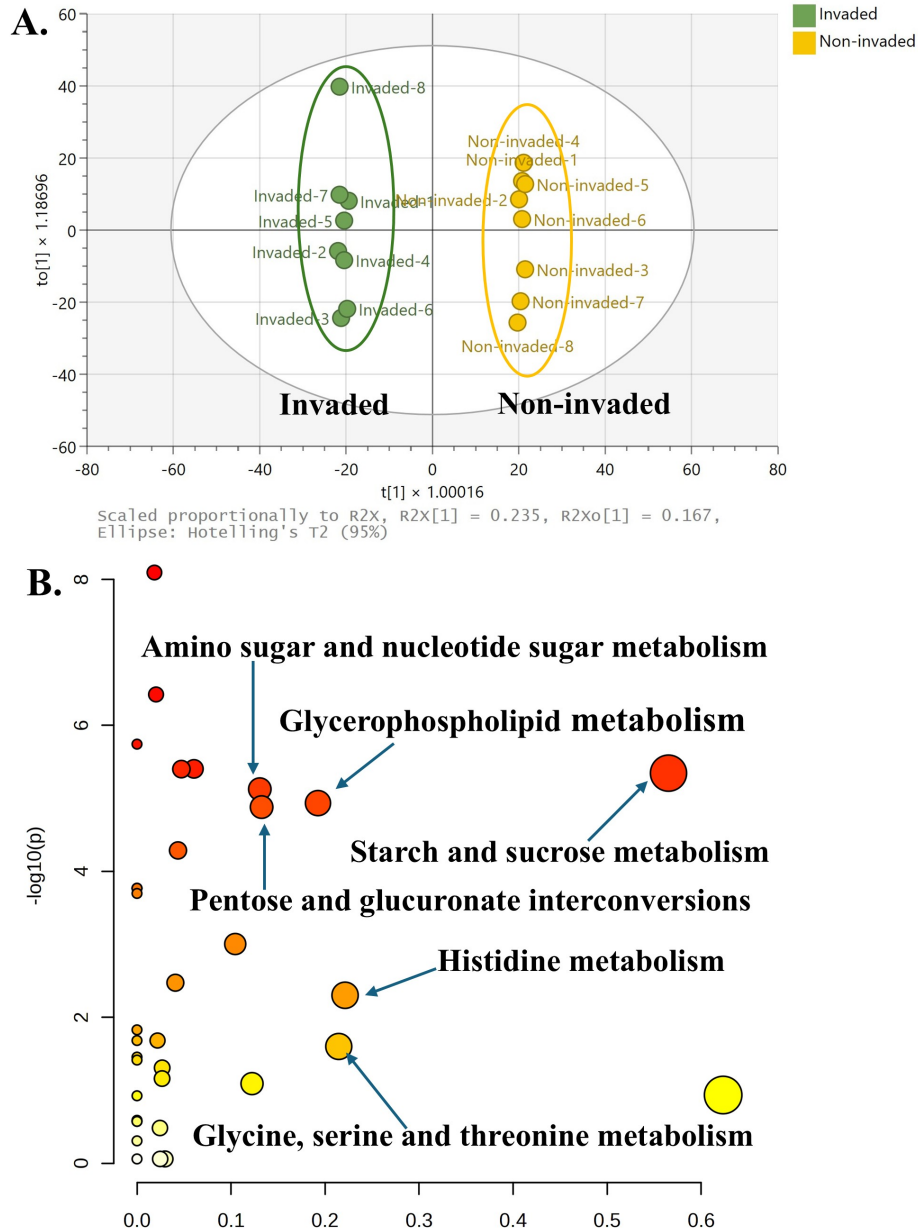


Figure 5.8. OPLS-DA scores plot and pathway analysis of invaded vs. non-invaded cells. (A): The cross-validation results were $R^2Y = 0.999$ and $Q^2 = 0.892$. (B): The colour represents the degree of significance: white (non-significant) to red (statistically significant). The impact factor represents the importance of an identified pathway within the whole metabolic network.

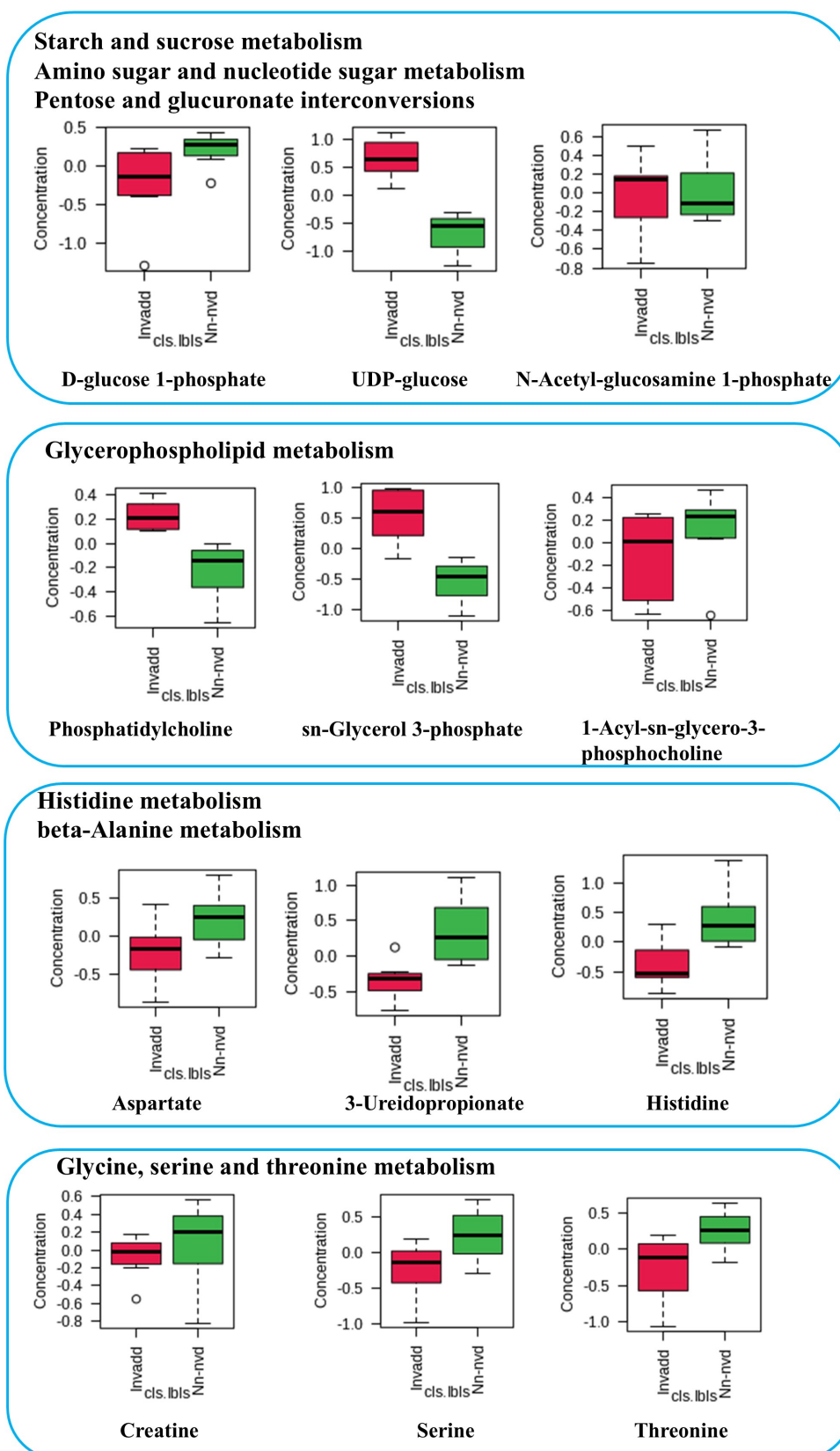


Figure 5.9. Metabolites and their abundance between invaded and non-invaded cells. The normalised abundances of identified metabolites are shown with invaded cells in red and non-invaded cells in green. The abundance of metabolites was exported from MetaboAnalyst v6.0, and reflects transformed and scaled data for visualisation.

signalling, and ECM formation²⁵⁵. Our results showed that invaded cells had higher level of UDP-glucose and GlcNAc-1-P, suggesting enhanced energy metabolism and ECM modification during GBM invasion.

Beta-alanine metabolism is responsible for the synthesis and degradation of carnosine, pantothenic acid, and coenzyme A. 3-ureidopropionate is a key precursor of carnosine, which helps regulate intracellular pH, reduce oxidative stress, and maintain cellular homeostasis^{256,257}. In our study, higher level of 3-ureidopropionate in non-invaded cell was observed, suggesting its tendency to maintain homeostasis during invasion.

Aspartate supports mitochondrial function by linking TCA cycle intermediates to amino acid biosynthesis, promoting energy production²⁵⁸. Higher aspartate levels detected by capillary-LC-MS and AP-MALDI-MS in non-invaded cells suggest that invaded cells may consume more aspartate to meet the energy demands of GBM invasion.

Histidine is converted into histamine by histidine decarboxylase, and histamine is associated with angiogenesis, immune evasion, and tumour migration²⁵⁹. In our study, histidine (identified by capillary-LC-MS) and histamine (identified by AP-MALDI-MS) were both more abundant in non-invaded cells, suggesting invaded cells may actively consume these metabolites to support invasive processes. The inhibition of histidine decarboxylase may be an approach for inhibiting GBM invasion.

Glycerophospholipid metabolism was identified as a statistically significant pathway in all three independent methods. Phosphatidylcholine, the most abundant phospholipid in cell membranes, showed higher levels in invaded cells, consistent with the results of 3D OrbiSIMS. Although its precursor choline phosphate was not detected in this study, it was found at higher levels in invaded cells by AP-MALDI and 3D OrbiSIMS. These results suggest that invaded cells may remodel membrane composition to facilitate GBM invasion. Moreover, the abundance of sn-glycerol-3-phosphate and phosphatidylcholine were found in higher abundance in cells derived from the GBM invasive margin compared to the tumour core (Chapter 2.3.4). These results strongly

suggested the role of glycerophospholipid metabolism in promoting GBM invasiveness.

5.4 Conclusion

The developed capillary-flow LC-MS method was applied to characterise metabolic mechanisms associated with GBM invasion. The developed method was demonstrated to be robust and statistically reliable.

Distinct metabolic profiles were observed between invaded and non-invaded cells. Our results suggested that energy metabolism, ECM modification and membrane remodelling support the invasive phenotype, while non-invaded cells favour pathways that maintain cellular homeostasis. These findings offer valuable insights into the biology of GBM invasion and may inform future research on therapeutic targets and biomarkers.

Nevertheless, our results reflect only changes in metabolite abundance and do not include corresponding enzyme activity or gene expression data. It would be beneficial to integrate with other omics data to better understand the underlying regulatory mechanisms. Additionally, validating these results in a larger sample set would strengthen the conclusions and improve their translational relevance.

Chapter 6

General discussion

6.1 Chapter 2

Glioblastoma (GBM) patients generally face poor prognosis despite receiving standard treatments, highlighting the urgent need for effective therapeutics.

Taking advantage of drug repurposing, compared with other repurposed candidates, Afuresertib was identified as the most potent candidate for GBM treatment, supported by its strong *in vitro* anti-GBM activity and well-established safety profile. However, its efficacy and capacity of penetrating BBB needs to be further evaluated in *in vivo* models. Moreover, given the heterogeneity observed in GBM and only IDH wild type of GBM tested in this study, its anti-tumour effects should be assessed across different GBM subtypes to determine its broader therapeutic potential.

Cellular metabolism is a direct readout of functional changes within cells, making it suitable for investigating drug-induced alterations. The metabolomics results revealed metabolic pathways associated with the anti-GBM effects of the repurposed candidates, contributing to the elucidation of their therapeutic mechanisms. Among these, glycerophospholipid metabolism was identified as an attractive pathway. The differential abundance of metabolites involved in this pathway between GINs and GCEs reflected intra-tumour heterogeneity, highlighting their potential as biomarkers to distinguish these cell lines and supporting the rationale behind their derivation. However, the applicability of this finding requires further validation in large-scale sample cohorts.

When combined with the invasion assay results, these findings led to the generation of a new hypothesis that glycerophospholipid metabolism may drive GBM invasiveness.

6.2 Chapter 3

To test the proposed hypothesis, metabolite profiling was performed directly on samples obtained from the Transwell invasion assay at the single-cell level, using 3D OrbiSIMS and AP-MALDI-MS.

These results extend previous work by showing that glycerophospholipid metabolism was a significant metabolic pathway between invaded and non-invaded cells, highlighting its potential role in promoting GBM invasion. Moreover, the consistent results of choline phosphate and choline across both methods suggested that choline kinase may become a therapeutic target for inhibiting GBM invasion.

Nevertheless, several challenges remain in this study. Generally, the technical challenges of surface analysis techniques, such as ion suppression and limited ionisation efficiency, can hinder reliable detection²⁶⁰. We cannot exclude the possibility that staining single cells for OrbiSIMS analysis may introduce chemical noise, suppress endogenous metabolites signals, potentially compromising data quality. Besides, neither 3D OrbiSIMS nor AP-MALDI-MS supported polarity switching, limiting the coverage of detectable metabolites from the same sample. Annotation is another challenge, arising from the reliance on exact mass alone and the multiple annotations for a single ion, reducing the reliability of biological interpretation. Lastly, for the AP-MALDI-MS results, identification of single-cell regions requires further optimisation, either through improved computational methods or enhanced spatial imaging resolution, to accurately distinguish and separate adjacent cells.

6.3 Chapter 4

As a complementary approach, a capillary-flow LC-MS-based metabolite profiling method was developed and optimised.

Compared to capillary/micro-flow methods, the poorer performance of the nano-flow method was evident by injection of both standard solutions and cell extracts. The limited loading capacity of the nano-column, together with solubility limitations at nano-flow rates, may contribute to the observed results, but the underlying reasons require further investigation.

The major challenge always lies in chromatographic separation, which may benefit from the use of columns with smaller particle sizes. Moreover, to improve peak shape, the ZIC-*p*HILIC column might be considered for future optimisation, as it offers enhanced tolerance to both acidic and alkaline conditions²⁴⁵.

A main drawback of the developed capillary-flow LC-MS method was its relatively long analysis cycle, limiting its application in high-throughput studies. Additionally, although the method was effectively optimised with the available consumables, further optimisation and validation are necessary to enhance its potential for studying GBM metabolome and to broaden its application to studies addressing limited sample input.

6.4 Chapter 5

The developed capillary-flow LC-MS method was applied to detect metabolic signatures associated with GBM invasion.

Compared to 3D OrbiSIMS and AP-MALDI-MS, this approach provided additional retention time and fragment information to enhance metabolite identification, with 37 metabolites identified as high confidence (Levels 1 and 2). Furthermore, it enabled simultaneous analysis of positive and negative ions within the same sample. In future studies, the integration of HILIC with RPLC could improve metabolite coverage.

The results across the three methods were consistent, with glycerophospholipid metabolism identified as statistically significant, strongly supporting its role in promoting GBM invasion. Besides, six additional pathways suggest that invaded cells reprogram metabolic pathways during the invasion process, including energy metabolism, ECM remodelling, and membrane composition, whereas non-invaded cells tend to maintain cellular homeostasis.

To further validate the role of glycerophospholipid metabolism in driving GBM invasion, it is further proposed to investigate whether inhibition of this pathway can suppress the invasion of GBM cells. The selection of inhibitors focus on targeting key enzymes involved in the biosynthesis, remodelling and degradation process of glycerophospholipids, summarised in Table 6.1. Hence, the anti-invasion effects of glycerophospholipid metabolism inhibition will be evaluated in patient-derived GBM cells using the selected inhibitors.

Table 6.1. Inhibitors of glycerophospholipid metabolism.

Process	Target	Inhibitor	Reference
Biosynthesis	GPAT	FSG67	261
	CK	RSM932A	262
	PLA ₂	MAFP	263
Remodelling & Degradation	PLC	U73122	264
	PLD	VU0155069	265

GPAT represents glycerol-3-phosphate acyltransferase. CK represents choline kinase. PLA₂ is phospholipase A₂. PLC is phospholipase C. PLD is phospholipase D.

Drug treatment was not incorporated into the single-cell metabolomics or capillary-flow LC–MS-based untargeted metabolomics analyses. Consequently, no direct evidence was obtained to demonstrate that the repurposed Afuresertib and Caledonine inhibit GBM invasion *in vitro* through disruption of glycerophospholipid metabolism, in comparison with Taxifolin. Future studies should therefore focus on investigating drug-induced metabolic alterations between invaded and non-invaded cells using 3D OrbiSIMS, AP-MALDI-MS and capillary-flow LC-MS methods. However, for drug-treated samples, a major challenge for capillary-flow LC–MS analysis is achieving the same number of invaded and non-invaded cells in Transwell assays, which is critical for reliable data normalisation. Therefore, drug concentration and cell seeding density should be carefully optimised.

Moreover, both single-cell metabolomics and capillary-flow LC-MS method lacked the ability to track the metabolic signatures of GBM invasion in real time, providing only static snapshots of metabolic state at the time of sample collection.

6.5 Further discussion

To further assess the anti-GBM potential of the repurposed candidates, their efficacy should be validated in patient-derived GBM xenograft models across different subtypes. In the case of Afuresertib, additional studies are required to evaluate its blood–brain barrier penetration *in vivo* using LC–MS/MS and to characterise its spatial distribution within GBM tissues by mass spectrometry imaging. Mechanistic validation should include the confirmation of on-target inhibition of the PI3K–AKT–mTOR signalling pathway using Western blot analysis. Moreover, combination studies with standard-of-care treatments, such as Temozolomide and radiotherapy, would be valuable for assessing potential synergistic therapeutic effects.

The studies primarily rely on mass spectrometry–based metabolomics data for pathway analysis. However, pathway analysis based solely on metabolite identity and relative abundance can be misleading or biologically uninformative results. This is because many metabolites can be rapidly transported between cells, tissues, or biofluids, meaning that observed concentration changes may reflect altered transport or redistribution rather than local biosynthesis²⁶⁶. In addition, alterations in a single metabolite can be attributed to multiple metabolic processes rather than to discrete and individual pathways, further complicating pathway-level interpretation²⁶⁷. Therefore, before drawing definitive conclusions regarding the significantly altered metabolic pathways, the current findings should be interpreted with caution and supported by appropriate biological validation.

Glycerophospholipid metabolism was identified as a discriminatory biomarker between GINs and GCEs, and it was further demonstrated to be involved in the GBM invasion process. These findings should be cross-validated by targeted metabolomics and lipidomics to confirm key metabolites, followed by stable-isotope tracing to assess pathway flux rather than steady-state pool changes. The mechanistic support could be further strengthened by evaluating the expression of relevant genes and the activity of key enzymes using qPCR and Western blot analyses, together with the pharmacological inhibition of glycerophospholipid metabolism (discussed in Table 6.1). In addition,

validation in GBM tissues and *in vivo* models would provide stronger evidence that the inferred pathway alterations reflect underlying tumour biology.

Finally, drug-induced metabolomic changes identified in this study should be integrated with other omics approaches to strengthen mechanistic interpretation. In particular, incorporation of transcriptomic and proteomic data would enable correlation of metabolite alterations with changes in gene expression and protein abundance, thereby reducing the ambiguity inherent to metabolomics-only pathway analysis. Moreover, these findings should be further validated using analytical and biological strategies, such as targeted metabolomics and pathway-specific biomarker analyses, to confirm their mechanistic relevance.

Chapter 7

Conclusion

Our study evaluated the potential of repurposed Afuresertib, Caledonine, and Taxifolin for GBM treatment, and provided valuable insights into the metabolic mechanisms underlying their anti-tumour effects, highlighting the potential of these agents for further preclinical and clinical evaluation as GBM therapy.

The observed intra-tumour heterogeneity between GINs and GCEs suggested that metabolites involved in glycerophospholipid metabolism may serve as potential biomarkers for distinguishing distinct GBM subpopulations. Identifying such metabolic biomarkers could promote the development of personalised therapeutic approaches targeting specific GBM cell phenotypes.

Our study also highlighted the potential of 3D OrbiSIMS and AP-MALDI-MS for single-cell metabolomics in GBM study, and demonstrated the utility of low-flow LC-MS for GBM metabolomics which address limited sample biomass. These techniques expand the methodological toolkit for the investigation of GBM metabolome.

Furthermore, metabolic signatures associated with GBM invasion were identified, providing strong evidence that glycerophospholipid metabolism plays a critical role in driving GBM invasion. To our knowledge, this is the first study to demonstrate the involvement of glycerophospholipid metabolism in GBM invasion using metabolomics-based approaches. These findings not only expand our knowledge of GBM pathobiology but also propose glycerophospholipid metabolism as a promising target for anti-invasion strategies.

It is acknowledged that the current studies capture only changes in metabolite types and abundances. To gain a deeper understanding of the molecular processes underlying treatment response and GBM progression, it is essential to link these metabolic alterations to upstream regulatory factors, such as gene expression, enzyme activity, and signalling pathways.

Ultimately, our work advances the field of GBM research by demonstrating the power of metabolomics to uncover function-relevant metabolic pathways, supporting the development of GBM treatments.

Bibliography

- [1] Mu Yingming, Luo Junchi, Xiong Tao, et al. Development and validation of nomogram model predicting overall survival and cancer specific survival in glioblastoma patients *Discover Oncology*. 2025;16:562.
- [2] Bello-Alvarez Claudia, Camacho-Arroyo Ignacio. Impact of sex in the prevalence and progression of glioblastomas: the role of gonadal steroid hormones *Biology of sex Differences*. 2021;12:28.
- [3] Asfaw Zerubabbel K, Hernandez-Marquez Gianina C, Naik Anant, et al. The intersection of race and social determinants of health on clinical outcome of glioblastoma patients *Journal of neurosurgery*. 2024;141:614–623.
- [4] Liu Elisa K, Yu Sharon, Sulman Erik P, Kurz Sylvia C. Racial and socioeconomic disparities differentially affect overall and cause-specific survival in glioblastoma *Journal of Neuro-Oncology*. 2020;149:55–64.
- [5] McKinnon Chris, Nandhabalan Meera, Murray Scott A, Plaha Puneet. Glioblastoma: clinical presentation, diagnosis, and management *Bmj*. 2021;374.
- [6] Kinzel Adrian, Ambrogi Michael, Varshaver Michael, Kirson Eilon D. Tumor treating fields for glioblastoma treatment: patient satisfaction and compliance with the second-generation Optune® system *Clinical Medicine Insights: Oncology*. 2019;13:1179554918825449.
- [7] Khagi Simon, Kotecha Rupesh, Gatson Na Tosha N, et al. Recent advances in Tumor Treating Fields (TTFields) therapy for glioblastoma *The Oncologist*. 2025;30:oyae227.
- [8] Stupp Roger, Taillibert Sophie, Kanner Andrew, et al. Effect of tumor-treating fields plus maintenance temozolomide vs maintenance temozolomide alone on survival in patients with glioblastoma: a randomized clinical trial *Jama*. 2017;318:2306–2316.
- [9] Brodbelt Mr Andrew, Williams Matthew, Thorpe Ms Antonia, et al. A pilot study of the acceptance and tolerability of tumour treating fields in adult glioblastoma patients *Neuro-Oncology*. 2018;20:v349.
- [10] Janjua Taskeen Iqbal, Rewatkar Prarthana, Ahmed-Cox Aria, et al. Frontiers in the treatment of glioblastoma: Past, present and emerging *Advanced drug delivery reviews*. 2021;171:108–138.

- [11] Hatoum Ahmad, Mohammed Rami, Zakieh Omar. The unique invasiveness of glioblastoma and possible drug targets on extracellular matrix *Cancer Management and Research*. 2019;11:1843–1855.
- [12] Liu Dongdong, Dai Xingliang, Ye Lei, et al. Nanotechnology meets glioblastoma multiforme: Emerging therapeutic strategies *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*. 2023;15:e1838.
- [13] Puente Pilar, Fetting Nicole, Luderer Micah J, et al. Injectable hydrogels for localized chemotherapy and radiotherapy in brain tumors *Journal of pharmaceutical sciences*. 2018;107:922–933.
- [14] Rahiminejad Ali, Dinarvand Rassoul, Johari Behrooz, et al. Preparation and investigation of indirubin-loaded SLN nanoparticles and their anti-cancer effects on human glioblastoma U87MG cells *Cell biology international*. 2019;43:2–11.
- [15] Nowak Maksymilian, Helgeson Matthew E, Mitragotri Samir. Delivery of nanoparticles and macromolecules across the blood–brain barrier *Advanced Therapeutics*. 2020;3:1900073.
- [16] Gilbert Mark, Zhang Peixin, Sloan Andrew, et al. ATIM-29. NRG BN002: SAFETY DATA FROM A PHASE I STUDY OF IPILIMUMAB (IPI), NIVOLUMAB (NIVO), AND THE COMBINATION FOR NEWLY DIAGNOSED GLIOBLASTOMA (GBM) *Neuro-Oncology*. 2018;20:vi7.
- [17] Liao Linda M, Ashkan Keyoumars, Tran David D, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma *Journal of translational medicine*. 2018;16:142.
- [18] Grabowski Matthew M, Sankey Eric W, Ryan Katherine J, et al. Immune suppression in gliomas *Journal of neuro-oncology*. 2021;151:3–12.
- [19] Min Hye-Young, Lee Ho-Young. Molecular targeted therapy for anticancer treatment *Experimental & molecular medicine*. 2022;54:1670–1694.
- [20] Crivii Carmen-Bianca, Boşca Adina Bianca, Melincovici Carmen Stanca, et al. Glioblastoma microenvironment and cellular interactions *Cancers*. 2022;14:1092.
- [21] Ganapathy-Kanniappan Shanmugasundaram, Geschwind Jean-Francois H. Tumor glycolysis as a target for cancer therapy: progress and prospects *Molecular cancer*. 2013;12:1–11.
- [22] Jürchott Kathrin, Guo Ke-Tai, Catchpole Gareth, et al. Comparison of metabolite profiles in U87 glioma cells and mesenchymal stem cells *Biosystems*. 2011;105:130–139.
- [23] Duraj Tomás, García-Romero Noemí, Carrión-Navarro Josefa, et al. Beyond the Warburg effect: oxidative and glycolytic phenotypes coexist within the metabolic heterogeneity of glioblastoma *Cells*. 2021;10:202.

- [24] Wolf Amparo, Agnihotri Sameer, Munoz Diana, Guha Abhijit. Developmental profile and regulation of the glycolytic enzyme hexokinase 2 in normal brain and glioblastoma multiforme *Neurobiology of disease*. 2011;44:84–91.
- [25] Xie Yuan, Bergström Tobias, Jiang Yiwen, et al. The human glioblastoma cell culture resource: validated cell models representing all molecular subtypes *EBioMedicine*. 2015;2:1351–1363.
- [26] Mergenthaler Philipp, Lindauer Ute, Dienel Gerald A, Meisel Andreas. Sugar for the brain: the role of glucose in physiological and pathological brain function *Trends in neurosciences*. 2013;36:587–597.
- [27] Chinnaiyan Prakash, Kensicki Elizabeth, Bloom Gregory, et al. The metabolomic signature of malignant glioma reflects accelerated anabolic metabolism *Cancer research*. 2012;72:5878–5888.
- [28] Liang Ji, Cao Ruixiu, Wang Xiongjun, et al. Mitochondrial PKM2 regulates oxidative stress-induced apoptosis by stabilizing Bcl2 *Cell research*. 2017;27:329–351.
- [29] Tateishi Kensuke, Iafrate A John, Ho Quan, et al. Myc-driven glycolysis is a therapeutic target in glioblastoma *Clinical Cancer Research*. 2016;22:4452–4465.
- [30] Zhang Yajuan, Yu Guanzhen, Chu Huiying, et al. Macrophage-associated PGK1 phosphorylation promotes aerobic glycolysis and tumorigenesis *Molecular cell*. 2018;71:201–215.
- [31] Wolf Amparo, Agnihotri Sameer, Micallef Johann, et al. Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme *Journal of Experimental Medicine*. 2011;208:313–326.
- [32] Patra Krushna C, Wang Qi, Bhaskar Prashanth T, et al. Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer *Cancer cell*. 2013;24:213–228.
- [33] Stein Mark, Lin Hongxia, Jeyamohan Chandrika, et al. Targeting tumor metabolism with 2-deoxyglucose in patients with castrate-resistant prostate cancer and advanced malignancies *The Prostate*. 2010;70:1388–1394.
- [34] Tataranni Tiziana, Piccoli Claudia. Dichloroacetate (DCA) and cancer: an overview towards clinical applications *Oxidative medicine and cellular longevity*. 2019;2019:8201079.
- [35] Garofano Luciano, Migliozi Simona, Oh Young Taek, et al. Pathway-based classification of glioblastoma uncovers a mitochondrial subtype with therapeutic vulnerabilities *Nature Cancer*. 2021;2:141–156.
- [36] Wu Min, Wang Tingting, Ji Nan, et al. Multi-omics and pharmacological characterization of patient-derived glioma cell lines *Nature Communications*. 2024;15:6740.

- [37] Wahl Daniel R, Dresser Joseph, Wilder-Romans Kari, et al. Glioblastoma therapy can be augmented by targeting IDH1-mediated NADPH biosynthesis *Cancer research*. 2017;77:960–970.
- [38] Maher Elizabeth A, Marin-Valencia Isaac, Bachoo Robert M, et al. Metabolism of [U-13C] glucose in human brain tumors in vivo *NMR in Biomedicine*. 2012;25:1234–1244.
- [39] Mashimo Tomoyuki, Pichumani Kumar, Vemireddy Vamsidhara, et al. Acetate is a bioenergetic substrate for human glioblastoma and brain metastases *Cell*. 2014;159:1603–1614.
- [40] Adams Seray, Braidy Nady, Bessesde Alban, et al. The kynurenine pathway in brain tumor pathogenesis *Cancer research*. 2012;72:5649–5657.
- [41] Larkin Paul B, Sathyaikumar Korrapati V, Notarangelo Francesca M, et al. Tryptophan 2, 3-dioxygenase and indoleamine 2, 3-dioxygenase 1 make separate, tissue-specific contributions to basal and inflammation-induced kynurenine pathway metabolism in mice *Biochimica et Biophysica Acta (BBA)-General Subjects*. 2016;1860:2345–2354.
- [42] Mitsuka Kentaro, Kawataki Tomoyuki, Satoh Eiji, Asahara Takayuki, Horikoshi Toru, Kinouchi Hiroyuki. Expression of indoleamine 2, 3-dioxygenase and correlation with pathological malignancy in gliomas *Neurosurgery*. 2013;72:1031–1039.
- [43] Bostian April CL, Eoff Robert L. Aberrant kynurenine signaling modulates DNA replication stress factors and promotes genomic instability in gliomas *Chemical research in toxicology*. 2016;29:1369–1380.
- [44] Prendergast George C, Malachowski William J, Mondal Arpita, Scherle Peggy, Muller Alexander J. Indoleamine 2, 3-dioxygenase and its therapeutic inhibition in cancer *International review of cell and molecular biology*. 2018;336:175–203.
- [45] Reardon David A, Desjardins Annick, Rixe Olivier, et al. A phase 1 study of PF-06840003, an oral indoleamine 2, 3-dioxygenase 1 (IDO1) inhibitor in patients with recurrent malignant glioma *Investigational New Drugs*. 2020;38:1784–1795.
- [46] Chinopoulos Christos, Seyfried Thomas N. Mitochondrial substrate-level phosphorylation as energy source for glioblastoma: review and hypothesis *ASN neuro*. 2018;10:1759091418818261.
- [47] Tanaka Kazuhiro, Sasayama Takashi, Irino Yasuhiro, et al. Compensatory glutamine metabolism promotes glioblastoma resistance to mTOR inhibitor treatment *The Journal of clinical investigation*. 2015;125:1591–1602.
- [48] Reitman Zachary J, Duncan Christopher G, Poteet Ethan, et al. Cancer-associated isocitrate dehydrogenase 1 (IDH1) R132H mutation and d-2-hydroxyglutarate stimulate glutamine metabolism under hypoxia *Journal of Biological Chemistry*. 2014;289:23318–23328.

- [49] Quinones Addison, Le Anne. The multifaceted glioblastoma: From genomic alterations to metabolic adaptations in *The Heterogeneity of Cancer Metabolism*:59–76Springer International Publishing Cham 2021.
- [50] Marin-Valencia Isaac, Yang Chendong, Mashimo Tomoyuki, et al. Analysis of tumor metabolism reveals mitochondrial glucose oxidation in genetically diverse human glioblastomas in the mouse brain in vivo *Cell metabolism*. 2012;15:827–837.
- [51] Tardito Saverio, Oudin Anaïs, Ahmed Shafiq U, et al. Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma *Nature cell biology*. 2015;17:1556–1568.
- [52] Rosati Anna, Poliani Pietro Luigi, Todeschini Alice, et al. Glutamine synthetase expression as a valuable marker of epilepsy and longer survival in newly diagnosed glioblastoma multiforme *Neuro-oncology*. 2013;15:618–625.
- [53] Cheng Tzuling, Sudderth Jessica, Yang Chendong, et al. Pyruvate carboxylase is required for glutamine-independent growth of tumor cells *Proceedings of the National Academy of Sciences*. 2011;108:8674–8679.
- [54] Khoury Oula, Ghazale Noura, Stone Everett, El-Sibai Mirvat, Frankel Arthur E, Abi-Habib Ralph J. Human recombinant arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-induced arginine depletion is selectively cytotoxic to human glioblastoma cells *Journal of neuro-oncology*. 2015;122:75–85.
- [55] Björkblom Benny, Wibom Carl, Eriksson Maria, et al. Distinct metabolic hallmarks of WHO classified adult glioma subtypes *Neuro-oncology*. 2022;24:1454–1468.
- [56] Guo Deliang, Bell Erica Hlavin, Chakravarti Arnab. Lipid metabolism emerges as a promising target for malignant glioma therapy *CNS oncology*. 2013;2:289–299.
- [57] Kant Shiva, Kesarwani Pravin, Prabhu Antony, et al. Enhanced fatty acid oxidation provides glioblastoma cells metabolic plasticity to accommodate to its dynamic nutrient microenvironment *Cell Death & Disease*. 2020;11:253.
- [58] Izquierdo-Garcia Jose L, Viswanath Pavithra, Eriksson Pia, et al. Metabolic reprogramming in mutant IDH1 glioma cells *PloS one*. 2015;10:e0118781.
- [59] Kahlert Ulf Dietrich, Koch Katharina, Suwala Abigail Kora, et al. The effect of neurosphere culture conditions on the cellular metabolism of glioma cells *Folia neuropathologica*. 2015;53:219–225.
- [60] Zhang Rui, Hu Peishan, Zang Qingce, et al. LC-MS-based metabolomics reveals metabolic signatures related to glioma stem-like cell self-renewal and differentiation *RSC advances*. 2017;7:24221–24232.
- [61] Huang Kai, Fang Chuan, Yi Kaikai, et al. The role of PTRF/Cavin1 as a biomarker in both glioma and serum exosomes *Theranostics*. 2018;8:1540.

- [62] Yi Kaikai, Zhan Qi, Wang Qixue, et al. PTRF/cavin-1 remodels phospholipid metabolism to promote tumor proliferation and suppress immune responses in glioblastoma by stabilizing cPLA2 *Neuro-oncology*. 2021;23:387–399.
- [63] Abuhusain Hazem J, Matin Azadeh, Qiao Qiao, et al. A metabolic shift favoring sphingosine 1-phosphate at the expense of ceramide controls glioblastoma angiogenesis *Journal of Biological Chemistry*. 2013;288:37355–37364.
- [64] Altwaairgi AK, Alghareeb W, Alnajjar F, et al. Phase II study of atorvastatin in combination with radiotherapy and temozolomide In patients with glioblastoma (ART): Interim analysis report *Annals of Oncology*. 2016;27:vi113.
- [65] Guo Deliang, Reinitz Felicia, Youssef Mary, et al. An LXR agonist promotes glioblastoma cell death through inhibition of an EGFR/AKT/SREBP-1/LDLR-dependent pathway *Cancer discovery*. 2011;1:442–456.
- [66] Wang Xiuxing, Yang Kailin, Xie Qi, et al. Purine synthesis promotes maintenance of brain tumor initiating cells in glioma *Nature neuroscience*. 2017;20:661–673.
- [67] Laks Dan R, Ta Lisa, Crisman Thomas J, et al. Inhibition of nucleotide synthesis targets brain tumor stem cells in a subset of glioblastoma *Molecular cancer therapeutics*. 2016;15:1271–1278.
- [68] Zhou Weihua, Wahl Daniel R. Metabolic abnormalities in glioblastoma and metabolic strategies to overcome treatment resistance *Cancers*. 2019;11:1231.
- [69] Sigmond J, Honeywell RJ, Postma TJ, et al. Gemcitabine uptake in glioblastoma multiforme: potential as a radiosensitizer *Annals of oncology*. 2009;20:182–187.
- [70] Bastiancich Chiara, Bastiat Guillaume, Lagarce Frederic. Gemcitabine and glioblastoma: challenges and current perspectives *Drug discovery today*. 2018;23:416–423.
- [71] Palanichamy Kamalakannan, Thirumoorthy Krishnan, Kanji Suman, et al. Methionine and kynurenine activate oncogenic kinases in glioblastoma, and methionine deprivation compromises proliferation *Clinical Cancer Research*. 2016;22:3513–3523.
- [72] Cerami Ethan, Gao Jianjiong, Dogrusoz Ugur, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data *Cancer discovery*. 2012;2:401–404.
- [73] Patro C Pawan K, Biswas Nupur, Pingle Sandeep C, et al. MTAP loss: a possible therapeutic approach for glioblastoma *Journal of Translational Medicine*. 2022;20:620.
- [74] Jamali Fatemeh, Lan Katherine, Daniel Paul, Petrecca Kevin, Sabri Siham, Abdulkarim Bassam. Synergistic Dual Targeting of Thioredoxin and Glutathione Systems Irrespective of p53 in Glioblastoma Stem Cells *Antioxidants*. 2024;13:1201.

- [75] Herzog Katharina, IJlst Lodewijk, Cruchten Arno G, et al. An UPLC-MS/MS assay to measure glutathione as marker for oxidative stress in cultured cells *Metabolites*. 2019;9:45.
- [76] Furnari Frank B, Cloughesy Timothy F, Cavenee Webster K, Mischel Paul S. Heterogeneity of epidermal growth factor receptor signalling networks in glioblastoma *Nature Reviews Cancer*. 2015;15:302–310.
- [77] Yang Weiwei, Xia Yan, Hawke David, et al. PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis *Cell*. 2012;150:685–696.
- [78] Babic Ivan, Anderson Erik S, Tanaka Kazuhiro, et al. EGFR mutation-induced alternative splicing of Max contributes to growth of glycolytic tumors in brain cancer *Cell metabolism*. 2013;17:1000–1008.
- [79] Griffiths Beatrice, Lewis Caroline A, Bensaad Karim, et al. Sterol regulatory element binding protein-dependent regulation of lipid synthesis supports cell survival and tumor growth *Cancer & metabolism*. 2013;1:1–21.
- [80] Villa Genaro R, Hulce Jonathan J, Zanca Ciro, et al. An LXR-cholesterol axis creates a metabolic co-dependency for brain cancers *Cancer cell*. 2016;30:683–693.
- [81] Brennan Cameron W, Verhaak Roel GW, McKenna Aaron, et al. The somatic genomic landscape of glioblastoma *cell*. 2013;155:462–477.
- [82] Masui Kenta, Tanaka Kazuhiro, Akhavan David, et al. mTOR complex 2 controls glycolytic metabolism in glioblastoma through FoxO acetylation and upregulation of c-Myc *Cell metabolism*. 2013;18:726–739.
- [83] Schulte Alexander, Liffers Katrin, Kathagen Annegret, et al. Erlotinib resistance in EGFR-amplified glioblastoma cells is associated with upregulation of EGFRvIII and PI3Kp110 δ *Neuro-oncology*. 2013;15:1289–1301.
- [84] Phillips Andrew C, Boghaert Erwin R, Vaidya Kedar S, et al. ABT-414, an antibody–drug conjugate targeting a tumor-selective EGFR epitope *Molecular cancer therapeutics*. 2016;15:661–669.
- [85] Weller Michael, Butowski Nicholas, Tran David D, et al. Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial *The lancet oncology*. 2017;18:1373–1385.
- [86] Metallo Christian M, Gameiro Paulo A, Bell Eric L, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia *Nature*. 2012;481:380–384.
- [87] Filipp Fabian V, Scott David A, Ronai Ze'ev A, Osterman Andrei L, Smith Jeffrey W. Reverse TCA cycle flux through isocitrate dehydrogenases 1 and 2 is required for lipogenesis in hypoxic melanoma cells *Pigment cell & melanoma research*. 2012;25:375–383.

- [88] Calvert Andrea E, Chalastanis Alexandra, Wu Yongfei, et al. Cancer-associated IDH1 promotes growth and resistance to targeted therapies in the absence of mutation *Cell reports*. 2017;19:1858–1873.
- [89] Du Xin, Hu Hai. The roles of 2-hydroxyglutarate *Frontiers in Cell and Developmental Biology*. 2021;9:651317.
- [90] Fu Yuejun, Zheng Shuhua, Zheng Yali, et al. Glioma derived isocitrate dehydrogenase-2 mutations induced up-regulation of HIF-1 α and β -catenin signaling: Possible impact on glioma cell metastasis and chemo-resistance *The international journal of biochemistry & cell biology*. 2012;44:770–775.
- [91] Zhang Xiang, Zhang WEI, Cao Wei-Dong, Cheng Gang, Zhang Yong-Qiang. Glioblastoma multiforme: Molecular characterization and current treatment strategy *Experimental and therapeutic medicine*. 2012;3:9–14.
- [92] Seltzer Meghan J, Bennett Bryson D, Joshi Avadhut D, et al. Inhibition of glutaminase preferentially slows growth of glioma cells with mutant IDH1 *Cancer research*. 2010;70:8981–8987.
- [93] McBrayer Samuel K, Mayers Jared R, DiNatale Gabriel J, et al. Transaminase inhibition by 2-hydroxyglutarate impairs glutamate biosynthesis and redox homeostasis in glioma *Cell*. 2018;175:101–116.
- [94] Rohle Dan, Popovici-Muller Janeta, Palaskas Nicolaos, et al. An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells *Science*. 2013;340:626–630.
- [95] Rivlin Noa, Brosh Ran, Oren Moshe, Rotter Varda. Mutations in the p53 tumor suppressor gene: important milestones at the various steps of tumorigenesis *Genes & cancer*. 2011;2:466–474.
- [96] Rodon J, Prenen H, Sacher A, et al. First-in-human study of AMG 193, an MTA-cooperative PRMT5 inhibitor, in patients with MTAP-deleted solid tumors: results from phase I dose exploration *Annals of Oncology*. 2024.
- [97] Engstrom Lars D, Aranda Ruth, Waters Laura, et al. MRTX1719 is an MTA-cooperative PRMT5 inhibitor that exhibits synthetic lethality in preclinical models and patients with MTAP-deleted cancer *Cancer discovery*. 2023;13:2412–2431.
- [98] Clish Clary B. Metabolomics: an emerging but powerful tool for precision medicine *Molecular Case Studies*. 2015;1:a000588.
- [99] Pandey Renu, Caflisch Laura, Lodi Alessia, Brenner Andrew J, Tiziani Stefano. Metabolomic signature of brain cancer *Molecular Carcinogenesis*. 2017;56:2355–2371.
- [100] Buragohain Lukumoni, Ghosh Mayukh, Kumar Rajesh, Dahiya Swati, Malik Yashpal Singh, Prasad Minakshi. Application of Proteomics and Metabolomics in Disease Diagnosis in *Advances in Animal Disease Diagnosis*:79–102CRC Press 2021.

- [101] Lelli Veronica, Belardo Antonio, Timperio Anna Maria. From targeted quantification to untargeted metabolomics *Metabolomics—Methodology and Applications in Medical Sciences and Life Sciences*. 2021;10.
- [102] Bruntz Ronald C, Lane Andrew N, Higashi Richard M, Fan Teresa W-M. Exploring cancer metabolism using stable isotope-resolved metabolomics (SIRM) *Journal of Biological Chemistry*. 2017;292:11601–11609.
- [103] Rončević Alen, Koruga Nenad, Soldo Koruga Anamarija, et al. MALDI Imaging Mass Spectrometry of High-Grade Gliomas: A Review of Recent Progress and Future Perspective *Current issues in molecular biology*. 2023;45:838–851.
- [104] Simoes R.V., Henriques R.N., Olesen J.L., et al. Deuterium metabolic imaging phenotypes mouse glioblastoma heterogeneity through glucose turnover kinetics *Elife*. 2025;13:RP100570.
- [105] Miggiels Paul, Wouters Bert, Westen Gerard JP, Dubbelman Anne-Charlotte, Hankemeier Thomas. Novel technologies for metabolomics: More for less *TrAC Trends in Analytical Chemistry*. 2019;120:115323.
- [106] Al Shboul Sofian, Singh Ashita, Kobetic Renata, et al. Mass spectrometry advances in analysis of glioblastoma *Mass spectrometry reviews*. 2024.
- [107] Glish Gary L, Vachet Richard W. The basics of mass spectrometry in the twenty-first century *Nature reviews drug discovery*. 2003;2:140–150.
- [108] Xu Xiangdong. Capillary electrophoresis-mass spectrometry for cancer metabolomics *Cancer Metabolomics: Methods and Applications*. 2021:189–200.
- [109] Damiano Fabrizio, De Benedetto Giuseppe E, Longo Serena, et al. Decanoic acid and not octanoic acid stimulates fatty acid synthesis in U87MG glioblastoma cells: a metabolomics study *Frontiers in neuroscience*. 2020;14:783.
- [110] Snyder Lloyd R, Kirkland Joseph J, Dolan John W. *Introduction to modern liquid chromatography*. John Wiley & Sons 2011.
- [111] Allwood J William, Goodacre Royston. An introduction to liquid chromatography–mass spectrometry instrumentation applied in plant metabolomic analyses *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*. 2010;21:33–47.
- [112] Chesnut Stephen M, Salisbury John J. The role of UHPLC in pharmaceutical development *Journal of separation science*. 2007;30:1183–1190.
- [113] Dejaegher Bieke, Vander Heyden Yvan. HILIC methods in pharmaceutical analysis *Journal of separation science*. 2010;33:698–715.
- [114] Contrepois Kévin, Jiang Lihua, Snyder Michael. Optimized analytical procedures for the untargeted metabolomic profiling of human urine and plasma by combining hydrophilic interaction (HILIC) and reverse-phase liquid chromatography (RPLC)–mass spectrometry *Molecular & Cellular Proteomics*. 2015;14:1684–1695.

- [115] Wernisch Stefanie, Pennathur Subramaniam. Evaluation of coverage, retention patterns, and selectivity of seven liquid chromatographic methods for metabolomics *Analytical and bioanalytical chemistry*. 2016;408:6079–6091.
- [116] Greco Giorgia, Letzel Thomas. Main interactions and influences of the chromatographic parameters in HILIC separations *Journal of chromatographic science*. 2013;51:684–693.
- [117] Jandera Pavel. Stationary and mobile phases in hydrophilic interaction chromatography: a review *Analytica chimica acta*. 2011;692:1–25.
- [118] Zhang Rong, Watson David G, Wang Lijie, Westrop Gareth D, Coombs Graham H, Zhang Tong. Evaluation of mobile phase characteristics on three zwitterionic columns in hydrophilic interaction liquid chromatography mode for liquid chromatography-high resolution mass spectrometry based untargeted metabolite profiling of Leishmania parasites *Journal of Chromatography A*. 2014;1362:168–179.
- [119] Šofranko Jakub, Gondáš Eduard, Murín Radovan. Application of the Hydrophilic Interaction Liquid Chromatography (HILIC-MS) Novel Protocol to Study the Metabolic Heterogeneity of Glioblastoma Cells *Metabolites*. 2024;14:297.
- [120] Pičmanová Martina, Moses Tessa, Cortada-Garcia Joan, et al. Rapid HILIC-Z ion mobility mass spectrometry (RHIMMS) method for untargeted metabolomics of complex biological samples *Metabolomics*. 2022;18:16.
- [121] Lee Ho-Joon, Kremer Daniel M, Sajjakulnukit Peter, Zhang Li, Lyssiotis Costas A. A large-scale analysis of targeted metabolomics data from heterogeneous biological samples provides insights into metabolite dynamics *Metabolomics*. 2019;15:1–13.
- [122] Luo Xian, Li Liang. Metabolomics of small numbers of cells: metabolomic profiling of 100, 1000, and 10000 human breast cancer cells *Analytical chemistry*. 2017;89:11664–11671.
- [123] Deng Jingjing, Zhang Guoan, Neubert Thomas A. Metabolomic Analysis of Glioma Cells Using Nanoflow Liquid Chromatography–Tandem Mass Spectrometry *Glioblastoma: Methods and Protocols*. 2018:125–134.
- [124] Langford Joel B, Lurie Ira S. Use of micro, capillary, and nano liquid chromatography for forensic analysis *Journal of Separation Science*. 2022;45:38–50.
- [125] Sanders Katherine L, Edwards James L. Nano-liquid chromatography-mass spectrometry and recent applications in omics investigations *Analytical Methods*. 2020;12:4404–4417.
- [126] Shan Lian, Jones Barry R. Nano-LC: An updated review *Biomedical Chromatography*. 2022;36:e5317.
- [127] Furlani Izadora L, Cruz Nunes Estéfane, Canuto Gisele AB, Macedo Adriana N, Oliveira Regina V. Liquid chromatography-mass spectrometry for clinical

- metabolomics: an overview *Separation Techniques Applied to Omics Sciences: From Principles to Relevant Applications*. 2021:179–213.
- [128] Kebarle Paul, Verkerk Udo H. Electrospray: from ions in solution to ions in the gas phase, what we know now *Mass spectrometry reviews*. 2009;28:898–917.
- [129] Lu Wenyun, Bennett Bryson D, Rabinowitz Joshua D. Analytical strategies for LC–MS-based targeted metabolomics *Journal of chromatography B*. 2008;871:236–242.
- [130] Pelander Anna, Decker Petra, Baessmann Carsten, Ojanperä Ilkka. Evaluation of a high resolving power time-of-flight mass spectrometer for drug analysis in terms of resolving power and acquisition rate *Journal of the American Society for Mass Spectrometry*. 2011;22:379–385.
- [131] Hu Qizhi, Noll Robert J, Li Hongyan, Makarov Alexander, Hardman Mark, Graham Cooks R. The Orbitrap: a new mass spectrometer *Journal of mass spectrometry*. 2005;40:430–443.
- [132] Zubarev Roman A Alexander. Orbitrap mass spectrometry 2013.
- [133] Zhou Zhiwei, Luo Mingdu, Chen Xi, et al. Ion mobility collision cross-section atlas for known and unknown metabolite annotation in untargeted metabolomics *Nature communications*. 2020;11:4334.
- [134] May Jody C, McLean John A. Integrating ion mobility into comprehensive multidimensional metabolomics workflows: critical considerations *Metabolomics*. 2022;18:104.
- [135] Zhang Xing, Chiu Veronica M, Stoica George, Lungu Gina, Schenk James O, Hill Jr Herbert H. Metabolic analysis of striatal tissues from Parkinson's disease-like rats by electrospray ionization ion mobility mass spectrometry *Analytical chemistry*. 2014;86:3075–3083.
- [136] Wang Xue, Yi Lian, Mukhitov Nikita, Schrell Adrian M, Dhumpa Raghuram, Roper Michael G. Microfluidics-to-mass spectrometry: a review of coupling methods and applications *Journal of Chromatography A*. 2015;1382:98–116.
- [137] Wetterhall Magnus, Nilsson Stefan, Markides Karin E, Bergquist Jonas. A conductive polymeric material used for nanospray needle and low-flow sheathless electrospray ionization applications *Analytical chemistry*. 2002;74:239–245.
- [138] Gibson Graham TT, Mugo Samuel M, Oleschuk Richard D. Nanoelectrospray emitters: trends and perspective *Mass Spectrometry Reviews*. 2009;28:918–936.
- [139] Randall Elizabeth C, Lopez Begoña GC, Peng Sen, et al. Localized metabolomic gradients in patient-derived xenograft models of glioblastoma *Cancer research*. 2020;80:1258–1267.
- [140] He Wenshi, Edney Max K, Paine Simon ML, et al. Untargeted metabolomic characterization of glioblastoma intra-tumor heterogeneity using OrbiSIMS *Analytical Chemistry*. 2023;95:5994–6001.

- [141] Kertesz Vilmos, Cahill John F. Spatially resolved absolute quantitation in thin tissue by mass spectrometry *Analytical and Bioanalytical Chemistry*. 2021;413:2619–2636.
- [142] Greco Viviana, Piras Cristian, Pieroni Luisa, et al. Applications of MALDI-TOF mass spectrometry in clinical proteomics *Expert review of proteomics*. 2018;15:683–696.
- [143] Keller Caitlin, Maeda Junko, Jayaraman Dhileepkumar, et al. Comparison of vacuum MALDI and AP-MALDI platforms for the mass spectrometry imaging of metabolites involved in salt stress in *Medicago truncatula* *Frontiers in plant science*. 2018;9:1238.
- [144] Kampa Judith M, Kellner Udo, Marsching Christian, et al. Glioblastoma multiforme: Metabolic differences to peritumoral tissue and IDH-mutated gliomas revealed by mass spectrometry imaging *Neuropathology*. 2020;40:546–558.
- [145] Baker Teesha C, Han Jun, Borchers Christoph H. Recent advancements in matrix-assisted laser desorption/ionization mass spectrometry imaging *Current opinion in biotechnology*. 2017;43:62–69.
- [146] Rappez Luca, Stadler Marlene, Triana Sergio, et al. SpaceM reveals metabolic states of single cells *Nature Methods*. 2021;18:799–805.
- [147] Buglakova Elena, Ekelöf Måns, Schwaiger-Haber Michaela, et al. Spatial single-cell isotope tracing reveals heterogeneity of de novo fatty acid synthesis in cancer *Nature Metabolism*. 2024;6:1695–1711.
- [148] Zhang Yaqi, Chen Panpan, Geng Haoyuan, et al. Development of a Single-Cell Spatial Metabolomics Method for the Characterization of Cell–Cell Metabolic Interactions *Analytical Chemistry*. 2025;97:7986–7994.
- [149] Passarelli Melissa K, Pirkel Alexander, Moellers Rudolf, et al. The 3D OrbiSIMS—label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power *Nature methods*. 2017;14:1175–1183.
- [150] McCrorie Phoebe, Rowlinson Jonathan, Scurr David J, Marlow Maria, Rahman Ruman. Detection of Label-Free Drugs within Brain Tissue Using Orbitrap Secondary Ion Mass Spectrometry as a Complement to Neuro-Oncological Drug Delivery *Pharmaceutics*. 2022;14:571.
- [151] Suvannapruk Waraporn, Edney Max K, Kim Dong-Hyun, Scurr David J, Ghaemmaghami Amir M, Alexander Morgan R. Single-cell metabolic profiling of macrophages using 3D OrbiSIMS: correlations with phenotype *Analytical Chemistry*. 2022;94:9389–9398.
- [152] Liu Ruoqi, Wei Lai, Zhang Ping. A deep learning framework for drug repurposing via emulating clinical trials on real-world patient data *Nature machine intelligence*. 2021;3:68–75.

- [153] Narayan Ravi S, Molenaar Piet, Teng Jian, et al. A cancer drug atlas enables synergistic targeting of independent drug vulnerabilities *Nature communications*. 2020;11:2935.
- [154] Van Tellingen O, Yetkin-Arik B, De Gooijer MC, Wesseling P, Wurdinger T, De Vries HE. Overcoming the blood–brain tumor barrier for effective glioblastoma treatment *Drug Resistance Updates*. 2015;19:1–12.
- [155] Gerlinger Marco, Rowan Andrew J, Horswell Stuart, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing *New England journal of medicine*. 2012;366:883–892.
- [156] Dirkse Anne, Golebiewska Anna, Buder Thomas, et al. Stem cell-associated heterogeneity in Glioblastoma results from intrinsic tumor plasticity shaped by the microenvironment *Nature communications*. 2019;10:1787.
- [157] Xu Can, Hou Pengyu, Li Xiang, et al. Comprehensive understanding of glioblastoma molecular phenotypes: classification, characteristics, and transition *Cancer Biology & Medicine*. 2024;21:363.
- [158] Fauziya , Gupta Akash, Nadaf Arif, et al. Dasatinib: a potential tyrosine kinase inhibitor to fight against multiple cancer malignancies *Medical Oncology*. 2023;40:173.
- [159] Qin Anna, Musket Anna, Musich Phillip R, Schweitzer John B, Xie Qian. Receptor tyrosine kinases as druggable targets in glioblastoma: Do signaling pathways matter? *Neuro-oncology advances*. 2021;3:vdab133.
- [160] Craveiro Rogerio B, Ehrhardt Michael, Velz Julia, et al. The anti-neoplastic activity of Vandetanib against high-risk medulloblastoma variants is profoundly enhanced by additional PI3K inhibition *Oncotarget*. 2017;8:46915.
- [161] Kreisl Teri N, McNeill Katharine A, Sul Joohee, Iwamoto Fabio M, Shih Joanna, Fine Howard A. A phase I/II trial of vandetanib for patients with recurrent malignant glioma *Neuro-oncology*. 2012;14:1519–1526.
- [162] Kubota N, Okada S, Inada T, Ohnishi K, Ohnishi T. Wortmannin sensitizes human glioblastoma cell lines carrying mutant and wild type TP53 gene to radiation *Cancer letters*. 2000;161:141–147.
- [163] Ghosh Jagadish C, Siegelin Markus D, Vaira Valentina, et al. Adaptive mitochondrial reprogramming and resistance to PI3K therapy *Journal of the National Cancer Institute*. 2015;107:dju502.
- [164] Wu Xianbo, Xu Yihua, Liang Qi, et al. Recent advances in dual PI3K/mTOR inhibitors for tumour treatment *Frontiers in Pharmacology*. 2022;13:875372.
- [165] Yoon Wan-Soo, Chang Jong Hee, Kim Jeong Hoon, et al. Efficacy and safety of metformin plus low-dose temozolomide in patients with recurrent or refractory glioblastoma: a randomized, prospective, multicenter, double-blind, controlled, phase 2 trial (KNOG-1501 study) *Discover Oncology*. 2023;14:90.

- [166] Zhao Wenpeng, Zhou Liwei, Zhao Wentao, et al. The combination of temozolomide and perifosine synergistically inhibit glioblastoma by impeding DNA repair and inducing apoptosis *Cell Death Discovery*. 2024;10:315.
- [167] Compter Inge, Eekers Danielle BP, Hoeben Ann, et al. Chloroquine combined with concurrent radiotherapy and temozolomide for newly diagnosed glioblastoma: a phase IB trial *Autophagy*. 2021;17:2604–2612.
- [168] Hsu Sanford PC, Kuo John S, Chiang Hsin-Chien, et al. Temozolomide, sirolimus and chloroquine is a new therapeutic combination that synergizes to disrupt lysosomal function and cholesterol homeostasis in GBM cells *Oncotarget*. 2018;9:6883.
- [169] Kaley Thomas J, Panageas Katherine S, Pentsova Elena I, et al. Phase I clinical trial of temsirolimus and perifosine for recurrent glioblastoma *Annals of clinical and translational neurology*. 2020;7:429–436.
- [170] Mattoo Abid R, Joun Alex, Jessup J Milburn. Repurposing of mTOR complex inhibitors attenuates MCL-1 and sensitizes to PARP inhibition *Molecular Cancer Research*. 2019;17:42–53.
- [171] Persico Michele, Abbruzzese Claudia, Matteoni Silvia, et al. Tackling the behavior of cancer cells: molecular bases for repurposing antipsychotic drugs in the treatment of glioblastoma *Cells*. 2022;11:263.
- [172] Liu Zhen-zhen, Liu Xiao-ning, Fan Rui-cheng, et al. Identification of pimavanserin tartrate as a potent Ca²⁺-calcineurin-NFAT pathway inhibitor for glioblastoma therapy *Acta Pharmacologica Sinica*. 2021;42:1860–1874.
- [173] Hayashi Keiichiro, Michiue Hiroyuki, Yamada Hiroshi, et al. Fluvoxamine, an anti-depressant, inhibits human glioblastoma invasion by disrupting actin polymerization *Scientific reports*. 2016;6:23372.
- [174] Zheng Mengzhu, Sun Weiguang, Gao Suyu, et al. Structure based discovery of clomifene as a potent inhibitor of cancer-associated mutant IDH1 *Oncotarget*. 2017;8:44255.
- [175] Datta Sandipan, Sears Thomas, Cortopassi Gino, Woolard Kevin, Angelastro James M. Repurposing FDA approved drugs inhibiting mitochondrial function for targeting glioma-stem like cells *Biomedicine & Pharmacotherapy*. 2021;133:111058.
- [176] Tomi-Andrino Claudio, Pandele Alina, Winzer Klaus, King John, Rahman Ruman, Kim Dong-Hyun. Metabolic modeling-based drug repurposing in Glioblastoma *Scientific reports*. 2022;12:11189.
- [177] Blagden Sarah P, Hamilton Anne L, Mileschkin Linda, et al. Phase IB dose escalation and expansion study of AKT inhibitor afuresertib with carboplatin and paclitaxel in recurrent platinum-resistant ovarian cancer *Clinical Cancer Research*. 2019;25:1472–1478.

- [178] Chen Christine I, Paul Harminder, Le Lisa W, et al. A phase 2 study of ofatumumab (Arzerra®) in combination with a pan-AKT inhibitor (afuresertib) in previously treated patients with chronic lymphocytic leukemia (CLL) *Leukemia & Lymphoma*. 2019;60:92–100.
- [179] Wang Jian, Xu Xiaqing, Wang Tingting, et al. Ceritinib increases sensitivity of AKT inhibitors to gastric cancer *European Journal of Pharmacology*. 2021;896:173879.
- [180] Sunil Christudas, Xu Baojun. An insight into the health-promoting effects of taxifolin (dihydroquercetin) *Phytochemistry*. 2019;166:112066.
- [181] Yao Weiqi, Gong Hongyun, Mei Heng, Shi Lei, Yu Jinming, Hu Yu. Taxifolin targets pi3k and mtor and inhibits glioblastoma multiforme *Journal of Oncology*. 2021;2021:5560915.
- [182] Shuai Yuye. *Isolation, Characterization and Anticancer Evaluation of Alkaloids from Eumachia montana (Rubiaceae)*. Ph.D. thesis, University of Nottingham 2025.
- [183] Shuai Yuye, Kim Dong-Hyun, Lim Kuan-Hon, et al. Isolation, Characterization, and Anticancer Evaluation of Alkaloids from Eumachia montana (Rubiaceae) *ACS omega*. 2025;10:35719–35737.
- [184] Weller Michael, Bent Martin, Preusser Matthias, et al. EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood *Nature reviews Clinical oncology*. 2021;18:170–186.
- [185] Lah Tamara T, Novak Metka, Breznik Barbara. Brain malignancies: Glioblastoma and brain metastases in *Seminars in cancer biology*;60:262–273Elsevier 2020.
- [186] Jaroch Karol, Modrakowska Paulina, Bojko Barbara. Glioblastoma metabolomics—in vitro studies *Metabolites*. 2021;11:315.
- [187] Perrin Sally L, Samuel Michael S, Koszyca Barbara, et al. Glioblastoma heterogeneity and the tumour microenvironment: implications for preclinical research and development of new treatments *Biochemical Society transactions*. 2019;47:625–638.
- [188] Rutherford Kayleigh D, Mazandu Gaston K, Mulder Nicola J. A systems-level analysis of drug–target–disease associations for drug repositioning *Briefings in functional genomics*. 2018;17:34–41.
- [189] Pushpakom Sudeep, Iorio Francesco, Eyers Patrick A, et al. Drug repurposing: progress, challenges and recommendations *Nature reviews Drug discovery*. 2019;18:41–58.
- [190] Barzegar Behrooz Amir, Talaie Zahra, Jusheghani Fatemeh, Łos Marek J, Klonisch Thomas, Ghavami Saeid. Wnt and PI3K/Akt/mTOR survival pathways as therapeutic targets in glioblastoma *International journal of molecular sciences*. 2022;23:1353.

- [191] Hütt-Cabezas Marianne, Karajannis Matthias A, Zagzag David, et al. Activation of mTORC1/mTORC2 signaling in pediatric low-grade glioma and pilocytic astrocytoma reveals mTOR as a therapeutic target *Neuro-oncology*. 2013;15:1604–1614.
- [192] Yang Rong, Yang Xinxing, Zhang Feng. New perspectives of taxifolin in neurodegenerative diseases *Current Neuropharmacology*. 2023;21:2097.
- [193] Liu Yang, Shi Xiaolu, Tian Ye, et al. An insight into novel therapeutic potentials of taxifolin *Frontiers in Pharmacology*. 2023;14:1173855.
- [194] Broadhurst David, Goodacre Royston, Reinke Stacey N, et al. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies *Metabolomics*. 2018;14:1–17.
- [195] Wishart David S, Guo AnChi, Oler Eponine, et al. HMDB 5.0: the human metabolome database for 2022 *Nucleic acids research*. 2022;50:D622–D631.
- [196] Sumner Lloyd W, Amberg Alexander, Barrett Dave, et al. Proposed minimum reporting standards for chemical analysis: chemical analysis working group (CAWG) metabolomics standards initiative (MSI) *Metabolomics*. 2007;3:211–221.
- [197] Sumner Lloyd W, Lei Zhentian, Nikolau Basil J, Saito Kazuki, Roessner Ute, Trengove Robert. Proposed quantitative and alphanumeric metabolite identification metrics *Metabolomics*. 2014;10:1047–1049.
- [198] Pang Zhiqiang, Zhou Guangyan, Ewald Jessica, et al. Using MetaboAnalyst 5.0 for LC–HRMS spectra processing, multi-omics integration and covariate adjustment of global metabolomics data *Nature protocols*. 2022;17:1735–1761.
- [199] Wieder Cecilia, Frainay Clément, Poupin Nathalie, et al. Pathway analysis in metabolomics: Recommendations for the use of over-representation analysis *PLoS computational biology*. 2021;17:e1009105.
- [200] García-Campos Miguel A, Espinal-Enríquez Jesús, Hernández-Lemus Enrique. Pathway analysis: state of the art *Frontiers in physiology*. 2015;6:383.
- [201] Chen Chengjie, Wu Ya, Li Jiawei, et al. TBtools-II: A “one for all, all for one” bioinformatics platform for biological big-data mining *Molecular plant*. 2023;16:1733–1742.
- [202] Masure Stefan, Haefner Burkhard, Wesselink Jan-Jaap, et al. Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3 *European journal of biochemistry*. 1999;265:353–360.
- [203] Smith Stuart James, Rowlinson Jonathan, Estevez-Cebrero Maria, et al. Metabolism-based isolation of invasive glioblastoma cells with specific gene signatures and tumorigenic potential *Neuro-oncology advances*. 2020;2:vdaa087.

- [204] Riedl Sabrina, Rinner Beate, Asslaber Martin, et al. In search of a novel target—Phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 2011;1808:2638–2645.
- [205] Xu Yunze, Dong Baijun, Wang Jianfeng, Zhang Jin, Xue Wei, Huang Yiran. Sphingosine kinase 1 overexpression contributes to sunitinib resistance in clear cell renal cell carcinoma *Oncoimmunology*. 2018;7:e1502130.
- [206] Ridgway Neale D. The role of phosphatidylcholine and choline metabolites to cell proliferation and survival *Critical reviews in biochemistry and molecular biology*. 2013;48:20–38.
- [207] Zhou Heling, Stafford Jason H, Hallac Rami R, et al. Phosphatidylserine-targeted molecular imaging of tumor vasculature by magnetic resonance imaging *Journal of biomedical nanotechnology*. 2014;10:846–855.
- [208] Furse Samuel, Brooks Nicholas J, Seddon Annela M, et al. Lipid membrane curvature induced by distearoyl phosphatidylinositol 4-phosphate *Soft Matter*. 2012;8:3090–3093.
- [209] Rajamanoharan Dayani, McCue Hannah V, Burgoyne Robert D, Haynes Lee P. Modulation of phosphatidylinositol 4-phosphate levels by CaBP7 controls cytokinesis in mammalian cells *Molecular biology of the cell*. 2015;26:1428–1439.
- [210] Chini Claudia CS, Zeidler Julianna D, Kashyap Sonu, Warner Gina, Chini Eduardo Nunes. Evolving concepts in NAD⁺ metabolism *Cell metabolism*. 2021;33:1076–1087.
- [211] Gao Peng, Yang Chunzhang, Nesvick Cody L, et al. Hypotaurine evokes a malignant phenotype in glioma through aberrant hypoxic signaling *Oncotarget*. 2016;7:15200.
- [212] Shen Dachuan, Tian Lili, Yang Fangyu, et al. ADO/hypotaurine: a novel metabolic pathway contributing to glioblastoma development *Cell death discovery*. 2021;7:21.
- [213] Rocha CRR, Garcia CCM, Vieira DB, et al. Erratum: Glutathione depletion sensitizes cisplatin-and temozolomide-resistant glioma cells in vitro and in vivo *Cell death & disease*. 2015;6:e1727–e1727.
- [214] Zhu Zhongling, Du Shuangshuang, Du Yibo, Ren Jing, Ying Guoguang, Yan Zhao. Glutathione reductase mediates drug resistance in glioblastoma cells by regulating redox homeostasis *Journal of neurochemistry*. 2018;144:93–104.
- [215] Spencer Andrew, Yoon Sung-Soo, Harrison Simon J, et al. The novel AKT inhibitor afuresertib shows favorable safety, pharmacokinetics, and clinical activity in multiple myeloma *Blood, The Journal of the American Society of Hematology*. 2014;124:2190–2195.
- [216] Orlova SV, Tatarinov VV, Nikitina EA, et al. Bioavailability and safety of dihydroquercetin *Pharmaceutical Chemistry Journal*. 2022;55:1133–1137.

- [217] Dietetic Products Nutrition, (NDA) Allergies, Turck Dominique, et al. Scientific Opinion on taxifolin-rich extract from Dahurian Larch (*Larix gmelinii*) *EFSA Journal*. 2017;15:e04682.
- [218] Wang Xiaodan, Xia Hongjun, Xing Feng, Deng Guifeng, Shen Qi, Zeng Su. A highly sensitive and robust UPLC–MS with electrospray ionization method for quantitation of taxifolin in rat plasma *Journal of Chromatography B*. 2009;877:1778–1786.
- [219] Herrero-Navarro Álvaro, Puche-Aroca Lorenzo, Moreno-Juan Verónica, et al. Astrocytes and neurons share region-specific transcriptional signatures that confer regional identity to neuronal reprogramming *Science Advances*. 2021;7:eabe8978.
- [220] Wood James, Smith Stuart J, Castellanos-Urbe Marcos, et al. Metabolomic characterisation of the glioblastoma invasive margin reveals a region-specific signature *Heliyon*. 2025;11.
- [221] Dong Yonghui, Heinig Uwe. Mass Spectrometry Imaging Data Analysis with ShinyCardinal 2024.
- [222] Bemis Kylie Ariel, Föll Melanie Christine, Guo Dan, Lakkimsetty Sai Srikanth, Vitek Olga. Cardinal v. 3: a versatile open-source software for mass spectrometry imaging analysis *Nature Methods*. 2023;20:1883–1886.
- [223] Edney Max K, Kotowska Anna M, Spanu Matteo, et al. Molecular formula prediction for chemical filtering of 3D OrbiSIMS Datasets *Analytical Chemistry*. 2022;94:4703–4711.
- [224] Pang Zhiqiang, Lu Yao, Zhou Guangyan, et al. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation *Nucleic Acids Research*. 2024;gkae253.
- [225] Kamiloglu Senem, Sari Gulce, Ozdal Tugba, Capanoglu Esra. Guidelines for cell viability assays *Food frontiers*. 2020;1:332–349.
- [226] Repetto Guillermo, Del Peso Ana, Zurita Jorge L. Neutral red uptake assay for the estimation of cell viability/cytotoxicity *Nature protocols*. 2008;3:1125–1131.
- [227] Bemis Kyle D, Harry April, Eberlin Livia S, et al. Probabilistic segmentation of mass spectrometry (MS) images helps select important ions and characterize confidence in the resulting segments *Molecular & Cellular Proteomics*. 2016;15:1761–1772.
- [228] Yamashita Daisuke, Bernstock Joshua D, Elsayed Galal, et al. Targeting glioma-initiating cells via the tyrosine metabolic pathway *Journal of neurosurgery*. 2020;134:721–732.
- [229] Garcia Joseph H, Akins Erin A, Jain Saket, et al. Multiomic screening of invasive GBM cells reveals targetable transsulfuration pathway alterations *The Journal of Clinical Investigation*. 2024;134.

- [230] Niechi Ignacio, Uribe-Ojeda Atenea, Erices José Ignacio, et al. Adenosine depletion as a new strategy to decrease glioblastoma stem-like cells aggressiveness *Cells*. 2019;8:1353.
- [231] Righi Valeria, Roda Jose M, Paz Jose, et al. ¹H HR-MAS and genomic analysis of human tumor biopsies discriminate between high and low grade astrocytomas *NMR in Biomedicine: An International Journal Devoted to the Development and Application of Magnetic Resonance In vivo*. 2009;22:629–637.
- [232] Martin-Perez Miguel, Urdiroz-Urricelqui Uxue, Bigas Claudia, Benitah Salvador Aznar. The role of lipids in cancer progression and metastasis *Cell metabolism*. 2022;34:1675–1699.
- [233] Koch Katharina, Hartmann Rudolf, Schröter Friederike, et al. Reciprocal regulation of the cholinic phenotype and epithelial-mesenchymal transition in glioblastoma cells *Oncotarget*. 2016;7:73414.
- [234] Kelly Claire Louise, Wydrzynska Martyna, Phelan Marie M, et al. Hypoxia Dependent Inhibition of Glioblastoma Cell Proliferation, Invasion, and Metabolism by the Choline-Kinase Inhibitor JAS239 *Metabolites*. 2025;15:76.
- [235] Zou Yourui, Huang Ling, Sun Shengyu, et al. Choline kinase alpha promoted glioma development by activating PI3K/AKT signaling pathway *Cancer Biotherapy and Radiopharmaceuticals*. 2021.
- [236] Chetwynd Andrew J, David Arthur, Hill Elizabeth M, Abdul-Sada Alaa. Evaluation of analytical performance and reliability of direct nanoLC-nanoESI-high resolution mass spectrometry for profiling the (xeno) metabolome *Journal of Mass Spectrometry*. 2014;49:1063–1069.
- [237] Gika Helen G, Theodoridis Georgios A, Plumb Robert S, Wilson Ian D. Current practice of liquid chromatography–mass spectrometry in metabolomics and metabonomics *Journal of pharmaceutical and biomedical analysis*. 2014;87:12–25.
- [238] Hemmer Selina, Manier Sascha K, Wagmann Lea, Meyer Markus R. Comparison of reversed-phase, hydrophilic interaction, and porous graphitic carbon chromatography columns for an untargeted toxicometabolomics study in pooled human liver microsomes, rat urine, and rat plasma *Metabolomics*. 2024;20:49.
- [239] Kumar Abhinav, Heaton James C, McCalley David V. Practical investigation of the factors that affect the selectivity in hydrophilic interaction chromatography *Journal of Chromatography a*. 2013;1276:33–46.
- [240] Ladd Mallory P, Giannone Richard J, Abraham Paul E, Wulschleger Stan D, Hettich Robert L. Evaluation of an untargeted nano-liquid chromatography-mass spectrometry approach to expand coverage of low molecular weight dissolved organic matter in Arctic soil *Scientific Reports*. 2019;9:5810.
- [241] Gika Helen, Virgiliou Christina, Theodoridis Georgios, Plumb Robert S, Wilson Ian D. Untargeted LC/MS-based metabolic phenotyping (metabonomics/metabolomics): The state of the art *Journal of Chromatography B*. 2019;1117:136–147.

- [242] Karhale Kiran, Kohale Nitin B, Rathod Suraj B, Jadhav RV. Overview an Nano Liquid Chromatography *Int. J. Humanit. Soc. Sci. and Man.* 2023;3:201–409.
- [243] Buszewski Bogusław, Noga Sylwia. Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique *Analytical and bioanalytical chemistry.* 2012;402:231–247.
- [244] Tang Dao-Quan, Zou Li, Yin Xiao-Xing, Ong Choon Nam. HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS *Mass spectrometry reviews.* 2016;35:574–600.
- [245] Teleki Attila, Sánchez-Kopper Andrés, Takors Ralf. Alkaline conditions in hydrophilic interaction liquid chromatography for intracellular metabolite quantification using tandem mass spectrometry *Analytical Biochemistry.* 2015;475:4–13.
- [246] Zhang Tong, Creek Darren J, Barrett Michael P, Blackburn Gavin, Watson David G. Evaluation of coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid chromatography with Orbitrap mass spectrometry for metabolomic studies of human urine *Analytical chemistry.* 2012;84:1994–2001.
- [247] Van Deemter JJ, Zuiderweg FJ, Klinkenberg A van. Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography *Chemical Engineering Science.* 1995;50:3869–3882.
- [248] King Amy CF, Giorio Chiara, Wolff Eric, et al. Direct injection liquid chromatography high-resolution mass spectrometry for determination of primary and secondary terrestrial and marine biomarkers in ice cores *Analytical chemistry.* 2019;91:5051–5057.
- [249] Fitz Veronika, El Abiead Yasin, Berger Daniel, Koellensperger Gunda. Systematic investigation of LC miniaturization to increase sensitivity in wide-target LC-MS-based trace bioanalysis of small molecules *Frontiers in molecular biosciences.* 2022;9:857505.
- [250] Dettmer Katja, Aronov Pavel A, Hammock Bruce D. Mass spectrometry-based metabolomics *Mass spectrometry reviews.* 2007;26:51–78.
- [251] Xiao Jun Feng, Zhou Bin, Resson Habtom W. Metabolite identification and quantitation in LC-MS/MS-based metabolomics *TrAC Trends in Analytical Chemistry.* 2012;32:1–14.
- [252] Zimmer Brenna M, Barycki Joseph J, Simpson Melanie A. Integration of sugar metabolism and proteoglycan synthesis by UDP-glucose dehydrogenase *Journal of Histochemistry & Cytochemistry.* 2021;69:13–23.
- [253] Trejo-Solis Cristina, Silva-Adaya Daniela, Serrano-García Norma, et al. Role of glycolytic and glutamine metabolism reprogramming on the proliferation, invasion, and apoptosis resistance through modulation of signaling pathways in glioblastoma *International journal of molecular sciences.* 2023;24:17633.

- [254] Oyinlade Olutobi, Wei Shuang, Lal Bachchu, et al. Targeting UDP- α -D-glucose 6-dehydrogenase inhibits glioblastoma growth and migration *Oncogene*. 2018;37:2615–2629.
- [255] Paneque Alysta, Fortus Harvey, Zheng Julia, Werlen Guy, Jacinto Estela. The hexosamine biosynthesis pathway: regulation and function *Genes*. 2023;14:933.
- [256] Turner Mark D, Sale Craig, Garner A Christopher, Hipkiss Alan R. Anti-cancer actions of carnosine and the restoration of normal cellular homeostasis *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 2021;1868:119117.
- [257] Caruso John, Charles Jessica, Unruh Kayla, Giebel Rachel, Learmonth Lexis, Potter William. Ergogenic effects of β -alanine and carnosine: proposed future research to quantify their efficacy *Nutrients*. 2012;4:585–601.
- [258] Gorgoglione Ruggiero, Impedovo Valeria, Riley Christopher L, et al. Glutamine-derived aspartate biosynthesis in cancer cells: role of mitochondrial transporters and new therapeutic perspectives *Cancers*. 2022;14:245.
- [259] Medina Vanina A, Rivera Elena S. Histamine receptors and cancer pharmacology *British journal of pharmacology*. 2010;161:755–767.
- [260] Zenobi Renato. Single-cell metabolomics: analytical and biological perspectives *Science*. 2013;342:1243259.
- [261] Zhou Yu, Zhao Huakan, Ren Ran, et al. GPAT3 is a potential therapeutic target to overcome sorafenib resistance in hepatocellular carcinoma *Theranostics*. 2024;14:3470.
- [262] Kumar Manoj, Arlauckas Sean P, Saksena Sona, et al. Magnetic resonance spectroscopy for detection of choline kinase inhibition in the treatment of brain tumors *Molecular cancer therapeutics*. 2015;14:899–908.
- [263] Aaltonen Niina, Singha Prosanta K, Jakupović Hermína, et al. High-Resolution Confocal Fluorescence Imaging of Serine Hydrolase Activity in Cryosections—Application to Glioma Brain Unveils Activity Hotspots Originating from Tumor-Associated Neutrophils *Biological Procedures Online*. 2020;22:6.
- [264] Khoshyomn Sami, Penar Paul L, Rossi John, Wells Alan, Abramson David L, Bhushan Alok. Inhibition of phospholipase C- γ 1 activation blocks glioma cell motility and invasion of fetal rat brain aggregates *Neurosurgery*. 1999;44:568–577.
- [265] Kang Dong Woo, Hwang Won Chan, Noh Yu Na, Park Kang Seo, Min Do Sik. Phospholipase D1 inhibition sensitizes glioblastoma to temozolomide and suppresses its tumorigenicity *The Journal of Pathology*. 2020;252:304–316.
- [266] Lee Kil Sun, Su Xiaoyang, Huan Tao. Metabolites are not genes—avoiding the misuse of pathway analysis in metabolomics *Nature Metabolism*. 2025:1–4.

- [267] Tsouka Sofia, Masoodi Mojgan. Metabolic pathway analysis: advantages and pitfalls for the functional interpretation of metabolomics and lipidomics data *Biomolecules*. 2023;13:244.

Appendix A

Tables

Table A.1. Significantly altered metabolites for GIN31 vs. GCE31 (adjusted *p* value < 0.05 and VIP > 1).

m/z	Formula	RT(min)	Δppm	Adducts	Putative metabolites	HMDB ID	Confidence	Log2 FC	Adj. <i>p</i> -value	VIP
207.0776	C ₁₀ H ₁₂ N ₂ O ₃	8.83	-1.41	[M-H] ⁻¹	Kynurenine	HMDB0000684	Level 1	-5.36	3.16E-09	1.39
104.1068	C ₅ H ₁₃ NO	17.98	-1.51	[M+H] ⁺¹	Choline	HMDB0000097	Level 2	-1.41	3.36E-08	1.39
214.0487	C ₅ H ₁₄ NO ₆ P	10.81	0.05	[M-H] ⁻¹	Glycerylphosphorylethanolamine	HMDB0000114	Level 2	1.77	8.94E-08	1.38
210.0286	C ₄ H ₁₀ N ₃ O ₅ P	10.15	-0.76	[M-H] ⁻¹	Phosphocreatine	HMDB0001511	Level 2	1.31	8.94E-08	1.39
204.1228	C ₉ H ₁₇ NO ₄	8.46	-1.13	[M+H] ⁺¹	Acetylcarnitine	HMDB0000201	Level 1	1.71	9.93E-08	1.37
174.0884	C ₆ H ₁₃ N ₃ O ₃	11.14	-0.52	[M-H] ⁻¹	Citrulline	HMDB0000904	Level 1	1.58	9.93E-08	1.38
162.1123	C ₇ H ₁₅ NO ₃	9.66	-1.13	[M+H] ⁺¹	Carnitine	HMDB0000062	Level 1	1.04	1.10E-07	1.38
131.1178	C ₆ H ₁₄ N ₂ O	17.70	-0.45	[M+H] ⁺¹	N-Acetylputrescine	HMDB0002064	Level 1	-0.62	1.26E-06	1.36
148.0603	C ₅ H ₉ NO ₄	10.03	-0.68	[M+H] ⁺¹	Glutamic acid	HMDB0000148	Level 1	0.41	1.58E-06	1.36
218.1034	C ₉ H ₁₇ NO ₅	4.74	-1.43	[M-H] ⁻¹	Pantothenic acid	HMDB0000210	Level 2	0.57	1.66E-06	1.36
114.0661	C ₄ H ₇ N ₃ O	8.29	-0.71	[M+H] ⁺¹	Creatinine	HMDB0000562	Level 1	1.02	3.62E-06	1.35
161.1283	C ₇ H ₁₆ N ₂ O ₂	16.72	-0.73	[M+H] ⁺¹	N(6)-Methyllysine	HMDB0002038	Level 1	-0.67	6.43E-06	1.34
258.1098	C ₈ H ₂₀ NO ₆ P	10.05	-1.15	[M+H] ⁺¹	Glycerophosphocholine	HMDB0000086	Level 1	1.49	6.63E-06	1.34
146.1174	C ₇ H ₁₅ NO ₂	9.64	-0.98	[M+H] ⁺¹	Acetylcholine	HMDB0000895	Level 1	0.55	8.39E-06	1.35
174.0408	C ₆ H ₉ NO ₅	9.96	-0.25	[M-H] ⁻¹	N-Acetyl-L-aspartic acid	HMDB0000812	Level 2	-0.65	1.14E-05	1.33
606.0748	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	10.04	0.56	[M-H] ⁻¹	UDP-GlcNAc	HMDB0000290	Level 2	-0.95	1.31E-05	1.34
148.0438	C ₅ H ₁₁ NO ₂ S	9.12	-0.65	[M-H] ⁻¹	Methionine	HMDB0000696	Level 1	0.57	1.35E-05	1.33
118.0611	C ₃ H ₇ N ₃ O ₂	11.46	-0.32	[M+H] ⁺¹	Guanidinoacetate	HMDB0000128	Level 1	1.02	2.66E-05	1.31
102.0561	C ₄ H ₉ NO ₂	10.06	0.05	[M-H] ⁻¹	Gamma-Aminobutyric acid	HMDB0000112	Level 2	0.49	3.64E-05	1.32
161.0456	C ₆ H ₁₀ O ₅	9.70	0.37	[M-H] ⁻¹	3-Hydroxymethylglutaric acid	HMDB0000355	Level 2	-0.41	4.39E-05	1.30
179.0562	C ₆ H ₁₂ O ₆	11.91	0.17	[M-H] ⁻¹	Fructose	HMDB0000660	Level 1	0.73	5.31E-05	1.31
129.0658	C ₅ H ₈ N ₂ O ₂	10.74	0.03	[M+H] ⁺¹	Dihydrothymine	HMDB0000079	Level 2	0.48	9.35E-05	1.29
244.0925	C ₉ H ₁₃ N ₃ O ₅	9.47	-1.08	[M+H] ⁺¹	Cytidine	HMDB0000089	Level 1	0.94	1.33E-04	1.29
128.0354	C ₅ H ₇ NO ₃	10.08	0.35	[M-H] ⁻¹	Pyroglutamic acid	HMDB0000267	Level 2	0.52	1.48E-04	1.27
145.0618	C ₅ H ₁₀ N ₂ O ₃	10.79	-0.66	[M-H] ⁻¹	Glutamine	HMDB0000641	Level 1	0.31	3.22E-04	1.26
116.0717	C ₅ H ₁₁ NO ₂	9.54	-1.00	[M-H] ⁻¹	Valine	HMDB0000883	Level 1	0.47	3.22E-04	1.24
100.0405	C ₄ H ₇ NO ₂	9.13	0.14	[M-H] ⁻¹	1-Aminocyclopropanecarboxylic acid	HMDB0036458	Level 2	0.37	3.35E-04	1.25
134.0447	C ₄ H ₇ NO ₄	10.31	-0.43	[M+H] ⁺¹	Aspartic acid	HMDB0000191	Level 1	0.20	5.83E-04	1.23
131.0462	C ₄ H ₈ N ₂ O ₃	9.53	0.09	[M-H] ⁻¹	Glycyl-glycine	HMDB0011733	Level 1	0.31	1.01E-03	1.21

Table A.1 (continued)

m/z	Formula	RT(min)	Δ ppm	Adducts	Putative metabolites	HMDB ID	Confidence	Log ₂ FC	Adj. <i>p</i> -value	VIP
175.0360	C ₅ H ₈ N ₂ O ₅	11.22	-0.05	[M-H] ⁻¹	Ureidosuccinic acid	HMDB0000828	Level 2	0.37	1.15E-03	1.19
134.0473	C ₅ H ₅ N ₅	7.84	0.26	[M-H] ⁻¹	Adenine	HMDB0000034	Level 2	1.09	1.30E-03	1.17
88.0392	C ₃ H ₅ NO ₂	10.31	-0.62	[M+H] ⁺¹	2-Aminoacrylic acid	HMDB0003609	Level 2	0.22	1.33E-03	1.20
88.0404	C ₃ H ₇ NO ₂	10.31	0.05	[M-H] ⁻¹	Alanine	HMDB0000056	Level 1	0.23	1.37E-03	1.19
268.1035	C ₁₀ H ₁₃ N ₅ O ₄	7.85	-1.78	[M+H] ⁺¹	Adenosine	HMDB0000050	Level 1	0.77	1.68E-03	1.16
203.1501	C ₈ H ₁₈ N ₄ O ₂	15.92	-0.68	[M+H] ⁺¹	Symmetric dimethylarginine	HMDB0003334	Level 2	0.43	2.18E-03	1.17
137.0458	C ₅ H ₄ N ₄ O	8.91	0.09	[M+H] ⁺¹	Hypoxanthine	HMDB0000157	Level 1	1.13	2.32E-03	1.18
245.0432	C ₆ H ₁₅ O ₈ P	9.02	-0.06	[M-H] ⁻¹	Glycerophosphoglycerol	HMDB0240316	Level 2	0.55	2.40E-03	1.15
184.0732	C ₅ H ₁₄ NO ₄ P	10.17	-0.83	[M+H] ⁺¹	Phosphorylcholine	HMDB0001565	Level 1	-0.25	2.53E-03	1.13
74.0248	C ₂ H ₅ NO ₂	10.51	0.03	[M-H] ⁻¹	Glycine	HMDB0000123	Level 2	0.34	2.63E-03	1.15
269.0876	C ₁₀ H ₁₂ N ₄ O ₅	8.91	-1.23	[M+H] ⁺¹	Inosine	HMDB0000195	Level 1	1.11	3.14E-03	1.16
133.0971	C ₅ H ₁₂ N ₂ O ₂	16.39	-0.31	[M+H] ⁺¹	Ornithine	HMDB0000214	Level 1	0.53	3.85E-03	1.12
222.0969	C ₈ H ₁₅ NO ₆	9.21	-1.30	[M+H] ⁺¹	N-Acetyl-D-glucosamine	HMDB0000215	Level 1	0.68	4.91E-03	1.11
243.0623	C ₉ H ₁₂ N ₂ O ₆	8.45	-0.52	[M-H] ⁻¹	Uridine	HMDB0000296	Level 1	1.34	5.99E-03	1.10
147.1126	C ₆ H ₁₄ N ₂ O ₂	17.20	-0.90	[M+H] ⁺¹	Lysine	HMDB0000182	Level 2	0.51	7.12E-03	1.06
195.0511	C ₆ H ₁₂ O ₇	9.88	0.10	[M-H] ⁻¹	Gluconic acid	HMDB0000625	Level 1	0.48	7.27E-03	1.06
214.1450	C ₁₁ H ₂₁ NO ₃	4.31	-0.91	[M-H] ⁻¹	N-Nonanoyl glycine	HMDB0013279	Level 2	1.16	7.35E-03	1.07
175.1188	C ₆ H ₁₄ N ₄ O ₂	18.67	-0.72	[M+H] ⁺¹	Arginine	HMDB0000517	Level 1	0.39	7.80E-03	1.06
189.1596	C ₉ H ₂₀ N ₂ O ₂	15.55	-0.71	[M+H] ⁺¹	N6,N6,N6-Trimethyl-L-lysine	HMDB0001325	Level 2	0.38	9.08E-03	1.04
197.1548	C ₁₂ H ₂₂ O ₂	3.59	0.56	[M-H] ⁻¹	11-Dodecenoic acid	HMDB0032248	Level 2	0.58	1.07E-02	1.03
106.0498	C ₃ H ₇ NO ₃	11.25	-0.29	[M+H] ⁺¹	Serine	HMDB0003406	Level 1	0.17	1.17E-02	1.04
89.0244	C ₃ H ₆ O ₃	7.29	0.15	[M-H] ⁻¹	Lactic acid	HMDB0000190	Level 1	0.62	1.18E-02	1.03
111.0201	C ₄ H ₄ N ₂ O ₂	8.45	0.18	[M-H] ⁻¹	Uracil	HMDB0000300	Level 2	1.28	1.21E-02	1.04

m/z is accurate mass. RT represents retention time. Δ ppm is mass error. Log₂ FC is short for Log₂ fold change. Adjusted *p* is generated using Benjamini-Hochberg correction. VIP represents variables importance in projection. Confidence is identification confidence: Level 1: RT, MS1, MS/MS; Level 2: MS1, MS/MS.

Table A.2. Significantly altered metabolites for GIN28 vs. GCE28 (adjusted *p* value < 0.05 and VIP > 1).

m/z	Formula	RT(min)	Δppm	Adducts	Putative metabolites	HMDB ID	Confidence	Log2 FC	Adj. <i>p</i> -value	VIP
114.0661	C ₄ H ₇ N ₃ O	8.28	-0.75	[M+H] ⁺	Creatinine	HMDB0000562	Level 1	0.63	3.33E-07	1.54
190.0543	C ₇ H ₁₃ NO ₃ S	4.64	-0.47	[M-H] ⁻	N-Acetyl-L-methionine	HMDB0011745	Level 2	-0.83	5.75E-06	1.53
110.0270	C ₂ H ₇ NO ₂ S	10.87	-0.62	[M+H] ⁺	Hypotaurine	HMDB0000965	Level 1	1.57	9.18E-06	1.52
703.5738	C ₃₉ H ₇₉ N ₂ O ₆ P	7.21	-1.48	[M+H] ⁺	SM(d18:1/16:0)	HMDB0010169	Level 2	-0.69	1.75E-05	1.51
161.1283	C ₇ H ₁₆ N ₂ O ₂	17.11	-1.18	[M+H] ⁺	N(6)-Methyllysine	HMDB0002038	Level 1	1.11	1.83E-05	1.51
131.1178	C ₆ H ₁₄ N ₂ O	18.89	-0.79	[M+H] ⁺	N-Acetylputrescine	HMDB0002064	Level 1	1.01	2.38E-05	1.50
146.0458	C ₅ H ₉ NO ₄	5.07	-0.56	[M-H] ⁻	N-Acetylserine	HMDB0002931	Level 2	-0.57	2.49E-05	1.49
246.1520	C ₁₂ H ₂₃ NO ₂ S	3.49	-0.50	[M+H] ⁺	S-3-oxodecanoyl cysteamine	HMDB0059773	Level 2	-1.18	2.49E-05	1.50
606.0744	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	9.89	0.00	[M-H] ⁻	UDP-GlcNAc	HMDB0000290	Level 2	-1.13	7.45E-05	1.47
284.0986	C ₁₀ H ₁₃ N ₅ O ₅	9.95	-1.04	[M+H] ⁺	Guanosine	HMDB0000133	Level 1	-3.67	8.83E-05	1.47
214.0486	C ₅ H ₁₄ NO ₆ P	10.76	-0.43	[M-H] ⁻	Glycerolphosphorylethanolamine	HMDB0000114	Level 2	0.50	1.31E-04	1.45
132.0656	C ₅ H ₉ NO ₃	10.53	0.11	[M+H] ⁺	4-Hydroxyproline	HMDB0000725	Level 2	0.57	1.41E-04	1.44
202.0721	C ₈ H ₁₃ NO ₅	9.92	-0.44	[M-H] ⁻	N-acetyl-L-2-amino adipic acid	HMDB0062715	Level 2	-0.82	1.76E-04	1.44
258.1099	C ₈ H ₂₀ NO ₆ P	10.05	-0.72	[M+H] ⁺	Glycerophosphocholine	HMDB0000086	Level 1	0.51	1.84E-04	1.44
126.0218	C ₂ H ₇ NO ₃ S	10.96	-0.78	[M+H] ⁺	Taurine	HMDB0000251	Level 1	0.33	1.86E-04	1.44
105.0193	C ₃ H ₆ O ₄	8.50	-0.71	[M-H] ⁻	Glyceric acid	HMDB0000139	Level 1	-2.36	2.38E-04	1.44
179.0560	C ₆ H ₁₂ O ₆	11.91	-0.43	[M-H] ⁻	myo-Inositol	HMDB0000211	Level 1	0.47	3.61E-04	1.41
112.0504	C ₄ H ₅ N ₃ O	9.45	-1.30	[M+H] ⁺	Cytosine	HMDB0000630	Level 1	-2.59	6.65E-04	1.39
244.0926	C ₉ H ₁₃ N ₃ O ₅	9.45	-0.90	[M+H] ⁺	Cytidine	HMDB0000089	Level 1	-2.73	7.00E-04	1.39
123.0551	C ₆ H ₆ N ₂ O	9.33	-1.00	[M+H] ⁺	Niacinamide	HMDB0001406	Level 2	-0.65	8.99E-04	1.38
268.1037	C ₁₀ H ₁₃ N ₅ O ₄	7.83	-1.12	[M+H] ⁺	Adenosine	HMDB0000050	Level 1	-1.41	9.10E-04	1.37
137.0457	C ₅ H ₄ N ₄ O	8.89	-0.98	[M+H] ⁺	Hypoxanthine	HMDB0000157	Level 1	-1.76	1.31E-03	1.36
136.0617	C ₅ H ₅ N ₅	8.21	-0.76	[M+H] ⁺	Adenine	HMDB0000034	Level 2	-1.73	1.39E-03	1.35
269.0877	C ₁₀ H ₁₂ N ₄ O ₅	8.88	-0.80	[M+H] ⁺	Inosine	HMDB0000195	Level 1	-1.68	1.39E-03	1.35
116.0705	C ₅ H ₉ NO ₂	9.70	-1.11	[M+H] ⁺	Proline	HMDB0003411	Level 1	-0.30	1.76E-03	1.34
664.1155	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	9.32	-0.83	[M+H] ⁺	NAD	HMDB0000902	Level 2	-0.68	1.85E-03	1.33
163.1076	C ₆ H ₁₄ N ₂ O ₃	16.28	-1.01	[M+H] ⁺	5-Hydroxylysine	HMDB0000450	Level 2	-1.84	2.07E-03	1.33
131.0461	C ₄ H ₈ N ₂ O ₃	10.83	-0.69	[M-H] ⁻	Asparagine	HMDB0000168	Level 1	-0.73	2.26E-03	1.32
747.5187	C ₄₀ H ₇₇ O ₁₀ P	3.25	0.45	[M-H] ⁻	PG (18:1(9Z)/16:0)	HMDB0010630	Level 2	0.48	2.26E-03	1.33

Table A.2 (continued)

m/z	Formula	RT(min)	Δ ppm	Adducts	Putative metabolites	HMDB ID	Confidence	Log2 FC	Adj. <i>p</i> -value	VIP
111.0199	C ₄ H ₄ N ₂ O ₂	8.43	-0.61	[M-H] ⁻¹	Uracil	HMDB0000300	Level 2	-1.79	2.73E-03	1.31
243.0621	C ₉ H ₁₂ N ₂ O ₆	8.43	-0.46	[M-H] ⁻¹	Uridine	HMDB0000296	Level 1	-1.82	3.28E-03	1.30
180.0665	C ₉ H ₁₁ NO ₃	10.00	-0.50	[M-H] ⁻¹	Tyrosine	HMDB0000158	Level 2	-0.29	3.54E-03	1.29
222.0970	C ₈ H ₁₅ NO ₆	9.18	-0.89	[M+H] ⁺¹	N-Acetyl-D-glucosamine	HMDB0000215	Level 1	-1.61	5.43E-03	1.26
145.0617	C ₅ H ₁₀ N ₂ O ₃	10.73	-0.88	[M-H] ⁻¹	Glutamine	HMDB0000641	Level 1	-0.35	8.62E-03	1.22
210.0285	C ₄ H ₁₀ N ₃ O ₅ P	9.98	-0.76	[M-H] ⁻¹	Phosphocreatine	HMDB0001511	Level 2	1.80	8.88E-03	1.22
132.0301	C ₄ H ₇ NO ₄	10.20	-0.93	[M-H] ⁻¹	Aspartic acid	HMDB0000191	Level 1	-0.27	1.11E-02	1.20
131.0349	C ₅ H ₈ O ₄	7.96	-0.39	[M-H] ⁻¹	Ethylmalonic acid	HMDB0000622	Level 2	-1.71	1.24E-02	1.17
184.0731	C ₅ H ₁₄ NO ₄ P	9.88	-1.01	[M+H] ⁺¹	Phosphorylcholine	HMDB0001565	Level 1	2.15	1.46E-02	1.17
130.0509	C ₅ H ₉ NO ₃	4.68	-0.73	[M-H] ⁻¹	N-Acetyl-L-alanine	HMDB0000766	Level 2	-0.19	1.63E-02	1.15
248.0234	C ₈ H ₁₁ NO ₆ S	12.08	-0.63	[M-H] ⁻¹	Norepinephrine sulfate	HMDB0002062	Level 2	-1.24	1.89E-02	1.12
128.0353	C ₅ H ₇ NO ₃	7.36	-0.57	[M-H] ⁻¹	4-Oxoproline	HMDB0304793	Level 2	0.22	2.66E-02	1.08
152.0022	C ₃ H ₇ NO ₄ S	9.96	-0.67	[M-H] ⁻¹	3-Sulfinioalanine	HMDB0000996	Level 2	-0.23	4.76E-02	1.02

m/z is accurate mass. RT represents retention time. Δ ppm is mass error. Log₂ FC is short for Log₂ fold change. Adjusted *p* is generated using Benjamini-Hochberg correction. VIP represents variables importance in projection. Confidence is identification confidence: Level 1: RT, MS1, MS/MS; Level 2: MS1, MS/MS.

Table A.3. Significantly altered metabolic pathways between untreated and treated GIN31 (adjusted *p* value < 0.05 and impact factor > 0.1).

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Afuresertib	Amino sugar and nucleotide sugar metabolism	37	8	2.56E-08	1.46E-06	0.12
	Glycerophospholipid metabolism	36	10	7.78E-08	4.36E-06	0.47
	Taurine and hypotaurine metabolism	8	4	4.84E-07	2.66E-05	1.00
	Pyrimidine metabolism	39	8	8.59E-07	4.55E-05	0.16
	Lysine degradation	25	8	1.47E-06	7.64E-05	0.36
	Cysteine and methionine metabolism	33	11	3.09E-06	1.51E-04	0.45
	Alanine, aspartate and glutamate metabolism	28	14	1.17E-05	5.60E-04	0.85
	Aminoacyl-tRNA biosynthesis	48	19	1.21E-04	5.70E-03	0.17
	Glutathione metabolism	28	8	1.71E-04	7.85E-03	0.48
	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	3.77E-04	1.70E-02	1.00
	Sphingolipid metabolism	21	5	6.61E-04	2.91E-02	0.45
	Pantothenate and CoA biosynthesis	19	7	7.71E-04	3.32E-02	0.25
	Arginine biosynthesis	14	8	1.14E-03	4.67E-02	0.48
	Histidine metabolism	16	7	1.26E-03	4.96E-02	0.36
Taxifolin	Glycerolipid metabolism	16	2	3.26E-07	1.89E-05	0.14
	Histidine metabolism	16	7	1.25E-06	7.02E-05	0.36
	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	3.30E-06	1.78E-04	1.00
	Glycerophospholipid metabolism	36	10	5.95E-06	3.15E-04	0.47
	Tyrosine metabolism	42	5	8.84E-06	4.60E-04	0.36
	Cysteine and methionine metabolism	33	11	1.50E-05	7.63E-04	0.45
	Nicotinate and nicotinamide metabolism	15	5	6.05E-05	3.03E-03	0.57
	Pantothenate and CoA biosynthesis	19	7	8.74E-05	4.28E-03	0.25
	Aminoacyl-tRNA biosynthesis	48	19	3.91E-04	1.88E-02	0.17
	Amino sugar and nucleotide sugar metabolism	37	8	4.52E-04	2.12E-02	0.12
	Glycine, serine and threonine metabolism	33	10	1.12E-03	4.58E-02	0.51
Caledonine	Taurine and hypotaurine metabolism	8	3	2.10E-07	1.01E-05	0.71
	Sphingolipid metabolism	21	4	4.43E-06	2.04E-04	0.42

Table A.3 (continued)						
Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Caledonine	Glycerophospholipid metabolism	36	7	2.38E-04	1.00E-02	0.32
	Cysteine and methionine metabolism	33	6	3.63E-04	1.48E-02	0.44
	Histidine metabolism	16	4	9.60E-04	3.74E-02	0.22

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted *p* is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

Table A.4. Significantly altered metabolic pathways between untreated and treated GCE31 (adjusted *p* value < 0.05 and impact factor > 0.1).

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Afuresertib	Alanine, aspartate and glutamate metabolism	28	14	3.30E-07	1.88E-05	0.85
	Glycerolipid metabolism	16	2	7.04E-06	3.87E-04	0.14
	Glycerophospholipid metabolism	36	10	1.34E-04	6.97E-03	0.47
	Cysteine and methionine metabolism	33	11	4.82E-04	2.36E-02	0.45
	Pyrimidine metabolism	39	8	5.88E-04	2.82E-02	0.16
	Histidine metabolism	16	7	6.21E-04	2.92E-02	0.36
Taxifolin	Histidine metabolism	16	7	7.67E-08	4.37E-06	0.36
	Cysteine and methionine metabolism	33	11	1.18E-05	6.38E-04	0.45
	Glycerophospholipid metabolism	36	10	1.82E-05	9.66E-04	0.47
	Pyrimidine metabolism	39	8	1.13E-04	5.87E-03	0.16
	Tryptophan metabolism	41	7	1.25E-04	6.38E-03	0.42
	Nicotinate and nicotinamide metabolism	15	5	2.25E-04	1.12E-02	0.57
	Glycerolipid metabolism	16	2	6.05E-04	2.91E-02	0.14
	Glycine, serine and threonine metabolism	33	10	8.74E-04	4.02E-02	0.51
	Arginine and proline metabolism	38	12	8.90E-04	4.02E-02	0.42
Caledonine	Taurine and hypotaurine metabolism	8	3	1.14E-11	5.26E-10	0.71
	Alanine, aspartate and glutamate metabolism	28	11	1.35E-09	5.96E-08	0.78
	Arginine biosynthesis	14	6	1.77E-08	7.63E-07	0.31
	Tryptophan metabolism	41	4	1.98E-08	8.30E-07	0.12
	Histidine metabolism	16	5	2.87E-08	1.18E-06	0.31
	Glycine, serine and threonine metabolism	33	6	6.66E-08	2.60E-06	0.29
	Glycerophospholipid metabolism	36	7	6.27E-07	2.38E-05	0.29
	Cysteine and methionine metabolism	33	7	1.55E-06	5.73E-05	0.50
	Arginine and proline metabolism	38	11	1.60E-06	5.74E-05	0.29
	Aminoacyl-tRNA biosynthesis	48	13	1.35E-05	4.46E-04	0.17
	Nicotinate and nicotinamide metabolism	15	2	2.87E-05	8.89E-04	0.23
	Citrate cycle (TCA cycle)	20	3	4.92E-05	1.48E-03	0.19
	Glutathione metabolism	28	5	9.23E-05	2.68E-03	0.36
	Pyrimidine metabolism	39	6	1.29E-04	3.35E-03	0.16

Table A.4 (continued)

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
	Sphingolipid metabolism	21	4	1.29E-04	3.35E-03	0.42
	Tyrosine metabolism	42	2	1.85E-04	4.26E-03	0.14
	D-Glutamine and D-glutamate metabolism	6	4	5.02E-04	1.10E-02	0.50

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted *p* is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

Table A.5. Significantly altered metabolic pathways between untreated and treated GIN28 (adjusted *p* value < 0.05 and impact factor > 0.1).

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Afuresertib	Cysteine and methionine metabolism	33	7	2.18E-09	1.13E-07	0.43
	Glycine, serine and threonine metabolism	33	9	2.84E-08	1.45E-06	0.32
	Alanine, aspartate and glutamate metabolism	28	12	4.43E-08	2.21E-06	0.76
	Arginine and proline metabolism	38	8	6.17E-08	3.02E-06	0.23
	Histidine metabolism	16	6	2.48E-07	1.19E-05	0.41
	Lysine degradation	25	7	2.55E-07	1.20E-05	0.21
	Arginine biosynthesis	14	7	8.92E-07	4.01E-05	0.35
	D-Glutamine and D-glutamate metabolism	6	3	1.10E-06	4.82E-05	0.50
	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	1.33E-06	5.71E-05	1.00
	Phenylalanine metabolism	10	2	1.33E-06	5.71E-05	0.36
	Aminoacyl-tRNA biosynthesis	48	15	1.43E-06	5.85E-05	0.17
	Glycerophospholipid metabolism	36	9	2.73E-06	1.09E-04	0.44
	Glutathione metabolism	28	5	3.06E-06	1.19E-04	0.36
	Tryptophan metabolism	41	4	4.36E-06	1.61E-04	0.37
	Tyrosine metabolism	42	5	7.99E-06	2.72E-04	0.26
	Inositol phosphate metabolism	30	1	8.55E-06	2.82E-04	0.13
	Amino sugar and nucleotide sugar metabolism	37	5	1.28E-05	3.83E-04	0.10
	Citrate cycle (TCA cycle)	20	3	1.82E-05	5.14E-04	0.18
	Sphingolipid metabolism	21	6	6.00E-05	1.50E-03	0.49
	Nicotinate and nicotinamide metabolism	15	3	6.69E-05	1.60E-03	0.43
	Glyoxylate and dicarboxylate metabolism	32	5	7.76E-05	1.78E-03	0.15
	Ascorbate and aldarate metabolism	8	2	1.82E-04	3.81E-03	0.50
	Pyrimidine metabolism	39	9	3.24E-04	5.83E-03	0.29
	Glycerolipid metabolism	16	2	3.32E-04	5.83E-03	0.14
	Vitamin B6 metabolism	9	2	3.48E-04	5.83E-03	0.57
	Taurine and hypotaurine metabolism	8	5	1.19E-03	1.43E-02	1.00
	Pentose and glucuronate interconversions	18	2	2.33E-03	2.57E-02	0.30
Taxifolin	Histidine metabolism	16	6	3.02E-07	1.60E-05	0.41

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Taxifolin	Nicotinate and nicotinamide metabolism	15	3	1.00E-05	5.10E-04	0.43
	Arginine and proline metabolism	38	8	1.03E-05	5.16E-04	0.23
	Glycine, serine and threonine metabolism	33	9	1.27E-05	6.20E-04	0.32
	Aminoacyl-tRNA biosynthesis	48	15	8.26E-05	3.97E-03	0.17
	Taurine and hypotaurine metabolism	8	5	1.88E-04	8.83E-03	1.00
	Glutathione metabolism	28	5	7.76E-04	3.34E-02	0.36
	Glycerophospholipid metabolism	36	9	7.84E-04	3.34E-02	0.44
	Cysteine and methionine metabolism	33	7	8.80E-04	3.61E-02	0.43
	D-Glutamine and D-glutamate metabolism	6	3	8.81E-04	3.61E-02	0.50
Caledonine	Alanine, aspartate and glutamate metabolism	28	12	1.05E-03	4.08E-02	0.76
	Glycerophospholipid metabolism	36	7	2.42E-07	1.06E-05	0.25
	Sphingolipid metabolism	21	4	8.44E-07	3.63E-05	0.42
	Alanine, aspartate and glutamate metabolism	28	10	1.20E-06	5.03E-05	0.56
	Tyrosine metabolism	42	3	5.12E-06	2.05E-04	0.15
	Aminoacyl-tRNA biosynthesis	48	13	5.53E-06	2.16E-04	0.17
	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	6.41E-06	2.44E-04	1.00
	Phenylalanine metabolism	10	2	6.41E-06	2.44E-04	0.36
	Arginine and proline metabolism	38	9	1.04E-05	3.73E-04	0.22
	Taurine and hypotaurine metabolism	8	4	6.52E-05	2.22E-03	1.00
	Amino sugar and nucleotide sugar metabolism	37	7	1.39E-04	4.32E-03	0.19
	Pentose and glucuronate interconversions	18	3	2.59E-04	7.49E-03	0.38
	Glycine, serine and threonine metabolism	33	8	3.23E-04	8.73E-03	0.56
	beta-Alanine metabolism	21	3	8.14E-04	2.03E-02	0.40
	Glycerolipid metabolism	16	2	1.63E-03	3.92E-02	0.14
	Cysteine and methionine metabolism	33	7	1.69E-03	3.92E-02	0.44

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted *p* is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

Table A.6. Significantly altered metabolic pathways between untreated and treated GCE28 (adjusted *p* value < 0.05 and impact factor > 0.1).

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Afuresertib	Taurine and hypotaurine metabolism	8	5	8.00E-13	4.24E-11	1.00
	Cysteine and methionine metabolism	33	7	1.03E-10	5.37E-09	0.43
	Sphingolipid metabolism	21	6	2.73E-10	1.39E-08	0.49
	Glutathione metabolism	28	5	5.63E-09	2.81E-07	0.36
	Alanine, aspartate and glutamate metabolism	28	12	1.98E-08	9.49E-07	0.76
	Glycine, serine and threonine metabolism	33	9	2.87E-08	1.35E-06	0.32
	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	3.21E-08	1.45E-06	1.00
	Phenylalanine metabolism	10	2	3.21E-08	1.45E-06	0.36
	Arginine biosynthesis	14	7	5.48E-08	2.36E-06	0.35
	Aminoacyl-tRNA biosynthesis	48	15	3.37E-07	1.42E-05	0.17
	Lysine degradation	25	7	3.88E-07	1.59E-05	0.21
	Histidine metabolism	16	6	2.56E-06	9.74E-05	0.41
	Arginine and proline metabolism	38	8	1.16E-05	4.06E-04	0.23
	Vitamin B6 metabolism	9	2	1.18E-05	4.06E-04	0.57
	Inositol phosphate metabolism	30	1	1.18E-05	4.06E-04	0.13
	Tyrosine metabolism	42	5	1.49E-05	4.48E-04	0.26
	Citrate cycle (TCA cycle)	20	3	3.02E-05	8.77E-04	0.18
	Glycerophospholipid metabolism	36	9	1.43E-04	3.58E-03	0.44
	Glyoxylate and dicarboxylate metabolism	32	5	4.03E-04	8.86E-03	0.15
	Glycerolipid metabolism	16	2	7.14E-04	1.50E-02	0.14
Taxifolin	Alanine, aspartate and glutamate metabolism	28	12	1.06E-06	5.49E-05	0.76
	Sphingolipid metabolism	21	6	1.32E-06	6.73E-05	0.49
	Taurine and hypotaurine metabolism	8	5	2.33E-06	1.16E-04	1.00
	Glutathione metabolism	28	5	3.90E-06	1.91E-04	0.36
	Glycerophospholipid metabolism	36	9	2.98E-05	1.40E-03	0.44
	Nicotinate and nicotinamide metabolism	15	3	1.26E-04	5.80E-03	0.43
Caledonine	Histidine metabolism	16	6	5.54E-04	2.44E-02	0.41
	Taurine and hypotaurine metabolism	8	3	1.14E-11	5.26E-10	0.71

Table A.6 (continued)

Treatment	Pathway name	Total compounds	Hits	Raw <i>p</i>	Adjusted <i>p</i>	Impact factor
Caledonine	Alanine, aspartate and glutamate metabolism	28	11	1.35E-09	5.96E-08	0.78
	Arginine biosynthesis	14	6	1.77E-08	7.63E-07	0.31
	Tryptophan metabolism	41	4	1.98E-08	8.30E-07	0.12
	Histidine metabolism	16	5	2.87E-08	1.18E-06	0.31
	Glycine, serine and threonine metabolism	33	6	6.66E-08	2.60E-06	0.29
	Glycerophospholipid metabolism	36	7	6.27E-07	2.38E-05	0.29
	Cysteine and methionine metabolism	33	7	1.55E-06	5.73E-05	0.50
	Arginine and proline metabolism	38	11	1.60E-06	5.74E-05	0.29
	Aminoacyl-tRNA biosynthesis	48	13	1.35E-05	4.46E-04	0.17
	Nicotinate and nicotinamide metabolism	15	2	2.87E-05	8.89E-04	0.23
	Citrate cycle (TCA cycle)	20	3	4.92E-05	1.48E-03	0.19
	Glutathione metabolism	28	5	9.23E-05	2.68E-03	0.36
	Pyrimidine metabolism	39	6	1.29E-04	3.35E-03	0.16
	Sphingolipid metabolism	21	4	1.29E-04	3.35E-03	0.42
	Tyrosine metabolism	42	2	1.85E-04	4.26E-03	0.14
	D-Glutamine and D-glutamate metabolism	6	4	5.02E-04	1.10E-02	0.50

"Total compounds" refers to the number of compounds involved in a specific pathway. Adjusted *p* is generated using Benjamini-Hochberg correction. Impact factor evaluates the relative importance of given pathway within the context of the whole metabolic network.

Table A.7. Separation conditions for the comparison of two ionisation sources.

Column	Nano-capillary ZIC [®] -HILIC column (5 µm, 0.075 × 150 mm)
Analyte	6 µM glutamine
Column temperature	50 °C
Injection volume	0.5 µL
Mobile phases	25 mM ammonium acetate (A) / acetonitrile (B)

Gradient table		
Time (min)	Flow rate (µL/min)	B%
0	1	95
20	1	40
25	1	5
26	1	95
30	1	95

UHPLC system was operated in capillary/nano-flow mode (flow rate < 5 µL/min) and EASY-Spray[™] micro-flow emitter was applied.

Table A.8. Detection parameters for the comparison of two ionisation sources.

MS parameters	TriVersa NanoMate[®]	EASY-Spray[™]
Resolution	70000 at m/z 200	
Scan range	m/z 70-1050	
Scan mode	Full MS-SIM	
Capillary temperature	275 °C	
Sheath gas	0	
Aux gas	0	
Sweep gas	0	
Spray voltage (+) kV	1.7	1.7
Spray voltage (-) kV	–	1.5

Table A.9. Separation conditions for the comparison of the C₁₈ and HILIC column.

Mobile phases	(A) 0.1% formic acid in water (B) 0.08% formic acid in acetonitrile				
Analytes	StdA solution				
Injection volume	1 µL				
Column temperature	45 °C				
Emitter	EASY-Spray™ nano-flow emitter (I.D. 10 µm)				
Strong wash	90% acetonitrile				
Weak wash	90% acetonitrile				

Gradient table					
Time (min)	C ₁₈		Time (min)	HILIC	
	Flow rate (µL/min)	B%		Flow rate (µL/min)	B%
0	0.3	5	0	0.3	80
30	0.3	95	30	0.3	20
40	0.3	95	31	0.3	5
45	0.3	5	46	0.3	5
60	0.3	5	47	0.3	80
–	–	–	62	0.3	80

Table A.10. Detection parameters for the comparison of the C₁₈ and HILIC column using EASY-Spray™ ionisation source.

MS parameters	C ₁₈	HILIC
Resolution	70000 at m/z 200	
Scan range	m/z 70-1050	
Scan mode	Full MS-SIM	
Capillary temperature	275 °C	
Sheath gas	0	
Aux gas	0	
Sweep gas	0	
Spray voltage (+) kV	1.7	
Spray voltage (-) kV	1.5	

Table A.11. Separation conditions for the optimisation of mobile phases at nano-flow rate.

Column	Nano-capillary ZIC [®] -HILIC column (5 μm, 0.075×150 mm)	
Analyte	StdA	
Column temperature	45 °C	
Injection volume	1 μL	
Emitter	EASY-Spray [™] nano-flow emitter (I.D. 10 μm)	
Gradient table		
Time (min)	Flow rate (μL/min)	B%
0	0.3	80
30	0.3	20
31	0.3	5
46	0.3	5
47	0.3	80
62	0.3	80
Re-equilibration		

Table A.12. Detection parameters for the optimisation of mobile phases at nano-flow rate.

MS parameters	EASY-Spray[™]
Resolution	70000 at m/z 200
Scan range	m/z 70-1050
Scan mode	Full MS-SIM
Capillary temperature	275 °C
Sheath gas	0
Aux gas	0
Sweep gas	0
Spray voltage (+) kV	1.7
Spray voltage (-) kV	1.7

Table A.13. Separation conditions for the optimisation of mobile phases at capillary-flow rate.

Column	ZIC®-HILIC capillary HPLC column (5 µm, 0.3×150 mm)	
Analyte	StdA-StdE	
Column temperature	45 °C	
Injection volume	1 µL	
Emitter	EASY-Spray™ micro-flow emitter (I.D. 20 µm)	
Gradient table		
Time (min)	Flow rate (µL/min)	B%
0	1	80
30	1	20
31	1	5
46	1	5
47	1	80
62	1	80
Re-equilibration		

Table A.14. Separation conditions for the optimisation of emitters at capillary-flow rate.

Column	ZIC [®] -HILIC capillary HPLC column (5 μm, 0.3×150 mm)	
Analyte	StdA	
Column temperature	45 °C	
Injection volume	1 μL	
Emitter	EASY-Spray [™] nano-flow emitter (I.D. 10 μm)	
	EASY-Spray [™] micro-flow emitter (I.D. 20 μm)	

Gradient table		
Time (min)	Flow rate (μL/min)	B%
0	1	80
30	1	20
31	1	5
41	1	5
42	1	80
50	1	80
	Re-equilibration	

Table A.15. Significantly altered metabolites between invaded cells and non-invaded cells analysed by capillary-flow LC-MS (adjusted p value < 0.05 and VIP > 1).

m/z	Formula	RT(min)	Δ ppm	Adducts	Putative metabolites	HMDB ID	Confidence	Log ₂ FC	Adj. p -value	VIP
243.0622	C ₉ H ₁₂ N ₂ O ₆	16.12	-0.68	[M-H] ⁻¹	Uridine	HMDB0000296	Level 1	1.93	4.43E-03	1.50
758.5657	C ₄₂ H ₈₀ NO ₈ P	14.97	-4.91	[M-H] ⁻¹	PC(14:0/20:2(11Z,14Z))	HMDB0007880	Level 2	-0.34	1.18E-02	1.54
703.5717	C ₃₉ H ₇₉ N ₂ O ₆ P	15.87	-4.54	[M-H] ⁻¹	Palmitoyl sphingomyelin	HMDB0010169	Level 2	-0.70	1.67E-03	1.73
524.3701	C ₂₆ H ₅₄ NO ₇ P	16.62	-1.86	[M+H] ⁺¹	LysoPC(18:0/0:0)	HMDB0010384	Level 2	-1.55	7.14E-04	1.75
496.3389	C ₂₄ H ₅₀ NO ₇ P	16.82	-1.66	[M+H] ⁺¹	LysoPC(16:0/0:0)	HMDB0010382	Level 2	-0.67	7.46E-03	1.67
171.0065	C ₃ H ₉ O ₆ P	25.11	0.36	[M-H] ⁻¹	Glycerol 3-phosphate	HMDB0000126	Level 1	-2.41	3.48E-03	1.40
112.0503	C ₄ H ₅ N ₃ O	20.80	-2.27	[M+H] ⁺¹	Cytosine	HMDB0000630	Level 1	-1.09	7.12E-03	1.40
244.0922	C ₉ H ₁₃ N ₃ O ₅	20.80	-2.35	[M+H] ⁺¹	Cytidine	HMDB0000089	Level 1	-1.35	2.97E-03	1.44
268.1035	C ₁₀ H ₁₃ N ₅ O ₄	15.69	-2.16	[M+H] ⁺¹	Adenosine	HMDB0000050	Level 1	5.07	1.90E-05	1.55

m/z is accurate mass. RT represents retention time. Δ ppm is mass error. Log₂ FC is short for Log₂ fold change. Adjusted p is generated using Benjamini-Hochberg correction. VIP represents variables importance in projection. Confidence is identification confidence: Level 1: RT, MS1, MS/MS; Level 2: MS1, MS/MS.

Appendix B

Figures

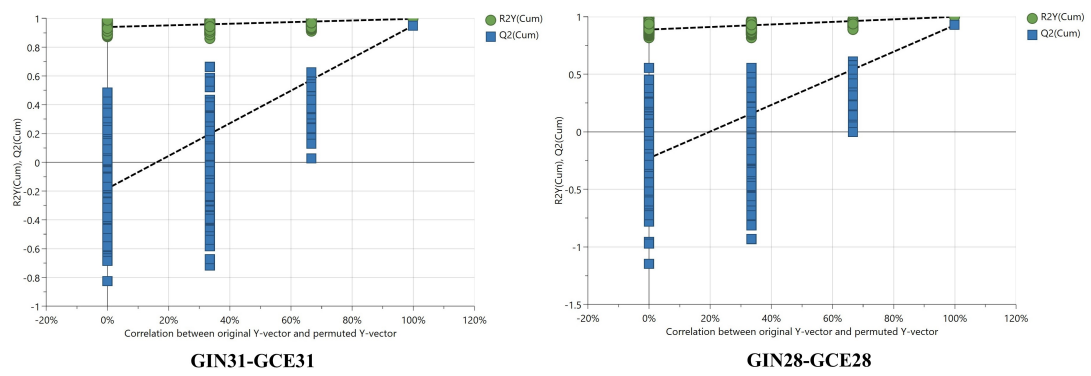


Figure B.1. Permutation tests of GINs vs. GCEs.

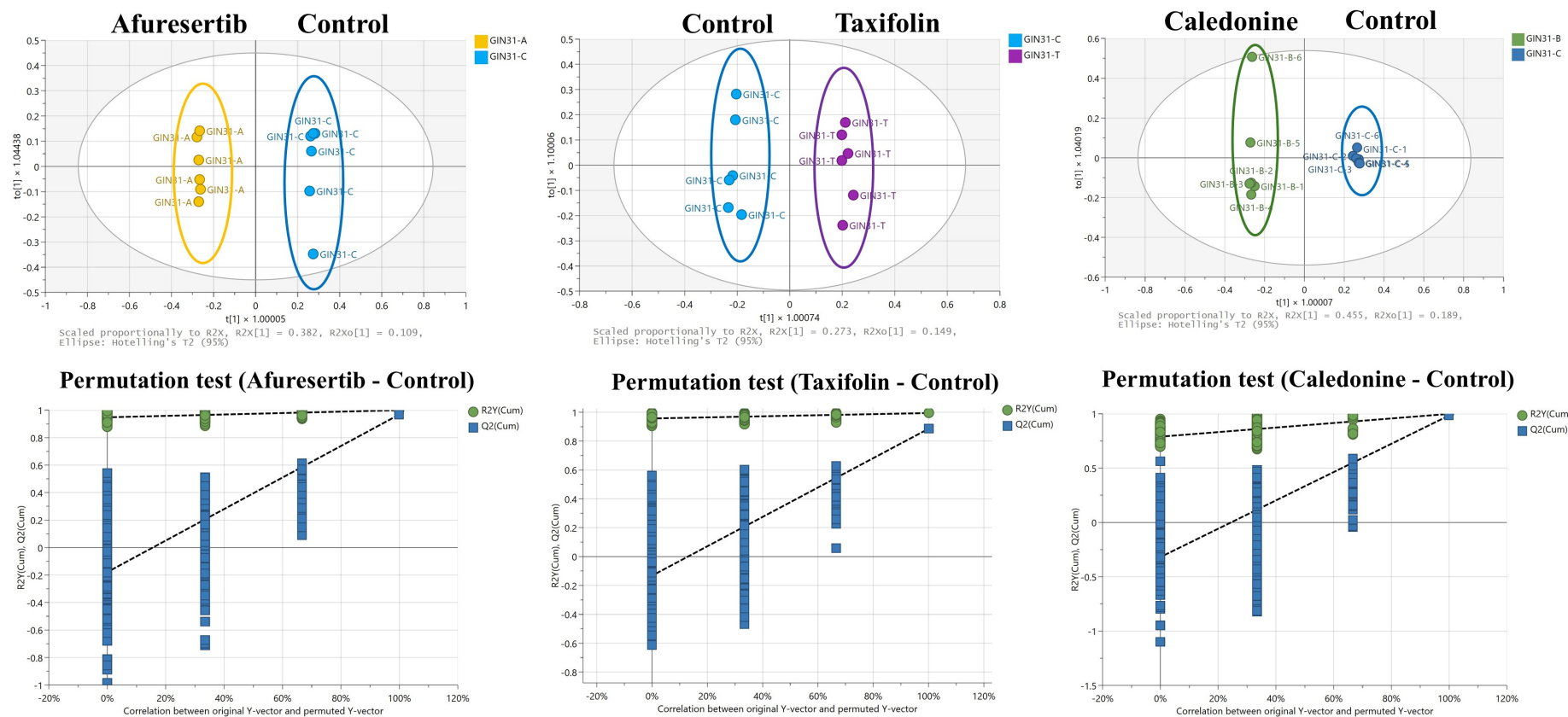


Figure B.2. OPLS-DA scores plots and corresponding permutation tests of treated vs. untreated GIN31.

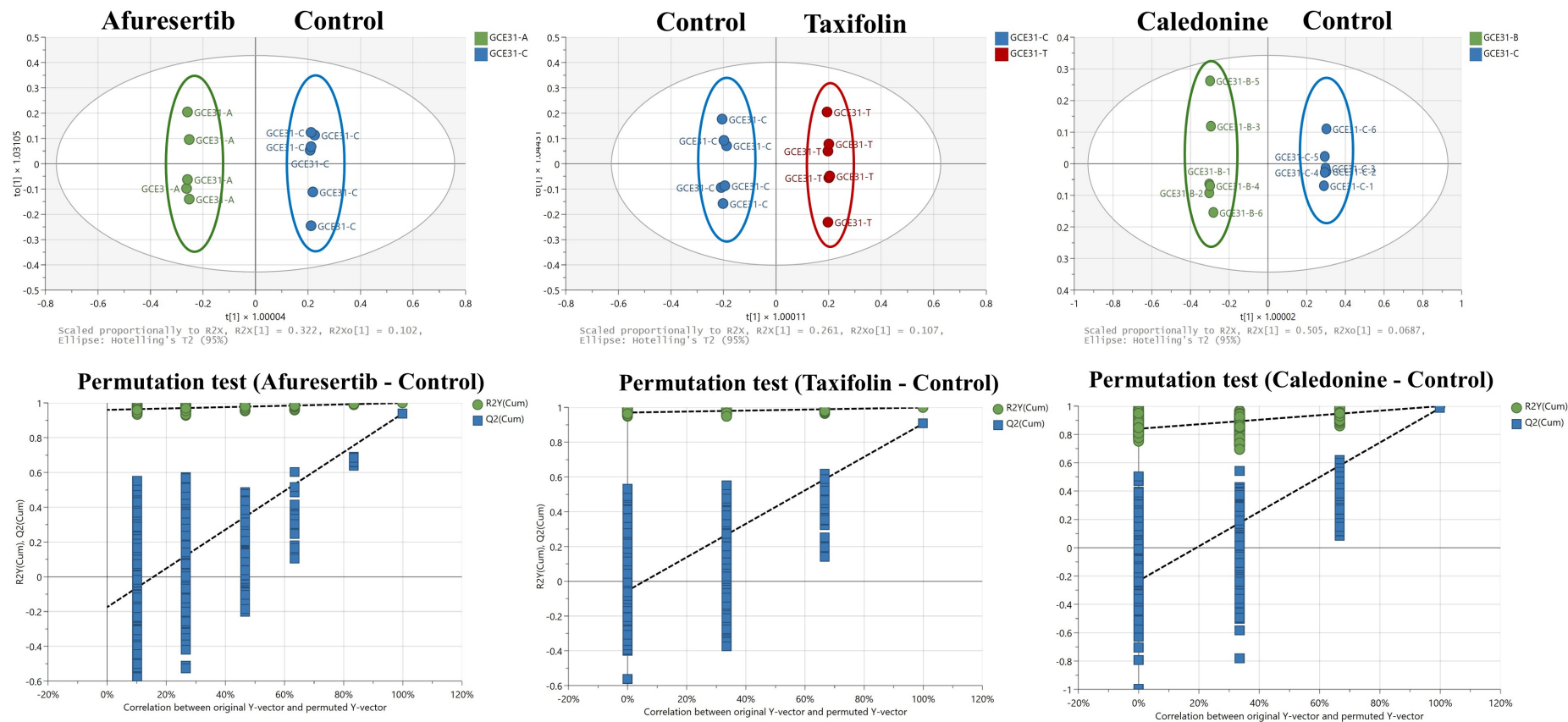


Figure B.3. OPLS-DA scores plots and corresponding permutation tests of treated vs. untreated GCE31.

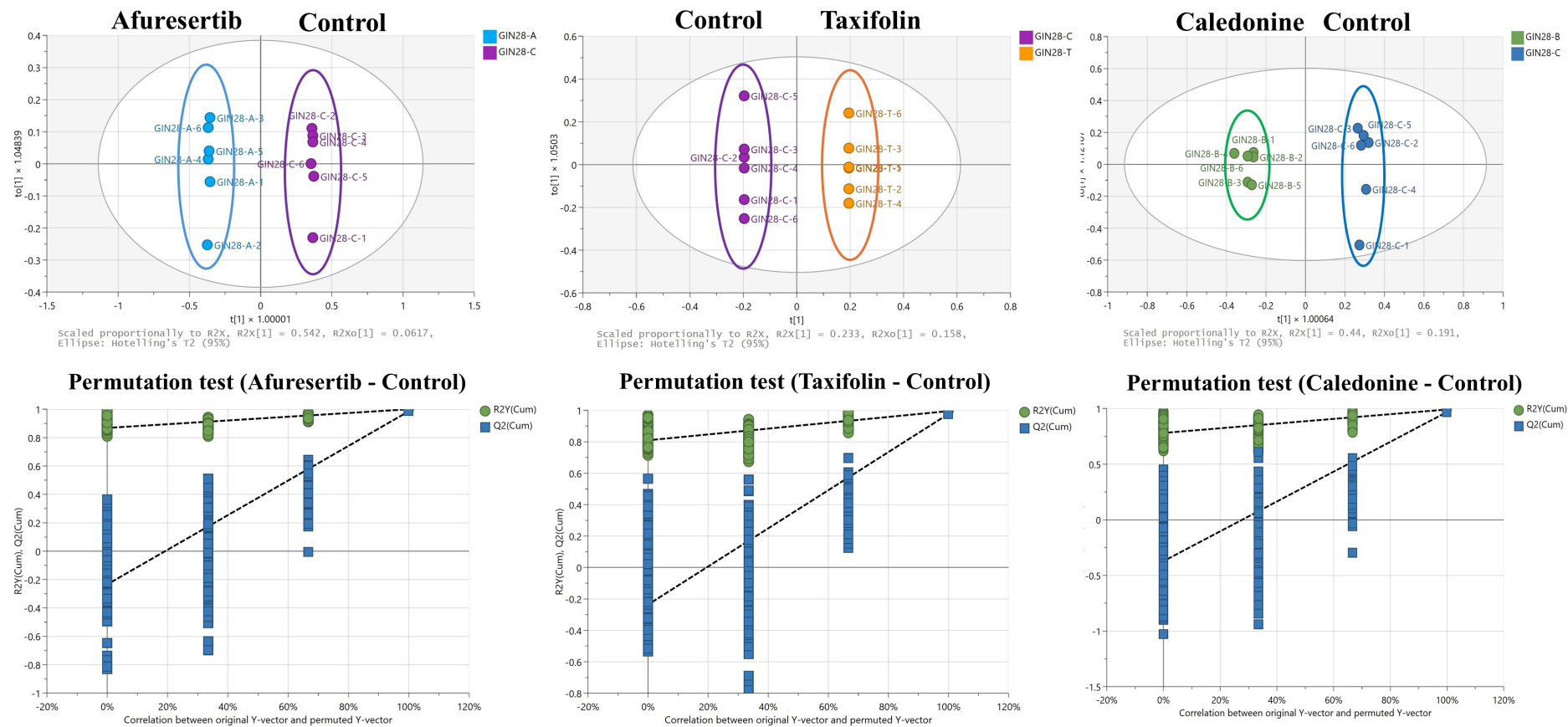


Figure B.4. OPLS-DA scores plots and corresponding permutation tests of treated vs. untreated GIN28.

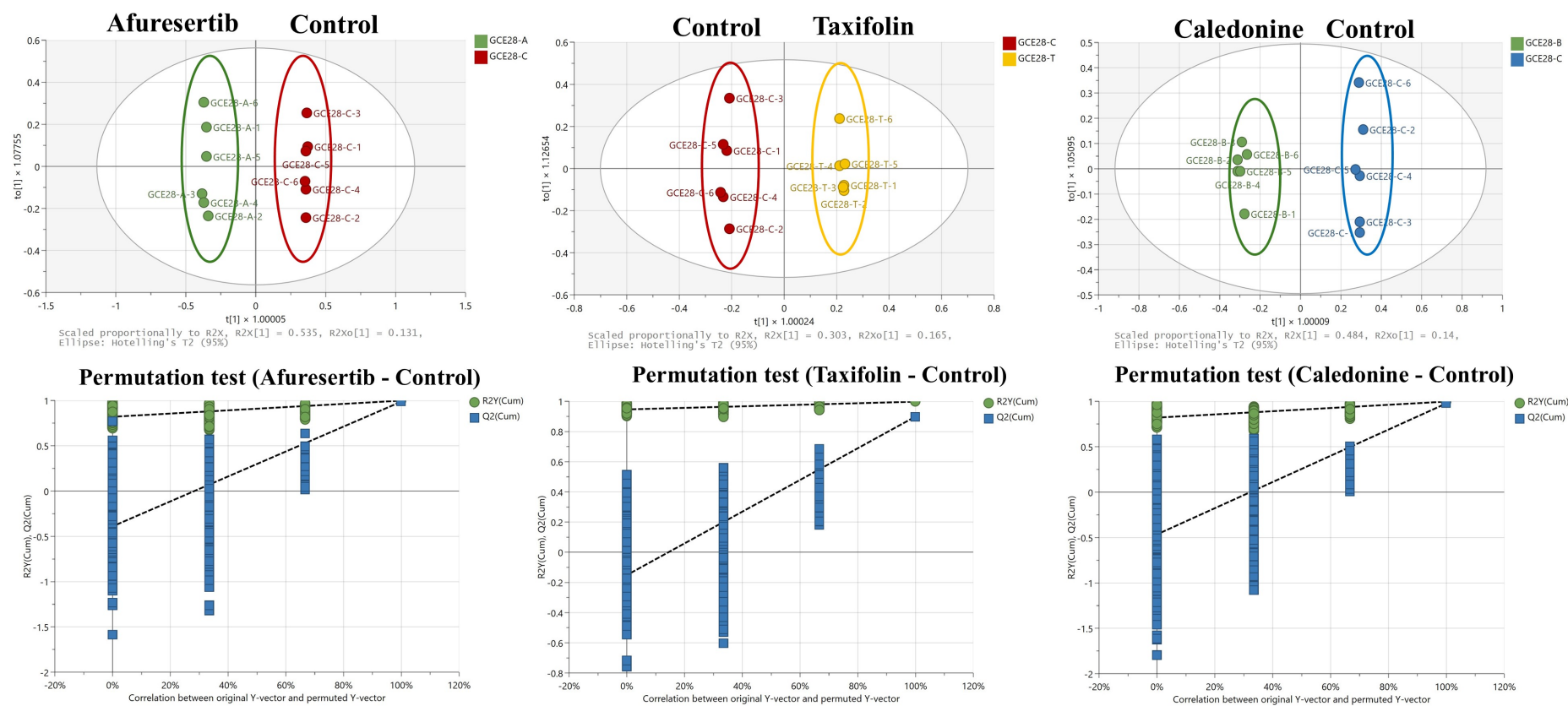


Figure B.5. OPLS-DA scores plots and corresponding permutation tests of treated vs. untreated GCE28.

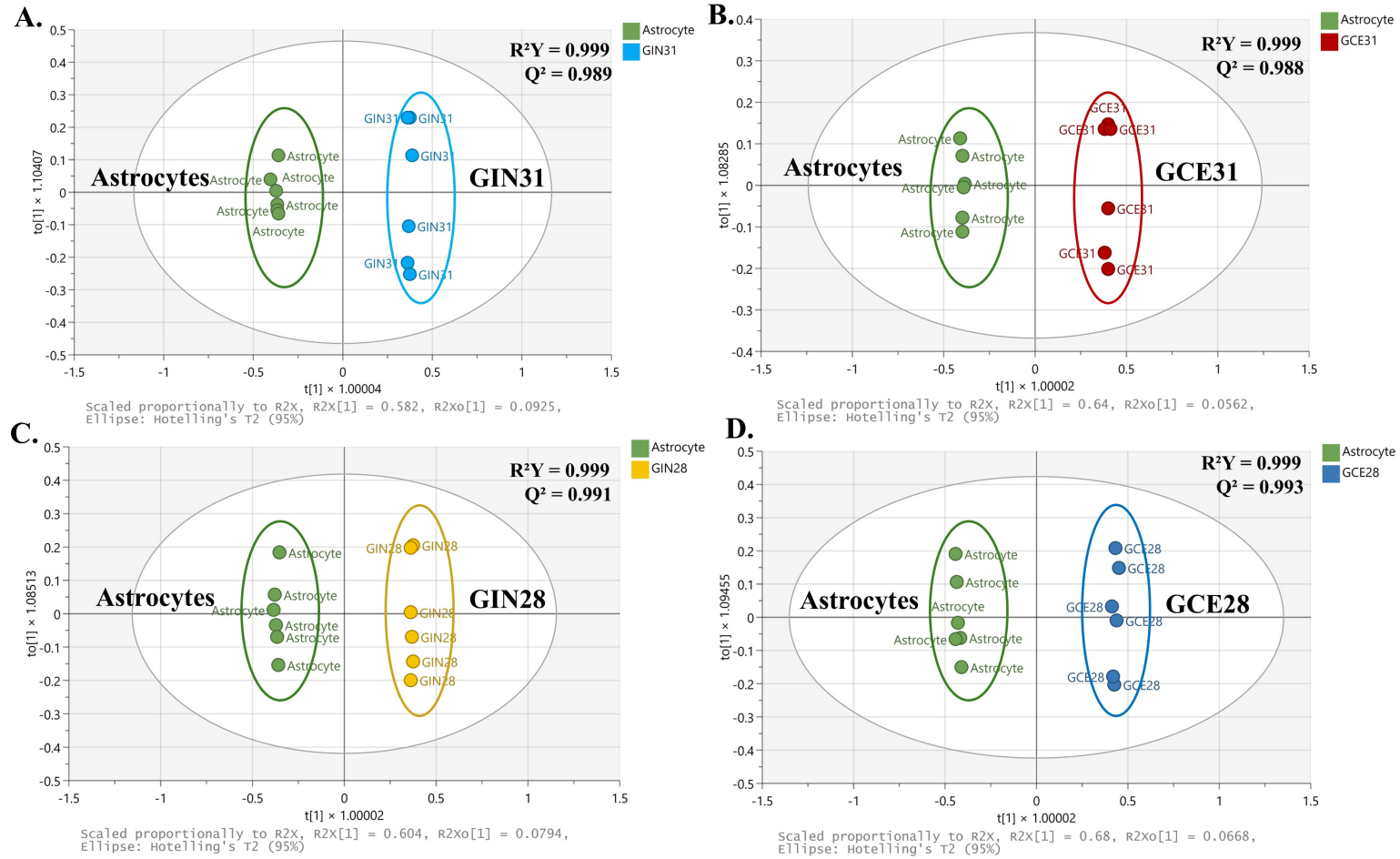


Figure B.6. OPLS-DA scores plots of untreated astrocytes vs. GBM cells.

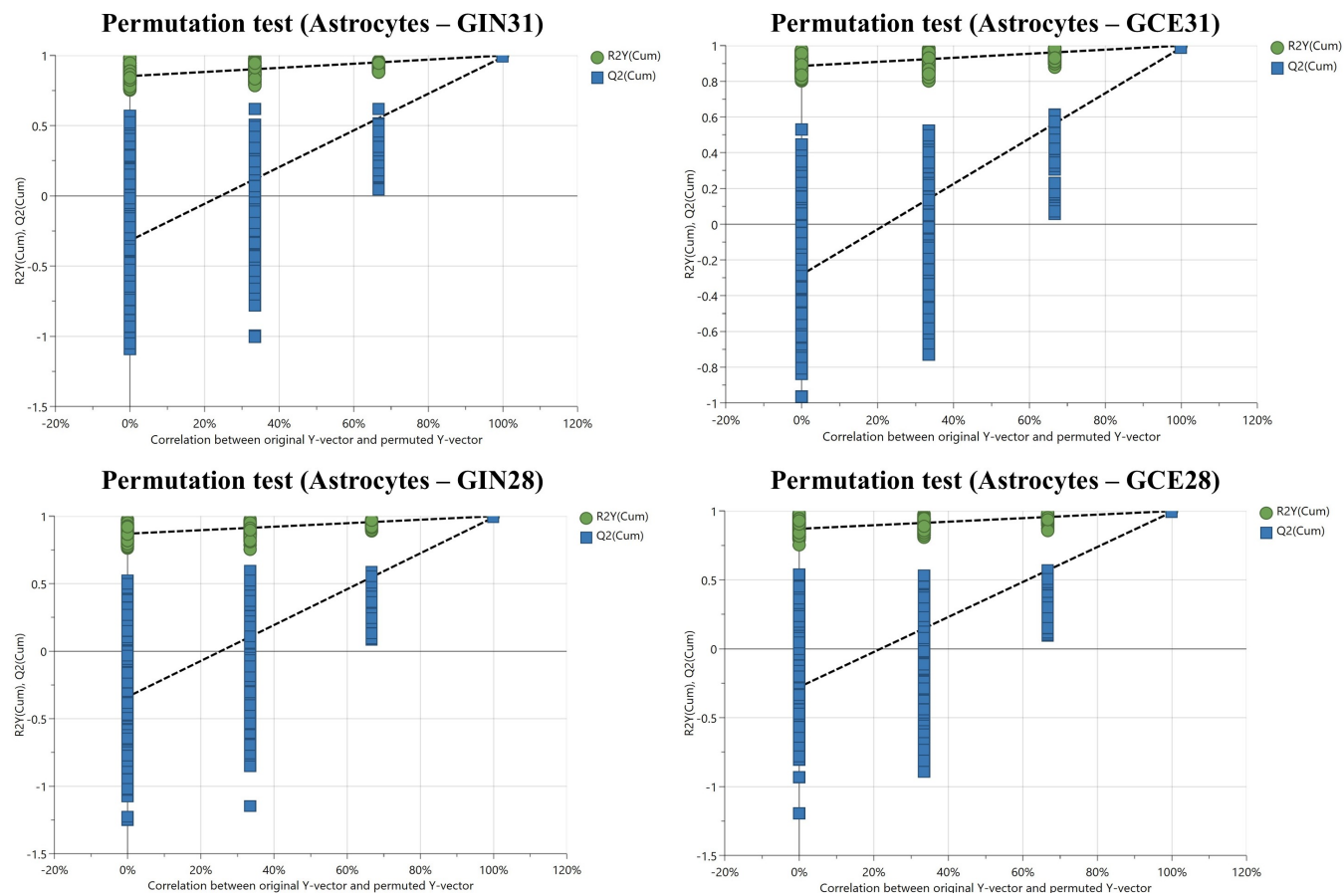


Figure B.7. Permutation tests of untreated astrocytes vs. GBM cells.

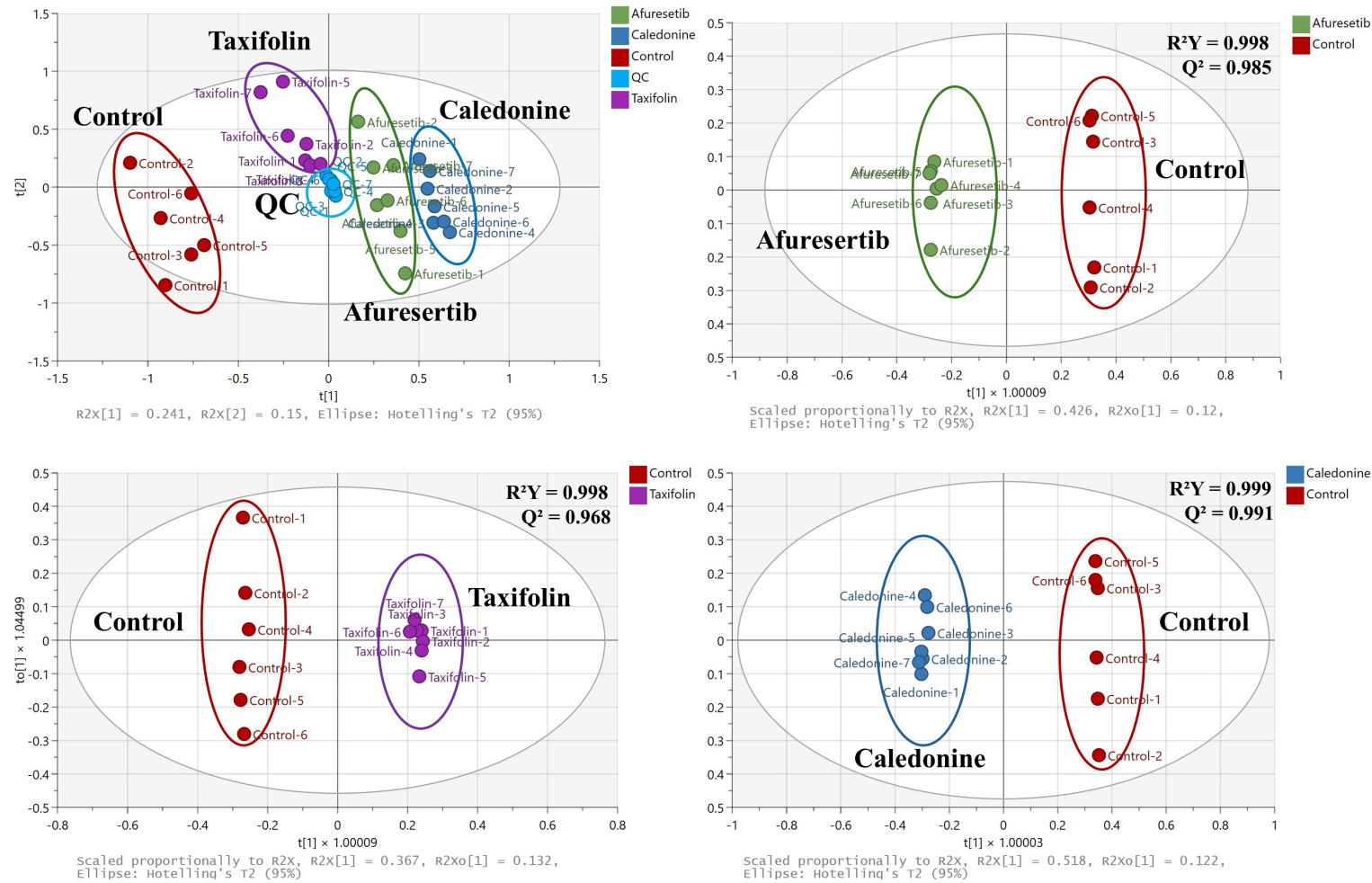


Figure B.8. PCA and OPLS-DA scores plots of untreated and treated astrocytes.

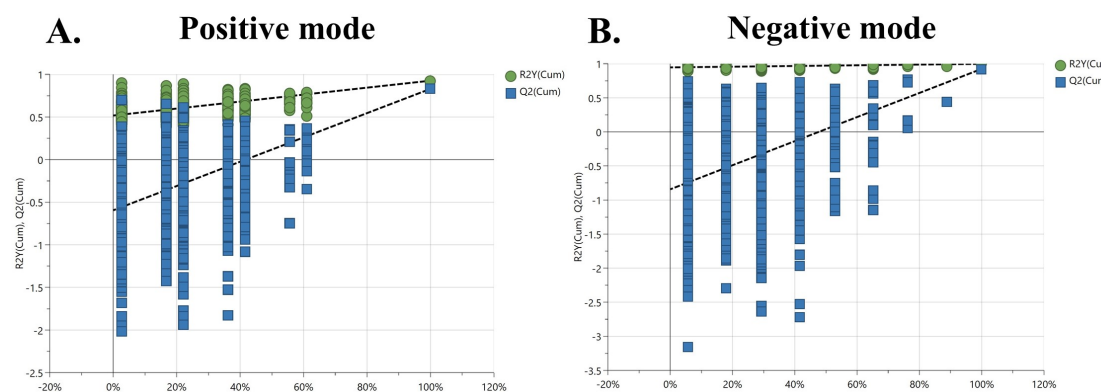


Figure B.9. Permutation tests of invaded vs. non-invaded cells analysed by 3D-OrbiSIMS.

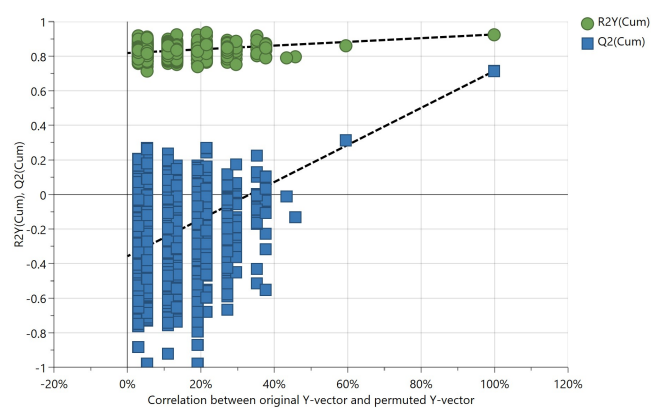


Figure B.10. Permutation test of invaded vs. non-invaded cells analysed by AP-MALDI-MS.

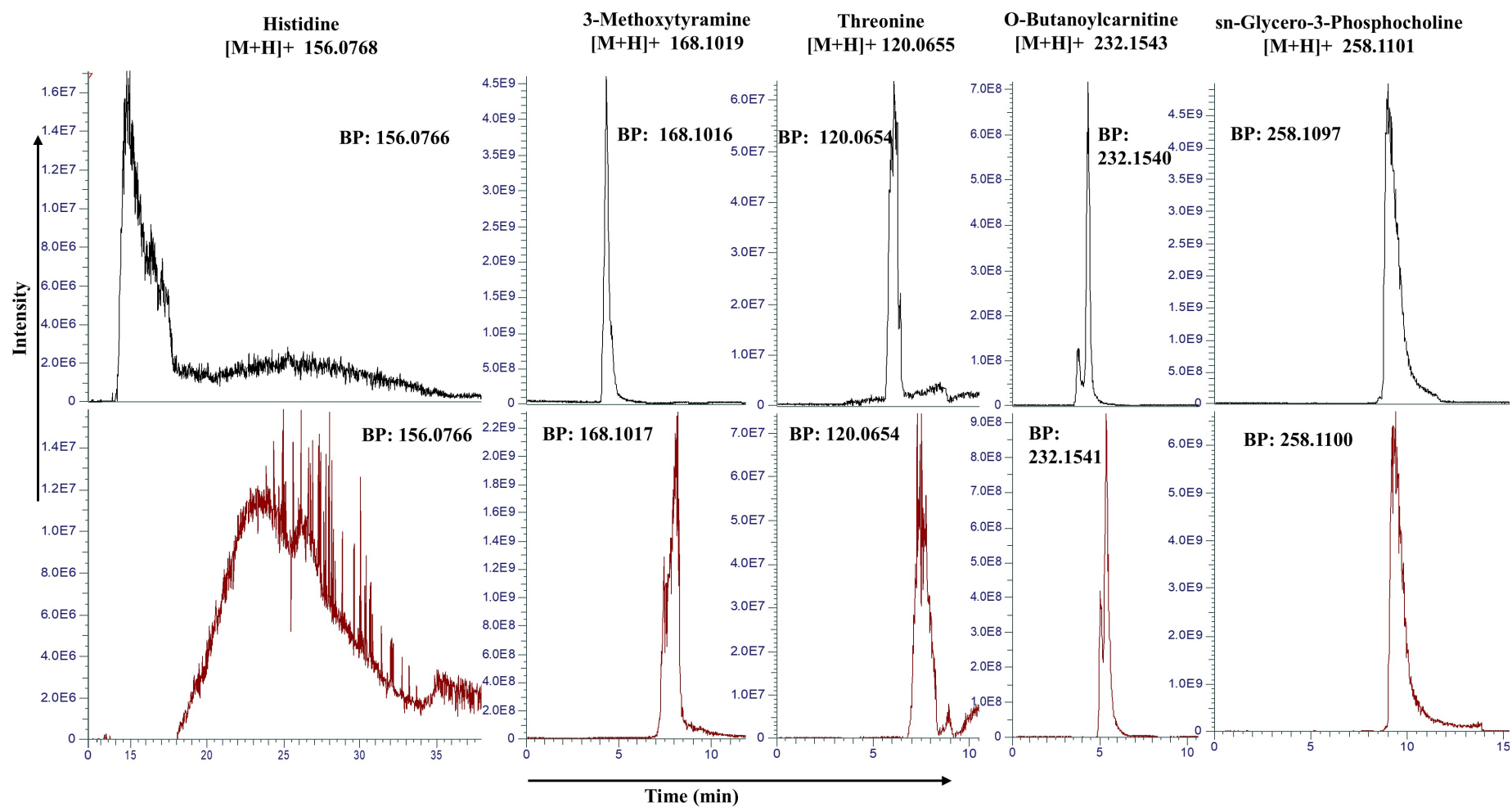


Figure B.11. Effect of formic acid in acetonitrile at nano-flow rate (300 nL/min). The EICs of standards with formic acid are shown in black, while these without formic acid are shown in red. BP (basic peak) refers to the most intense ion observed on the spectrum.

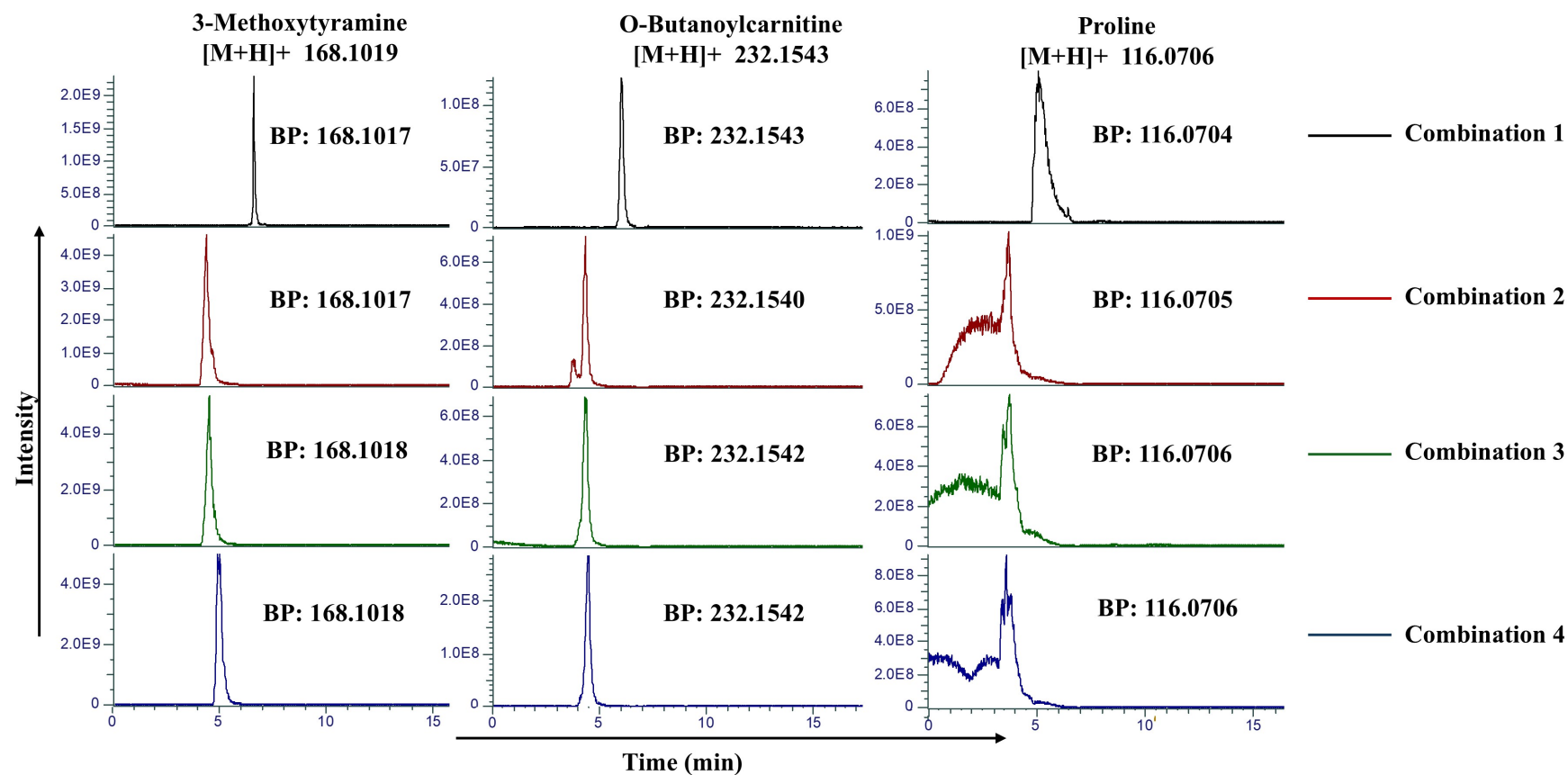


Figure B.12. Comparison of different mobile phases at nano-flow rate (300 nL/min). The EICs of 3-methoxytyramine, O-butanoylcarnitine and proline were separated by different mobile phases. BP (basic peak) refers to the most intense ion observed on the spectrum.

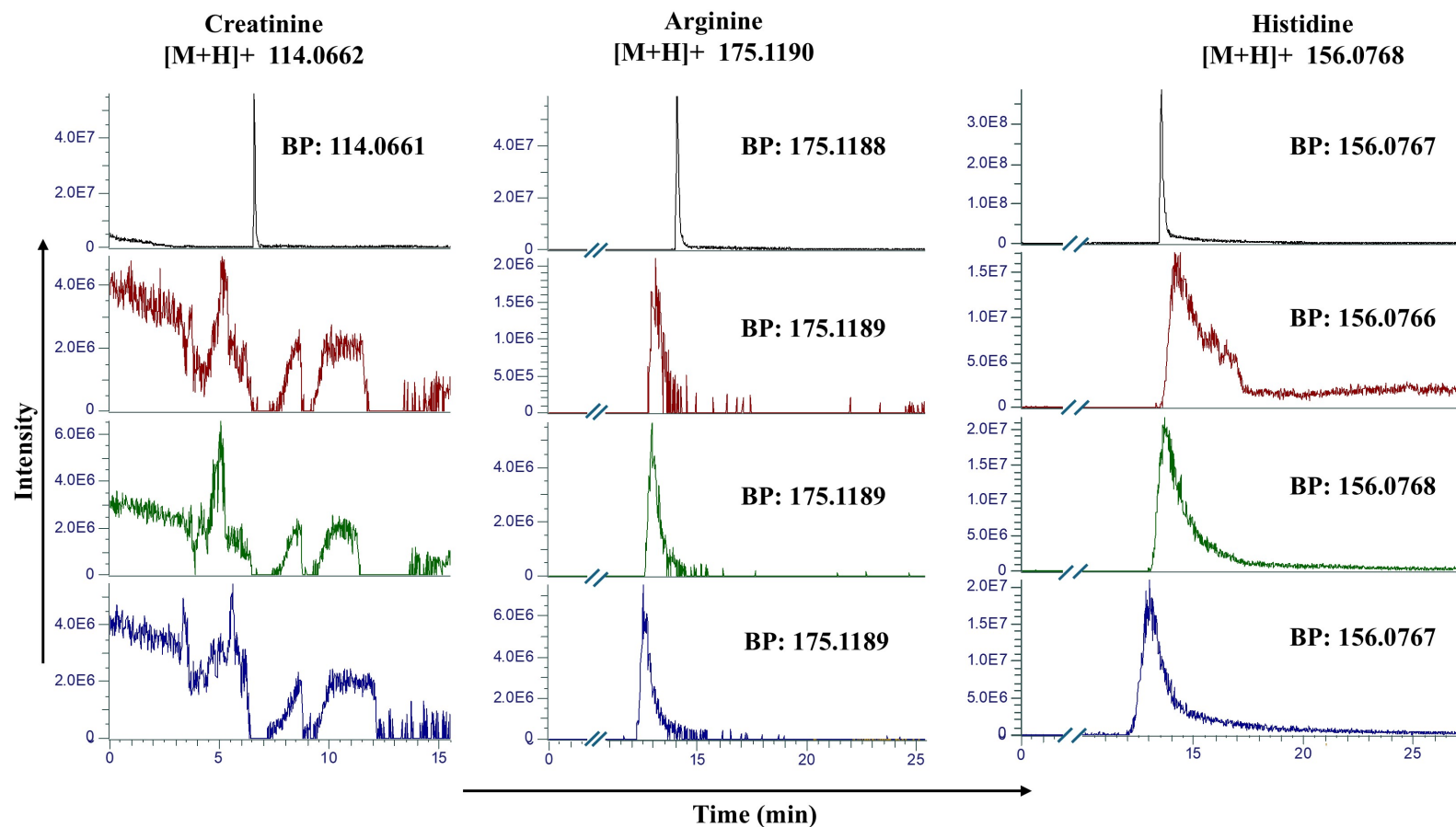


Figure B.13. Comparison of different mobile phases at nano-flow rate (300 nL/min) (continued). The EICs of creatinine, arginine and histidine were separated by different mobile phases. BP (basic peak) refers to the most intense ion observed on the spectrum.

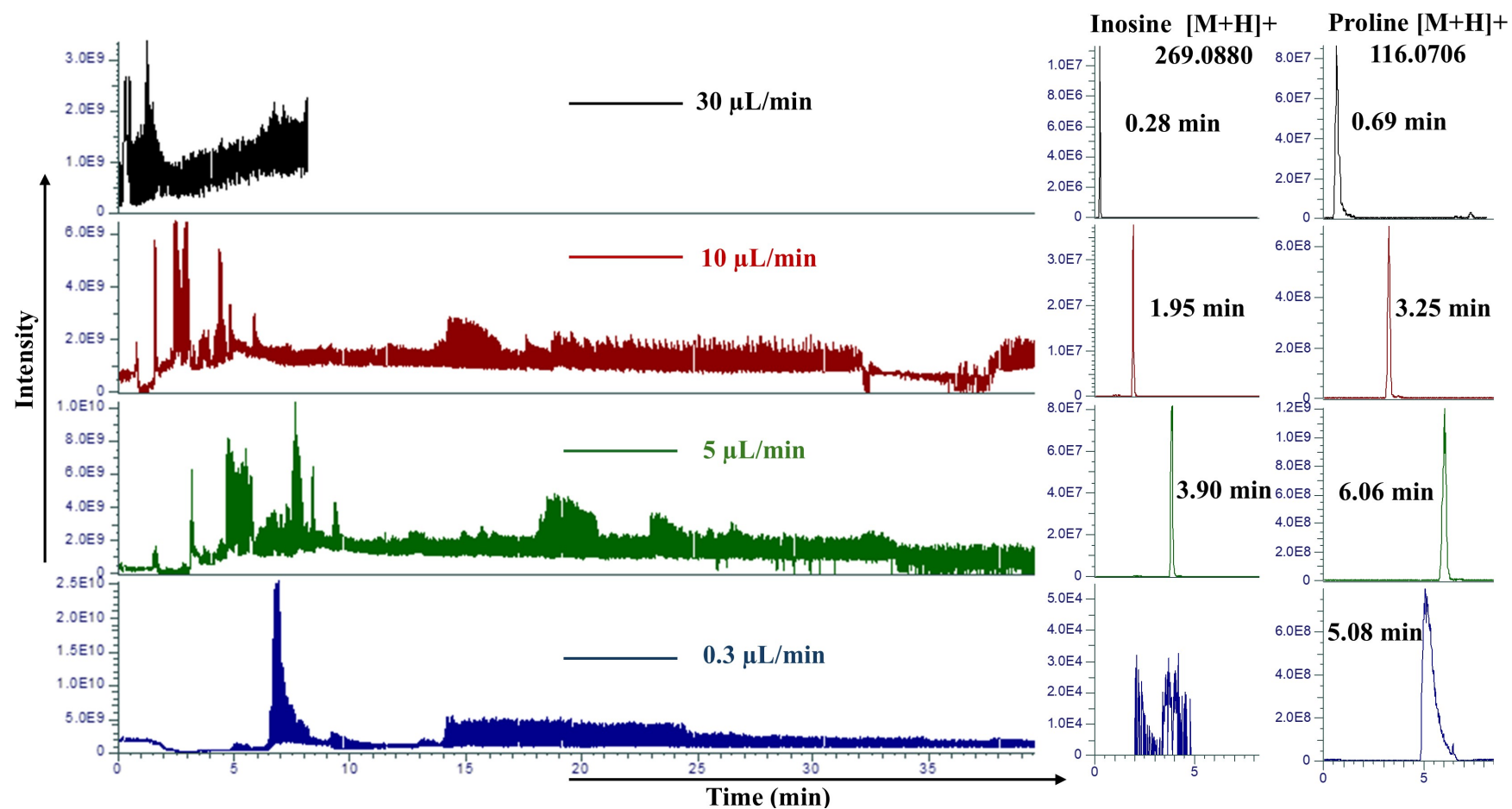


Figure B.14. Separation performance at nano-flow and micro-flow rate. The TICs of StdA were separated at nano-flow rate (300 nL/min) and micro-flow rate (5 $\mu\text{L}/\text{min}$, 10 $\mu\text{L}/\text{min}$ and 30 $\mu\text{L}/\text{min}$).

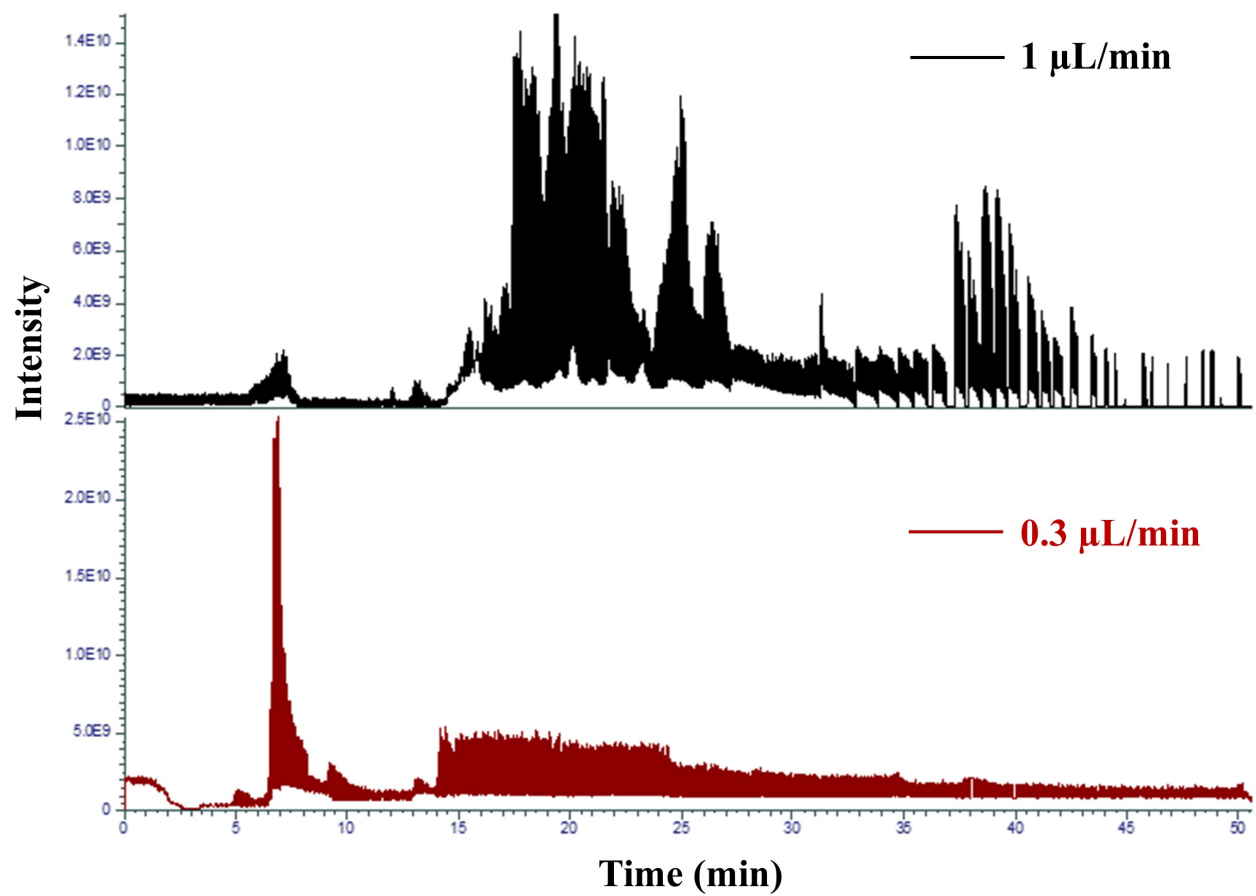


Figure B.15. Separation performance at nano-flow and capillary-flow rate (StdA). The TICs of StdA were separated at nano-flow rate (300 nL/min) and capillary-flow rate (1 µL/min).

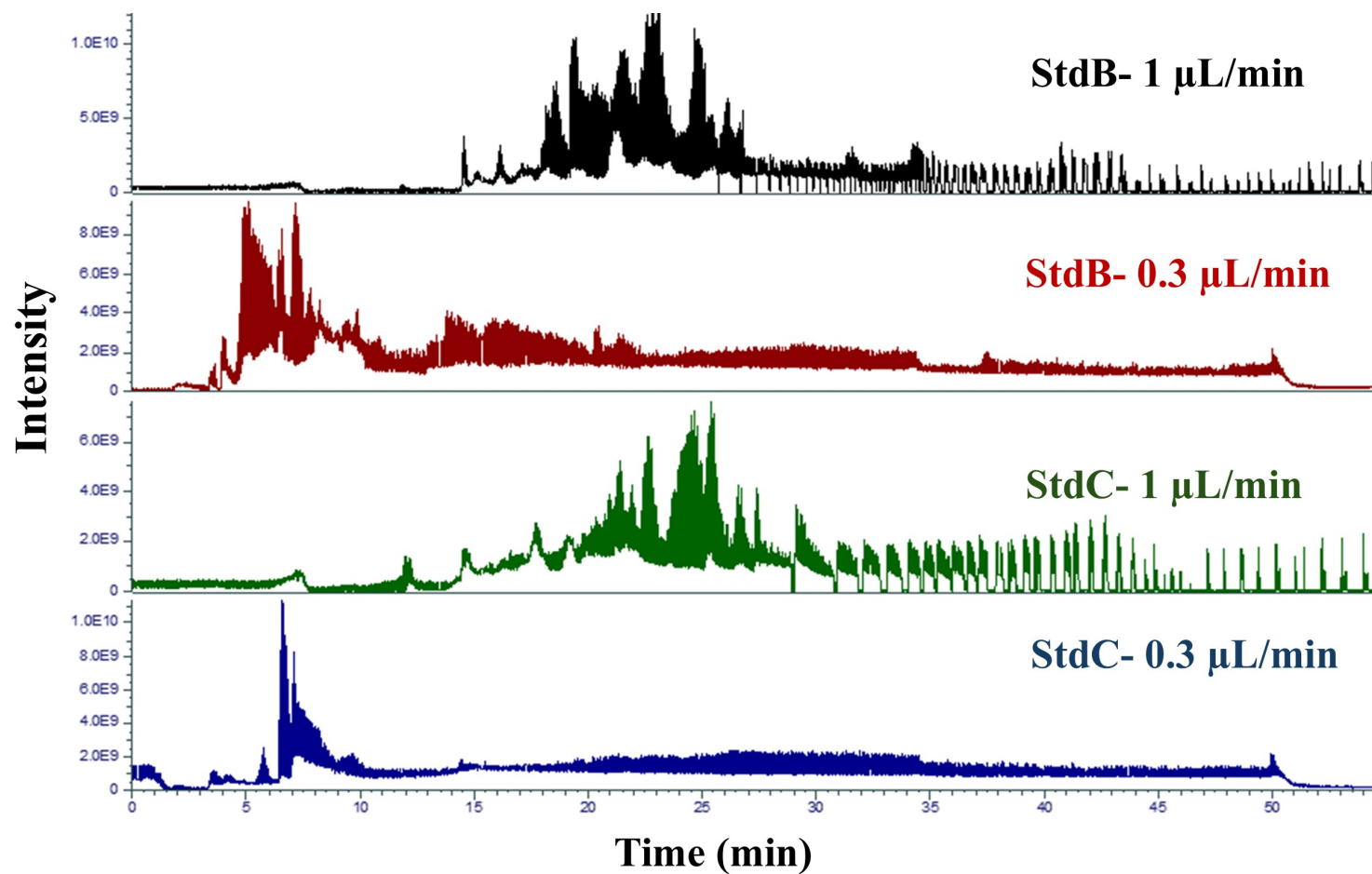


Figure B.16. Separation performance at nano-flow and capillary-flow rate (StdB and StdC). The TICs of StdB and StdC were separated at 1 $\mu\text{L}/\text{min}$ and 300 nL/min.

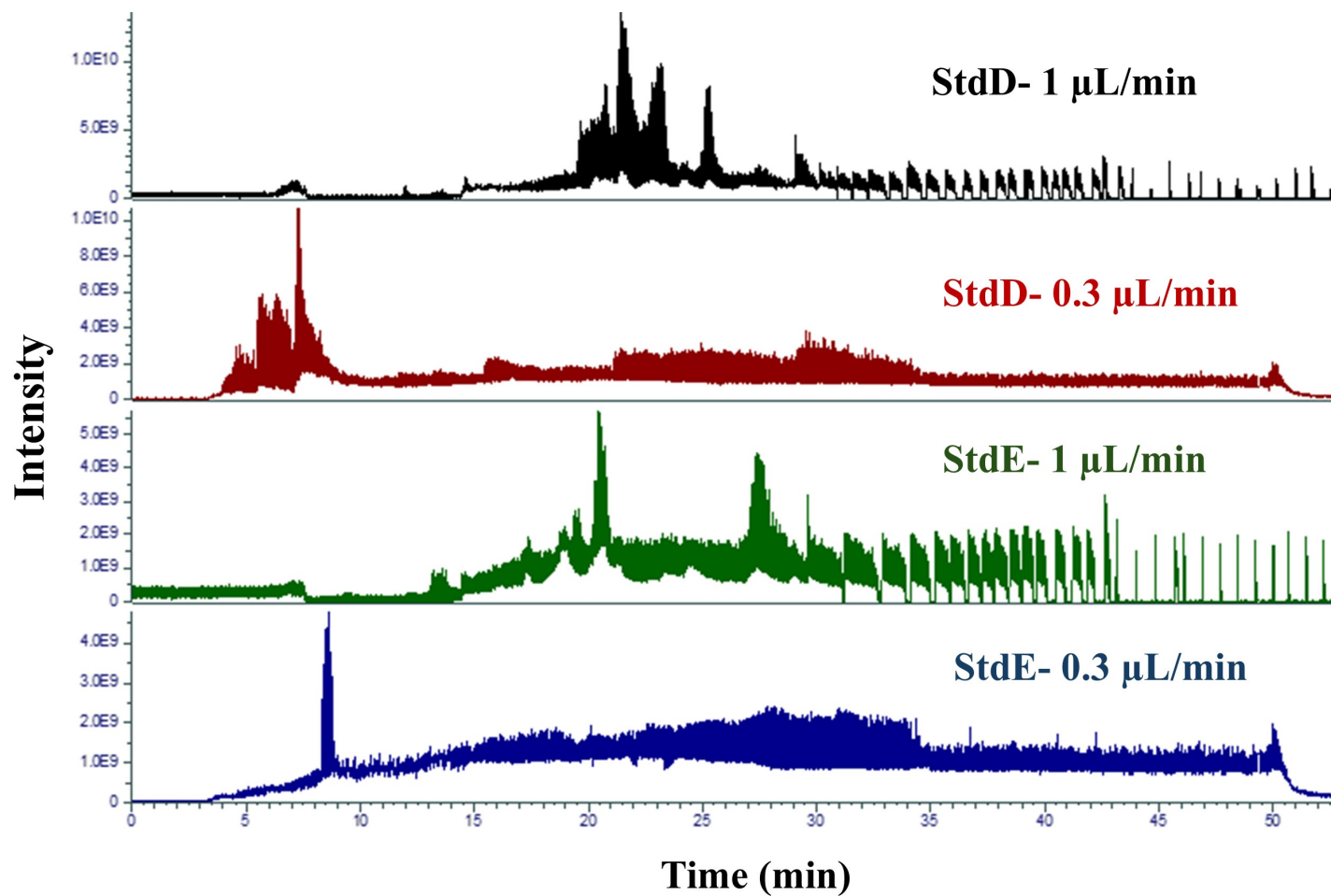


Figure B.17. Separation performance at nano-flow and capillary-flow rate (StdD and StdE). The TICs of StdD and StdE were separated at 1 $\mu\text{L}/\text{min}$ and 300 nL/min.

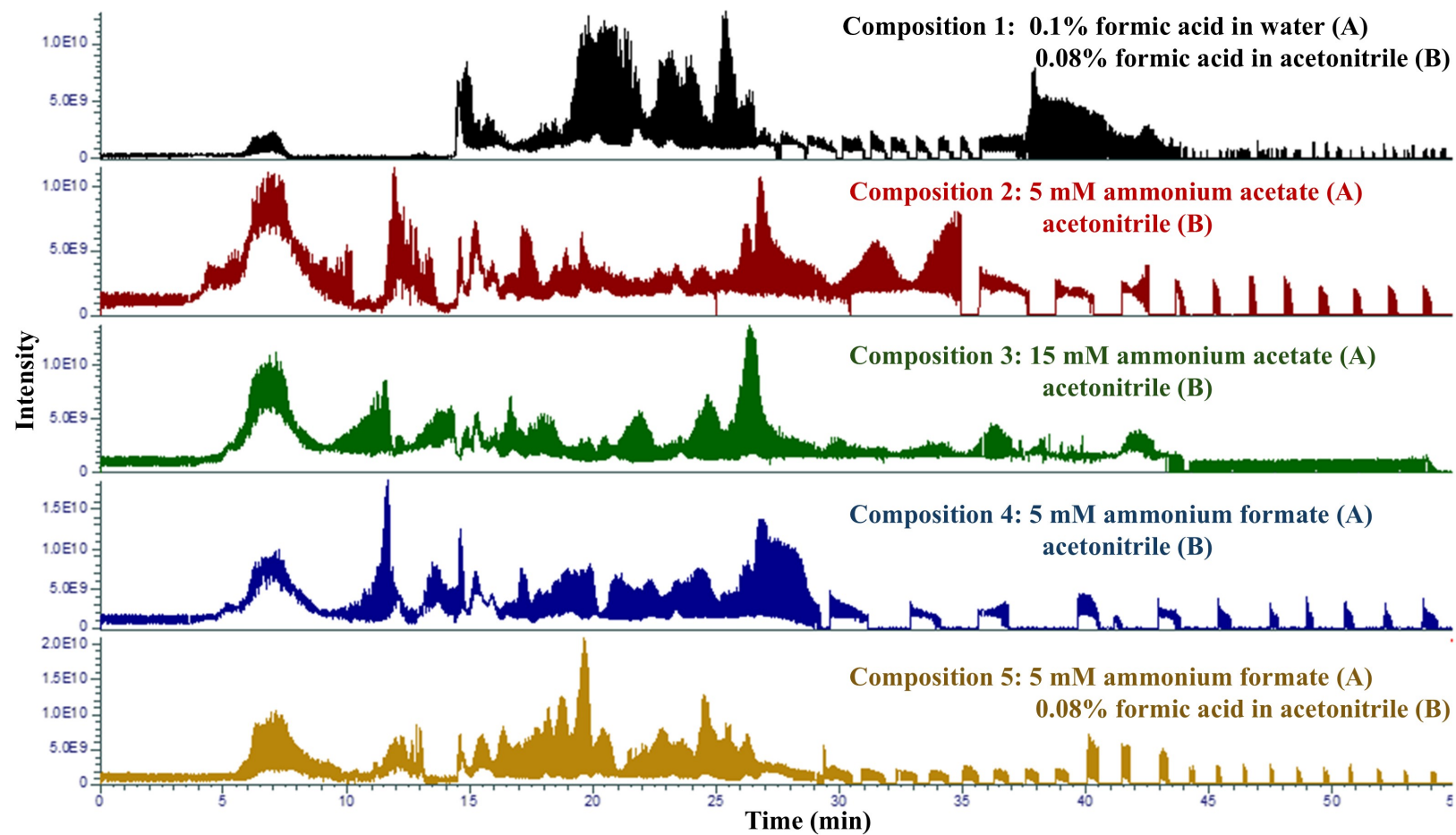


Figure B.18. Comparisons of different mobile phases at capillary flow rate (1 $\mu\text{L}/\text{min}$). The TICs of StdA were separated by the mobile phases with different additives.

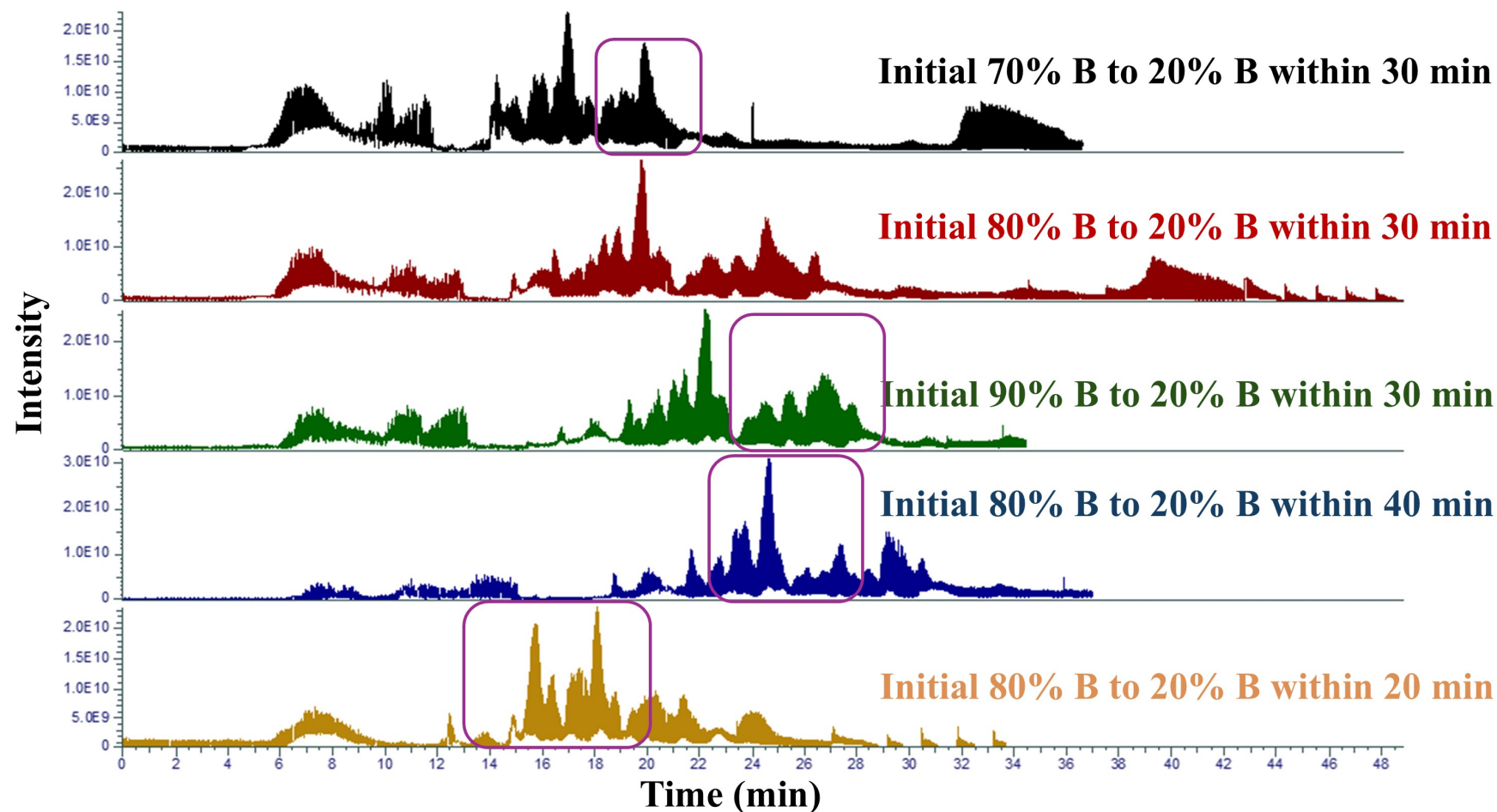


Figure B.19. Optimisation of gradient profile at capillary flow rate (1 $\mu\text{L}/\text{min}$). The TICs of StdA were separated by different gradient.

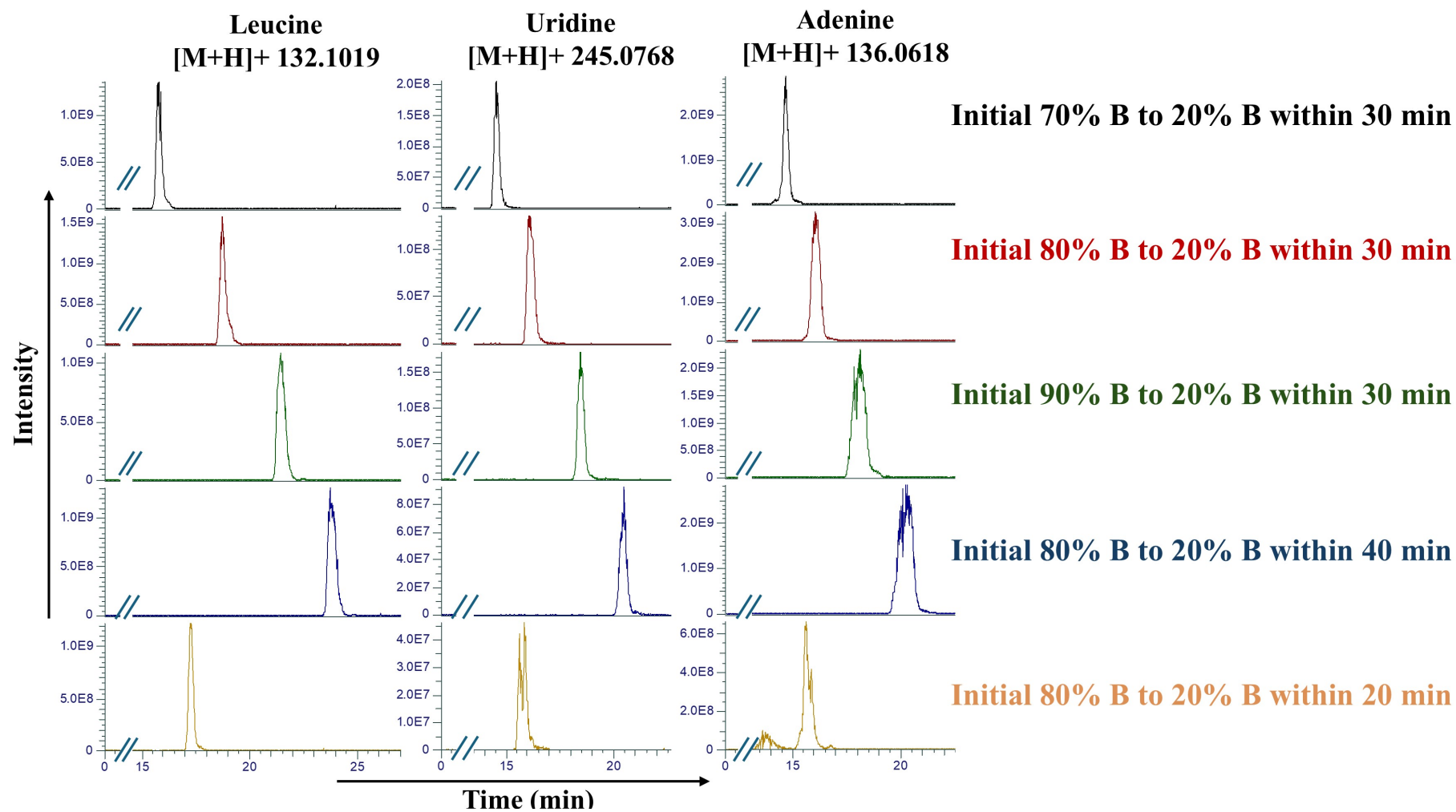


Figure B.20. Optimisation of gradient profile at capillary flow rate (continued). The EICs of leucine, uridine and adenine were separated by different gradient.

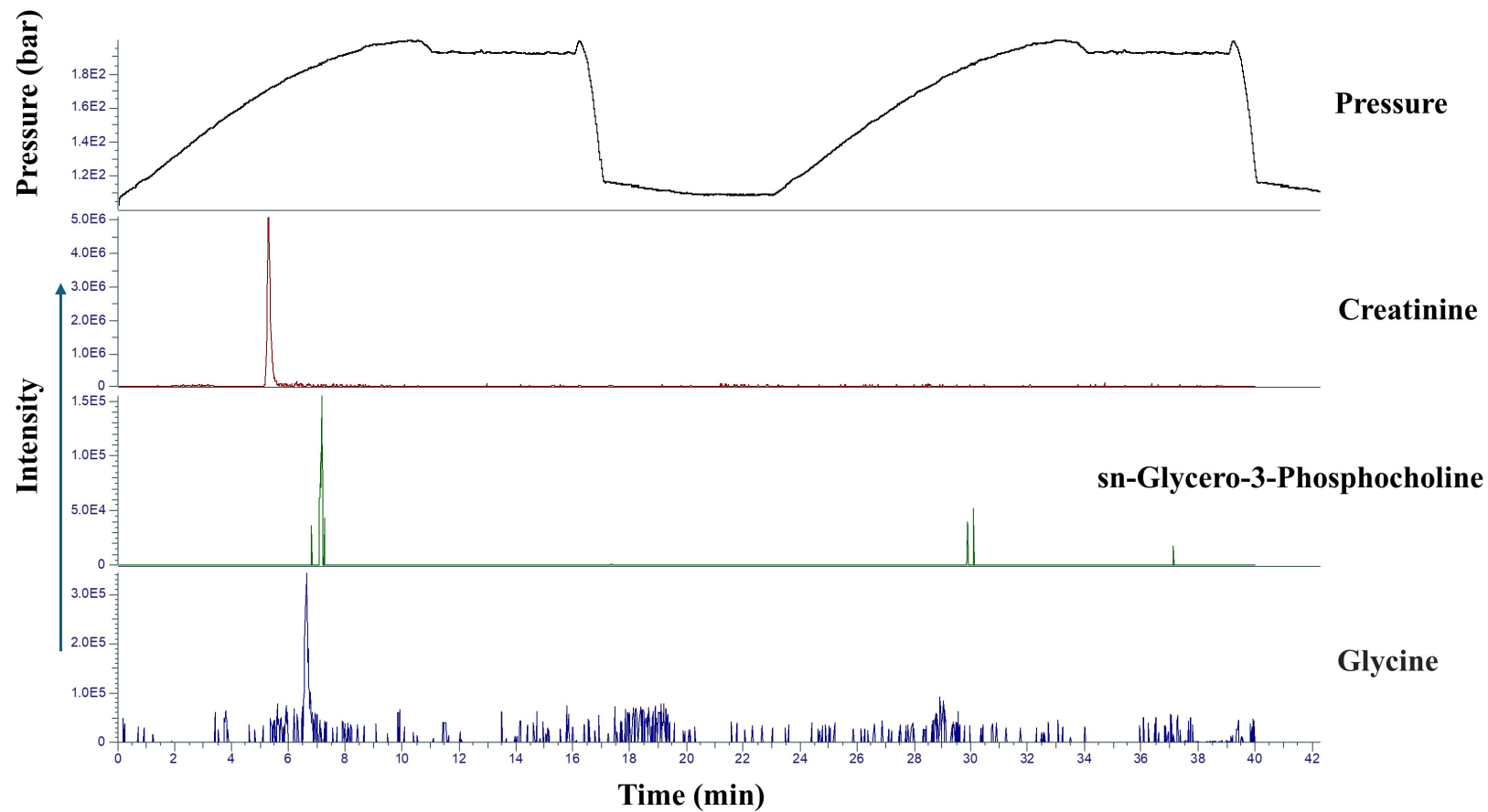


Figure B.21. Residuals of standards in the sampler. The system pressure of the solvent-blank sample after the injection of StdA, along with the EICs of creatine, sn-Glycero-3-phosphocholine, and glycine in the solvent-blank sample.

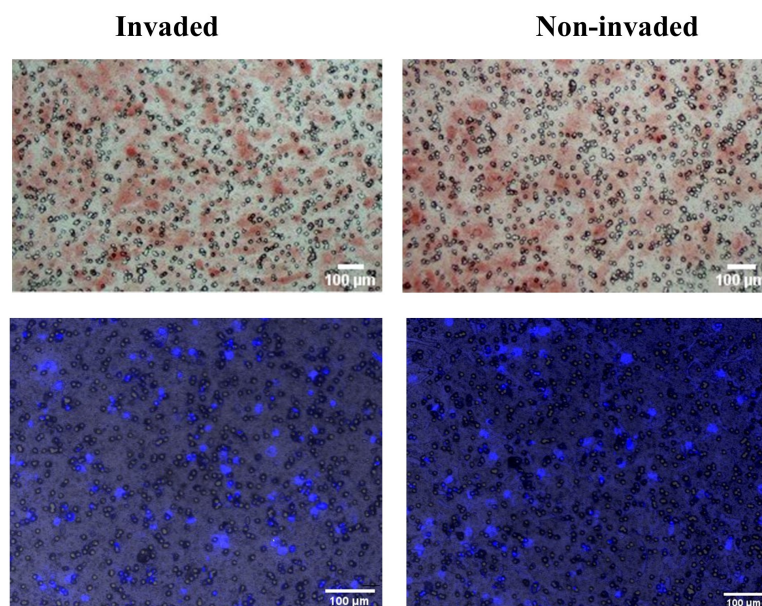


Figure B.22. Images of invaded cells and non-invaded generated from bright-field and confocal microscopy. Images from four fields of view were taken under a bright-field microscope (Brunel N-510) with 10X magnification len, and at a confocal microscope (Leica DMI4000B) at 360/470 nm with 10X magnification len.

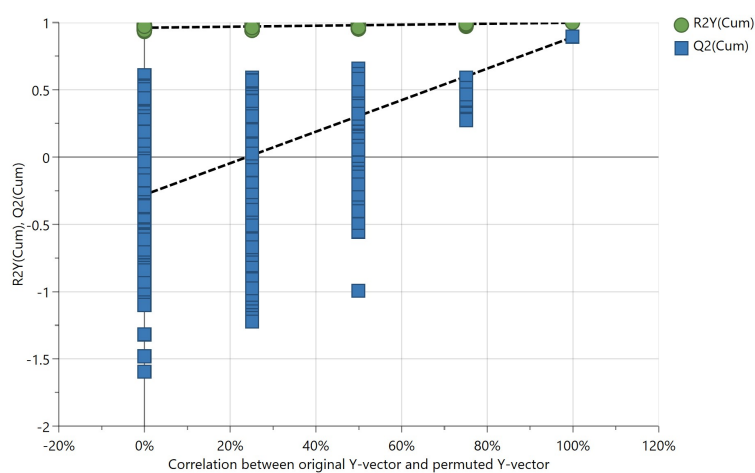


Figure B.23. Permutation test of invaded vs. non-invaded cells analysed by capillary-flow LC-MS.