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**Mapping Natural Variability in Human Intestinal
Fluid to Simulated Media: Implications for
Caco-2 Permeability Studies**

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A thesis presented in fulfilment of the requirements for the
degree of Doctor of Philosophy

2026

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A handwritten signature in black ink, appearing to be 'Em' followed by a stylized flourish.

Date: 31 March 2026

Acknowledgements

It is true when they say, “It takes a village”, I am thankful for everyone who has shaped me into who I am today.

To Prof. Hannah Batchelor, for the opportunity to do my PhD under your supervision. Your knowledge, guidance and understanding have made the past three years not only productive but highly enjoyable. I looked forward to every Tuesday meeting wither that be to troubleshoot new problems (but often persisting ones), your ability to stay calm and thoughtful approach was valued greatly. I am grateful for your understanding on sometimes a demanding athletics schedule. Your trust I would be able to balance both commitments, and confidence in me to still deliver high-quality work, has given me space to grow both personally and professionally.

The team within GSK who gave me the opportunity and funding to fulfil this PhD— Kosta, Nena, and James — whose scientific insight, thoughtful guidance, and willingness to challenge ideas consistently strengthened the quality and direction of this work.

Thanks to Dr. Lina Akil and Gillian Farrell for your patience and sharing of Mass Spec expertise. Both of your ability to troubleshoot a mass spec and find the root cause of the problem has allowed me to complete this work but also strengthened my own development as a researcher. To Prof. Nik Rattray for the use of your equipment and for always having a door open to talk, the willingness to discuss scientific conversation has been invaluable.

To Strath’s Finest, Callum, Trisha, Maria, and Rana, I couldn’t have asked for better people to go through this journey beside. The ability to talk about PhD troubles and real-life messiness often gave me clarity when needing it most.

My most precious besties, Tegan, Clare and Amy (Jaffa)- a job that none of you applied for. Your support has carried me from every corner of the globe throughout my entire academic journey. The way you listened you me complain and panic about my research, despite having no idea what I was talking about has kept me sane and anchored. From day one you have all been a source of laughter and welcomed distractions, I couldn’t have wished for better friends-I am endlessly grateful for each of you.

Team McAll — my second family and the people I spend most of my time with. Thank you for being my escape from the chaos of research and for turning the track into a place of rest bite. And to Ryan, who has been not just a coach but a big-brother figure: your stories, guidance, and much-needed life advice have shaped me far beyond athletics. Your influence has driven success both on and off the track.

To my partner Andy, a constant throughout the process. You've supported me through every high and every unravelled low, grounding me when research felt overwhelming and celebrating every small win as if it were your own. I couldn't have done this PhD without everything you've quietly and consistently done for me.

My thanks to my wee brother Oliver, who—often without realising it—became the unwilling audience to many of my research frustrations. His silent endurance deserves an honorary degree of its own (or monetary reward).

Finally, to my parents, who greeted the news of a PhD with the unforgettable line: 'Three more years of education... excellent.' Thank you for instilling in me the drive, persistence, and stubborn determination that have carried me through every stage of this journey. You taught me hard work and belief in myself even when the path ahead looked anything but straightforward. There are truly no words that capture how grateful I am to you both. You have both been a constant which has given me every opportunity to grow, to learn, and to chase ambitions that once felt impossibly far away.

As Sheldon Cooper would say:

'BSc, MSc, PhD... OMG, am I right?'

Abstract

Biopharmaceutics is the study of how the physical and chemical properties of a drug influence its absorption, distribution, metabolism and excretion (ADME) within the human body, with the aim of optimising the drug development pipeline so that therapeutic benefit is maximised, and adverse effects minimised. Central to this is the accurate prediction of oral absorption, which depends on reliable measurements of both solubility and permeability. While considerable progress has been made in generating biorelevant solubility data, permeability is still routinely assessed in simple buffer systems that bear little resemblance to the colloidal environment of the small intestine. As a consequence, the permeability values used as inputs into predictive software may not reflect the conditions drugs encounter *in vivo*.

This thesis addresses that gap by applying a suite of simulated intestinal fluids (SIF), developed through statistical evaluation of human intestinal fluid composition, to the Caco-2 model, with the aim of generating more physiologically representative permeability data. Four objectives were pursued. Chapter 2 investigates the development of a single gas chromatography-mass spectrometry method capable of quantifying the components of these media alongside the drug within the same sample. Chapter 3 examines the capacity of Caco-2 monolayers to withstand the stress imposed by compositionally complex media, identifying which formulations are compatible with the model and can be carried forward. Chapter 4 applies the selected media to the Caco-2 Transwell™ system, determining the apparent permeability of drugs spanning different classes of the Biopharmaceutics Classification System (BCS) and examining the variability that arises when a simple buffer is replaced by a colloidal medium. Chapter 5 turns to variability within the model itself, using absolute protein quantification by QconCat analysis to characterise how transporter and enzyme expression changes with passage number, thereby defining the passage range appropriate for experimental use, and comparing these profiles with paediatric biopsy data to assess the suitability of the model for predicting absorption in paediatric populations.

Together, this work expands the limited data available on drug permeability in simulated intestinal fluids and provides both a method and a mechanistic basis for assessing drug performance under physiologically realistic gastrointestinal conditions.

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List of Abbreviations

ABC – ATP-Binding Cassette

ADME – Absorption, Distribution, Metabolism and Excretion

API – Active Pharmaceutical Ingredient

BCRP – Breast Cancer Resistance Protein

BCS – Biopharmaceutical Classification System

BSA – Bovine Serum Albumin

BSEP – Bile Salt Export Pump

Caco-2 – Human Colorectal Adenocarcinoma Cell Line

CMC – Critical Micelle Concentration

CYP3A4 – Cytochrome P450 3A4

DCS – Developability Classification System

DMEM – Dulbecco's Modified Eagle Medium

DoE – Design of Experiments

ELISA – Enzyme-Linked Immunosorbent Assay

FaHIF – Fasted-State Human Intestinal Fluid

FaSSIF – Fasted-State Simulated Intestinal Fluid

FeSSIF – Fed-State Simulated Intestinal Fluid

FFA – Free Fatty Acid

GC-MS – Gas Chromatography–Mass Spectrometry

GEFs – Guanine Nucleotide Exchange Factors

HBSS – Hank's Balanced Salt Solution

HIF – Human Intestinal Fluid I

VIVE – *In vitro*–*in vivo* Extrapolation

JAMs – Junctional Adhesion Molecules

LC/MS/MS – Liquid Chromatography–Tandem Mass Spectrometry

LRP – Lung Resistance-Related Protein

MCT1 – Monocarboxylate Transporter 1

MDCK – Madin–Darby Canine Kidney Cells

MRP1–6 – Multidrug Resistance-Associated Proteins 1–6

MTT – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

OATP-A / OATP-B – Organic Anion Transporting Polypeptides

OCT1 / OCTN2 – Organic Cation Transporters

PAMPA – Parallel Artificial Membrane Permeability Assay

PBPK – Physiologically Based Pharmacokinetic

P-gp / MDR1 – P-glycoprotein / Multidrug Resistance Protein 1

SIF – Simulated Intestinal Fluid

TAG – Triacylglycerides

TEER – Transepithelial Electrical Resistance

UWL – Unstirred Water Layer

ZO-1 / ZO-2 – Zonula Occludens Proteins 1 and 2

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Chapter 1- Introduction

1.1. Principle of Biopharmaceutics

Biopharmaceutics links the physicochemical properties of an active pharmaceutical ingredient (API) and its dosage form with drug performance in the living organism. It encompasses pharmacokinetics, pharmaceutical technology and physiology. Through an understanding of absorption, distribution, metabolism and excretion (ADME), biopharmaceutical scientists can guide formulation design and support the development of medicines with effective therapeutic performance¹.

Absorption is a key determinant of an API's systemic exposure and, as such, a central concern in biopharmaceutics¹. Formulation design plays a critical role in determining the solubility, dissolution, and permeability characteristics of a drug, all of which directly influence the extent and rate of its absorption². Through rational formulation strategies, limitations in solubility, dissolution and permeability can be addressed^{4,5}. Enhancing these properties can improve API bioavailability by increasing the proportion of drug that reaches the systemic circulation and, ultimately, the target site, thereby optimising therapeutic efficacy⁸.

The Biopharmaceutics Classification System (BCS) was introduced by Amidon and colleagues in 1995 to establish relationships between *in vitro* dissolution, gastrointestinal permeability and *in vivo* bioavailability². The system classifies drugs according to their aqueous solubility and intestinal permeability, two key determinants of oral drug absorption and pharmacokinetic performance. Based on these properties, drugs are assigned to one of four classes: Class I, high solubility and high permeability; Class II, low solubility and high permeability; Class III, high solubility and low permeability; and Class IV, low solubility and low permeability². A drug is considered highly soluble when its highest therapeutic dose dissolves in 250 mL or less of aqueous media across the physiological pH range, reflecting the approximate volume of fluid typically administered with an oral dosage form. High permeability is generally defined as intestinal absorption of at least 85% of the administered dose².

Under the BCS, a drug is considered highly soluble when its highest therapeutic dose dissolves completely in 250 mL or less of aqueous media across the physiological pH range

(1.2–6.8). This volume reflects the glass of water typically taken with an oral dosage form. A drug is considered highly permeable when at least 85% of the administered dose is absorbed from the intestine.³ Following the development of the BCS, the Developability Classification System (DCS) was introduced to extend the BCS framework and better support formulation development. It uses biorelevant media (FaSSIF) and a more physiological intestinal fluid volume (500 mL) to estimate solubility, and it divides BCS Class II compounds into dissolution rate-limited (IIa) and solubility-limited (IIb) subclasses. By considering how permeability can compensate for low solubility, the DCS provides a more practical basis for selecting formulation strategies⁴. A key distinction is that the DCS incorporates biorelevant media for solubility assessment and further subdivides BCS Class II drugs according to whether absorption is primarily solubility-limited or dissolution rate-limited³.

The DCS was further refined in 2018 to account for supersaturation and precipitation of drugs within the gastrointestinal tract⁵. Incorporating these processes supports formulation development where such phenomena present a risk and contributes to more accurate prediction of *in vivo* performance.

1.2. Solubility

Solubility is a critical parameter in pharmaceutical development because it directly influences dissolution, absorption and, consequently, bioavailability⁶. The solubility of an API is determined by its physicochemical properties and the characteristics of the surrounding medium. At saturation, a stable state may be reached in which the concentration of dissolved solute remains constant over time. This occurs when the rates of dissolution and precipitation are equal, a condition known as dynamic equilibrium^{7,8}. The solubility measured under these conditions is referred to as equilibrium solubility. By contrast, kinetic solubility describes a metastable, non-equilibrium state in which the solute concentration temporarily exceeds equilibrium solubility before precipitation occurs⁷.

If the concentration of dissolved drug exceeds its equilibrium solubility, for example because of rapid dissolution, pH shifts or formulation effects, the solution becomes supersaturated.

Supersaturated solutions are thermodynamically unstable, and the excess dissolved drug may precipitate, potentially leading to crystallisation⁸.

Advances in drug discovery, including high-throughput screening, have increased the number of compounds with poor aqueous solubility. This has emphasised the need for early risk assessment and informed decision-making during drug development. Aqueous solubility describes the amount of solute that can dissolve in water; compounds with limited interaction with water, including highly lipophilic APIs, are commonly associated with poor aqueous solubility⁸. However, aqueous solubility alone omits important physiological factors, including pH, bile acids and interactions with other luminal components, and is therefore an incomplete predictor of drug solubility within the gastrointestinal tract⁸.

The solubilisation of weak acids and weak bases is strongly influenced by environmental pH. A compound's pK_a indicates the pH at which 50% of the compound is ionised and 50% is unionised⁹. In the ionised state, compounds generally show greater aqueous solubility because increased polarity facilitates interaction with the surrounding medium. This highlights the importance of pH-solubility profiling during early drug development. The gastrointestinal tract does not maintain a constant pH, and regional pH fluctuations can therefore alter drug solubility^{10,11}. This is particularly important for weakly basic drugs, which often show reduced ionisation and solubility in the small intestine, a major site of API absorption. Consequently, assessment of APIs under pH-relevant and pH-controlled conditions is essential.

Poor solubility often contributes to reduced absorption, although the relationship is drug-dependent and cannot be defined solely by rules such as Lipinski's rule of five¹². For example, poorly permeable drugs may require higher solubility to achieve adequate absorption, whereas highly permeable drugs may achieve sufficient absorption at lower solubility values¹³. Given this complexity, simple aqueous buffers may not adequately reflect gastrointestinal solubility. More biorelevant media are therefore desirable because they better replicate the chemical and physicochemical environment of the gastrointestinal tract. Markopoulos *et al.* proposed a tiered approach for simulating luminal conditions, ranging

from simple to more complex media¹⁴. Level 0 media consist of simple buffer systems adjusted to gastrointestinal pH. Level 1 media incorporate gastrointestinal buffering capacity, which is commonly approximated using bicarbonate-based systems. Level 2 media further replicate pH, buffer capacity, osmolarity and solubilisation capacity through the inclusion of bile salts, lipids and digestion products¹⁴. These components can form micellar structures that increase the apparent solubility of some compounds. Finally, Level 3 media incorporate proteins and enzymes to provide the highest degree of biorelevance.

The complexity of the gastrointestinal tract means that relying solely on Level 0 or Level 1 media may provide an incomplete understanding of API solubility behaviour. In 1998, Dressman *et al.* introduced a biorelevant fasted-state medium containing bile salt and lecithin to mimic key aspects of the fasted intestinal environment¹⁵. This formulation evolved to commercially available dissolution media showing the importance of a major step towards more meaningful physiologically relevant *in vitro* testing and highlighting the consideration which must be taken to select an appropriate media to predict the *in vivo* behaviour.

1.3. Drug absorption and Bioavailability

Drug absorption is a critical determinant of API bioavailability, which is defined as the fraction of the administered dose that reaches the systemic circulation. For orally administered drugs, bioavailability is often less than 100% because absorption is influenced by both API properties and gastrointestinal physiology¹⁶. Only the free fraction of drug is available to permeate across the intestinal epithelium, and this process is governed by several transport pathways, including transcellular transport, paracellular transport, carrier-mediated transport and endocytosis¹⁶. Absorption is controlled by multiple interrelated factors, including solubility, which determines the ability of an API to dissolve in gastrointestinal fluid, and permeability, which describes the capacity of a compound to cross the epithelial membrane¹⁷.

1.3.1. Transcellular Transport

Active and passive mechanisms determine how readily a compound can cross the cellular membrane. Active transport requires an energy source, such as ATP, whereas passive diffusion is an unassisted process driven by concentration or electrochemical gradients. API solubility, molecular size and ionisation state all influence absorption and therefore bioavailability¹⁸. Passive mechanisms, including simple diffusion and facilitated diffusion, are important routes of drug transport across the intestinal epithelium¹⁸. In simple diffusion, molecules move directly through the lipid bilayer, whereas facilitated diffusion occurs through solute-specific carriers or channels located within the membrane¹⁸. Solute-specific transport proteins can be broadly categorised as carrier proteins or gated channels. Carrier proteins possess substrate-binding sites and undergo conformational changes that enable transport across the membrane. For example, members of the GLUT family facilitate glucose transport through substrate-induced conformational changes rather than direct ATP hydrolysis¹⁸. Gated channels, including sodium channels, may be voltage-gated or ligand-gated, opening in response to changes in membrane potential or ligand binding at a receptor site on the channel (Figure 1).

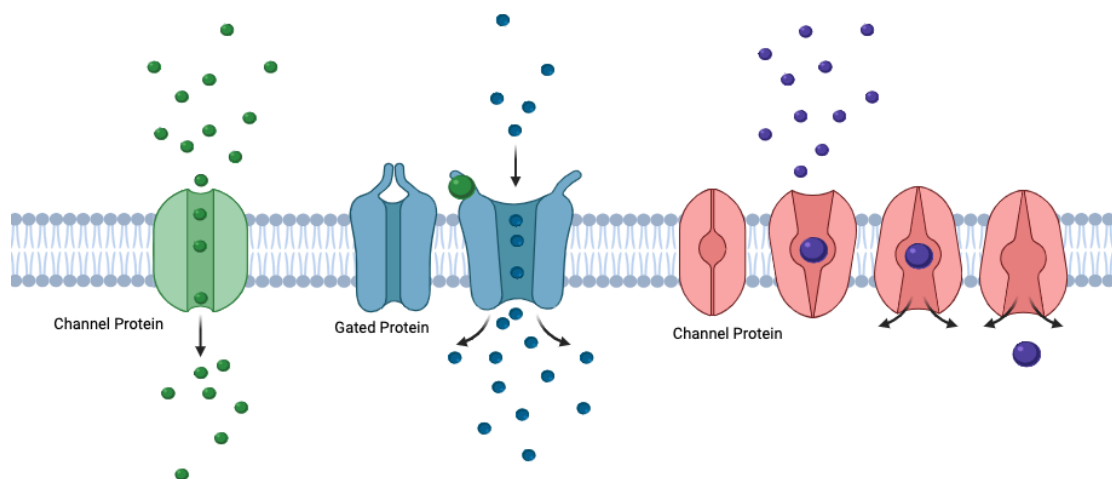


Figure 1 - Schematic representation of the principal membrane transport mechanisms involved in intestinal drug absorption.

The figure illustrates three major classes of membrane transport proteins which facilitate movement of molecules across the epithelial membrane. **Left:** Channel proteins form constitutently open aqueous pores that permit the passive diffusion of specific ions or small molecules down their electrochemical gradient without requiring conformational changes or energy transfer. **Centre:** Gated protein regulated substrate transport through conformational changes triggered by external stimuli allowing the selective movement of molecules. **Right:** Carrier proteins, these bind specific substrates and undergo subsequent conformational changes to translocate molecules across the membrane. Carrier-mediate transport occurs via facilitates diffusion, in which substrates move down the concentration gradient, or via active transport where the movement occurs against the concentration gradient through coupling to ATP hydrolysis or ion gradient. Created with BioRender.com.

Active transport requires energy, often in the form of ATP, to move solutes across the cell, frequently against a concentration or electrochemical gradient¹⁹. Calcium pumps are examples of membrane transport systems that use energy to regulate intracellular calcium concentrations. Although active transcellular transport is not the dominant route for most APIs, molecules that resemble endogenous substrates, such as peptides or vitamins, may use active transport pathways to enter epithelial cells and subsequently reach the systemic circulation¹⁹.

1.3.2. Paracellular Transport

Tight junctions are a common structural feature of mammalian epithelial cells and regulate paracellular transport of small molecules, including ions, sugars and amino acids²⁰. They are maintained through networks of protein-protein interactions that create close contacts between adjacent epithelial cells²⁰.

These junctional complexes include tight junction and adherens junction proteins, terms that reflect their roles in restricting the intercellular space and maintaining adhesion between neighbouring cells, respectively. Collectively, these proteins regulate paracellular pore size and form a key barrier to diffusion²¹. The paracellular route accounts for only a small fraction of overall transport for many hydrophilic small molecules.

These absorption mechanisms collectively determine the bioavailability of APIs. For example, a highly soluble molecule may still exhibit low bioavailability if it interacts poorly with intestinal transport pathways or cannot effectively cross the epithelial barrier. Conversely, a compound with favourable membrane transport characteristics may show limited bioavailability if poor solubility prevents sufficient dissolution prior to absorption. Bioavailability is therefore dependent on the interplay between drug solubility, permeability, transport mechanisms and gastrointestinal physiology, highlighting the need for a comprehensive understanding of drug absorption.

1.4. Gastrointestinal Fluids

The composition of gastrointestinal fluids is complex and reflects the diverse origins of their constituents. Gastric secretions pass into the intestinal tract following gastric emptying, where they mix with duodenal secretions, including bile-derived components. Early characterisation of human intestinal fluid (HIF) was limited and required further refinement^{15,22}. Riethorst *et al.* addressed this gap in 2016 by providing a more detailed physiological characterisation of HIF²³. Such characterisation is particularly important for predicting drug dissolution and absorption, as intestinal fluid contains a complex mixture of bile salts, cholesterol, phospholipids and enzymes that contribute to the digestion,

solubilisation and absorption of nutrients and drugs within the intestinal lumen²³. Bile salts are released from the gallbladder into the small intestine and enhance the solubilisation of lipophilic drugs and dietary fats. They are derived from cholesterol, primarily through the neutral pathway, and alterations in bile salt synthesis or secretion can influence cholesterol homeostasis²⁴ (Figure 2). Bile salt synthesis and activity are closely linked to dietary intake; consequently, higher bile salt concentrations are typically observed in the fed state, increasing the solubilisation capacity for lipophilic drugs.

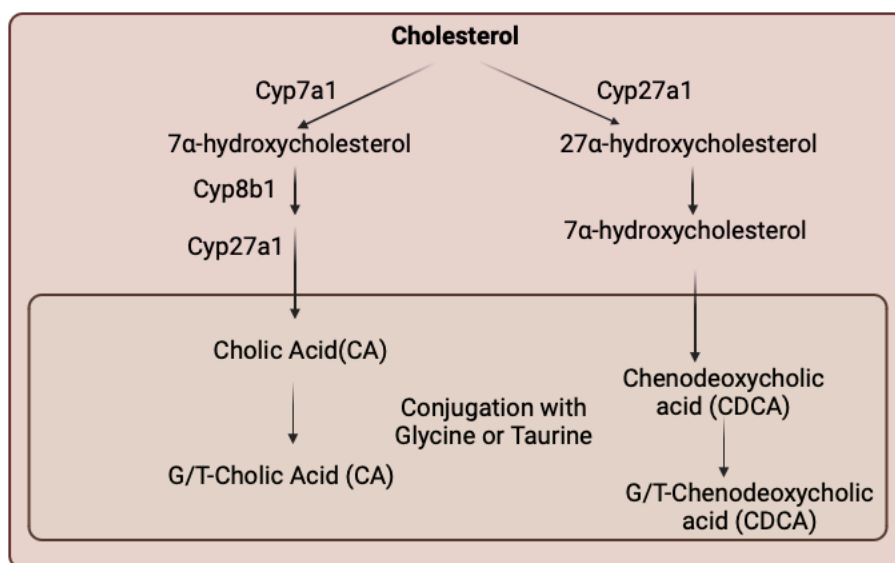


Figure 2 - Simplified overview of the classical and alternative pathways of bile acid synthesis from cholesterol.

Cholesterol serves as the precursor for primary bile acid synthesis via two distinct metabolic pathways. The classical pathway, cholesterol is converted to 7 α -hydroxycholesterol by CYP7a1 which is the rate limiter in bile acid synthesis. Where this is then hydroxylated by CYP27A1 resulting in the formation of cholic acid. Whereas in the alternative pathway cholesterol is initially hydroxylated by CYP21A1 to produce 21 α -hydroxycholesterol, which is converted into 7 α -hydroxycholesterol leading to the synthesis of chenodeoxycholic acid. Prior to its secretion from the bile ducts is it conjugated with either a glycine or taurine to enhance solubility and amphipathic properties of bile acid.

In simulated intestinal fluids, sodium taurocholate is commonly used as a representative bile salt to investigate drug behaviour *in vitro*. The polar functional groups of sodium taurocholate

increase its interaction with water, while its hydrophobic steroid backbone contributes to detergent-like behaviour and micelle formation²⁵.

Cholesterol within the small intestine also contributes to drug solubilisation. It can interact with other components of the intestinal matrix to form mixed micelles²⁶. Micelles containing cholesterol have been reported to improve API release from drug delivery systems²⁶. This may be attributed to the ability of cholesterol to enhance the structural stability and hydrophobicity of mixed micelles, thereby increasing their capacity to solubilise lipophilic compounds and promote partitioning into intestinal fluid. Cholesterol may also influence absorption through its interaction with cellular membranes²⁷. Incorporation of cholesterol into cellular membranes can alter membrane fluidity and phase behaviour, which may affect membrane permeability and absorptive capacity²⁸.

Alongside cholesterol, phospholipids are important constituents of gastrointestinal fluids because they support the formation of micellar and colloidal structures that contribute to API solubilisation and absorption. Biologically, phospholipids play an essential role in the digestion and absorption of dietary lipids and facilitate their transport to the intestinal epithelium²⁹. Lipids within the small intestine can solubilise poorly water-soluble compounds into mixed micelles, increasing the apparent surface area available for absorption. The extent of this enhancement depends on physiological conditions, including bile salt concentration, lipid composition and whether the intestine is in the fasted or fed state. Consequently, lipid-based formulation strategies are widely used in modern drug development to improve drug solubilisation and absorption. However, their performance is influenced by endogenous intestinal lipids, which can introduce variability into drug delivery.

Enzymatic hydrolysis of dietary lipids generates free fatty acids (FFAs), which further contribute to the solubilisation capacity of mixed micelles within the intestinal lumen²³. The physicochemical properties of FFAs vary with chain length; longer-chain fatty acids are generally less water-soluble and more lipophilic, influencing their incorporation into mixed micelles and their interactions with drug molecules. The presence of FFAs can increase the hydrophobic core of micelles and thereby enhance the solubilisation of hydrophobic drugs.

FFAs may also promote interactions between micelles and cellular membranes, facilitating drug absorption³⁰. Similar to phospholipids, FFAs can affect interactions between intestinal luminal components and epithelial membranes. However, unlike phospholipids, which contribute to membrane structure and may produce more gradual permeability effects, FFAs can disrupt lipid packing and lead to more pronounced permeability enhancement³¹.

Overall, the volume and composition of gastrointestinal fluid, including bile salts, phospholipids, free fatty acids and cholesterol, play a critical role in governing the solubilisation capacity of the intestinal environment. Variation in these parameters directly influences micelle formation and stability, and therefore API dissolution and absorption. However, their dynamic and physiologically variable nature presents significant challenges for achieving consistent drug bioavailability and for designing representative *in vitro* systems.

1.5. Simulated intestinal fluid

Although the composition and volume of gastrointestinal (GI) fluids play a critical role in determining API solubilisation and absorption *in vivo*, their dynamic and variable nature presents challenges for systematic experimental investigation. Simulated intestinal fluids (SIFs) have therefore been developed to reproduce key physicochemical characteristics of the intestinal environment under controlled conditions. These media were first proposed in the late 1990s by Dressman *et al.*, who incorporated sodium taurocholate and soybean lecithin into dissolution media to represent bile salts and phospholipids present within the intestinal lumen. Subsequent modifications were introduced by Galia *et al.*³² and Jantrai *et al.*²². However, an important advance was made by Riethorst *et al.* in 2016, who provided a detailed characterisation of human duodenal fluids in both fasted and fed states²³. Their work showed that food intake triggers substantial increases in bile salts, phospholipids, lipid digestion products and digestive enzymes. The study also revealed pronounced intra- and inter-individual variability across measured parameters, including wide ranges in pH and bile salt concentration, emphasising the dynamic nature of intestinal fluids²³. Collectively, these findings indicate that the fed state can enhance solubilisation capacity, but that physiological

variability remains a critical consideration in drug absorption prediction and in the design of biorelevant *in vitro* systems.

Pyper *et al.* subsequently applied statistical analysis to the Riethorst *et al.* dataset in 2020, using a multidimensional approach to evaluate HIF composition. The Riethorst dataset included 152 fasted-state and 172 fed-state measurements across five variables: total bile salt, phospholipid, total free fatty acid, cholesterol and pH, measured in time-dependent HIF samples from 20 volunteers³³. Pyper and colleagues demonstrated that these components did not vary independently; instead, they were correlated and distributed within a complex multidimensional space, described as an ellipsoid rather than as a series of single average values³³. This analysis highlighted that conventional SIF formulations may fail to capture the full extent of physiological variability and may not fully represent biorelevant intestinal conditions. The authors proposed that a nine-point media design could capture approximately 95% of the natural variability in intestinal fluid composition³³. Use of such media could provide a more realistic framework for representing intestinal conditions, improving *in vitro* study design and enhancing prediction of drug solubilisation behaviour.

1.6. Measuring permeability

The measurement of absorption is critical in drug development because it determines how effectively a drug can cross biological membranes and reach the systemic circulation. Absorption is strongly influenced by membrane permeability, which describes the ability of a compound to pass through biological barriers such as the intestinal epithelium². Accurate permeability measurements enable researchers to predict oral bioavailability and identify potential limitations of an API, including poor passive diffusion or active efflux. These data can therefore inform formulation optimisation and support decision-making during early drug development³⁴.

Direct measurement of human intestinal absorption is not usually feasible during early drug development. Therefore, *in vitro* laboratory models that simulate biological barriers are commonly used to assess permeability. Cell-based assays, including Caco-2 and MDCK cell

monolayers, are widely regarded as standard approaches for evaluating intestinal epithelial permeability. In contrast, the parallel artificial membrane permeability assay (PAMPA) uses an artificial membrane system and is primarily used to assess passive diffusion³⁵.

The Caco-2 cell model uses human colorectal adenocarcinoma cells which, following seeding, differentiate over approximately 21 days to form a polarised monolayer with well-developed tight junctions that resemble key features of the intestinal epithelium. Additional characteristics include microvilli on the apical surface and expression of transport proteins and metabolic enzymes, including efflux transporters such as P-glycoprotein and CYP enzymes. Consequently, this model is widely used to evaluate drug permeability, absorption mechanisms and potential drug-drug interactions³⁶. Another widely used epithelial cell line is the Madin-Darby Canine Kidney (MDCK) model³⁷. MDCK cells originate from canine kidney tissue and, like Caco-2 cells, polarise to form monolayers with tight junctions, enabling assessment of both transcellular and paracellular transport³⁷. A key advantage of MDCK cells is their shorter culture time of approximately 3-5 days, which makes them suitable for higher-throughput screening. MDCK cells can also be genetically modified to knock down or overexpress specific transporters implicated in drug-drug interactions³⁸.

Cell-based permeability assays are typically conducted by seeding Caco-2 or MDCK cells onto Transwell™ inserts and incubating them for the required duration to allow differentiation and monolayer formation. API permeability is measured from the rate of transport across the monolayer, commonly expressed as the apparent permeability coefficient (P_{app}). Transport is often assessed from the apical chamber to the basolateral chamber, representing movement from the intestinal lumen towards the systemic circulation (Figure 3). These studies are usually performed using physiological transport buffers, such as Hanks' Balanced Salt Solution (HBSS) or phosphate-buffered saline (PBS), maintained at approximately pH 7.4 to support physiological experimental conditions.

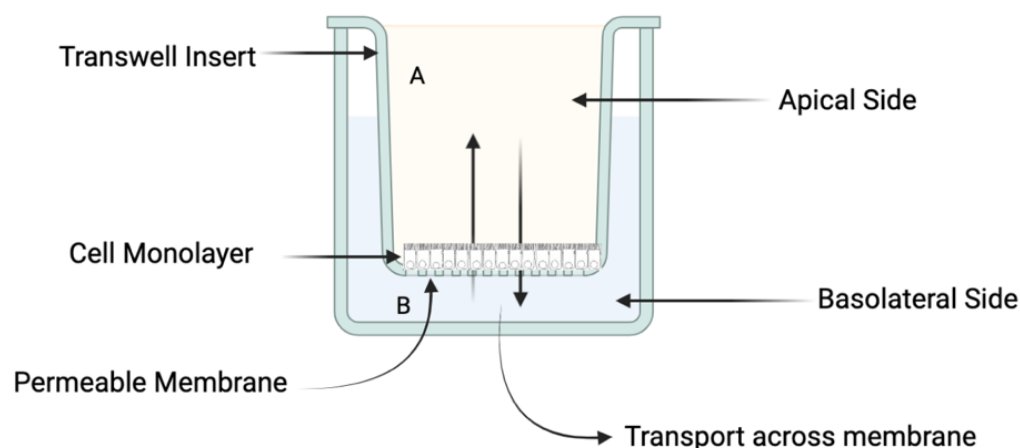


Figure 3 - Schematic representation of the Transwell® permeability system used for Caco-2 transport studies.

The Transwell™ insert consists of a porous, permeable membrane upon which a confluence Caco-2 monolayer is cultured, separating the apical and basolateral compartments. The apical chamber represents the intestinal lumen where drug formulations or transport media are applied, while the basolateral chamber represents the systemic circulation. During permeability studies transport of API is monitored over time. Bidirectional transport studies can be performed by assessing movement from apical-to-basolateral representing intestinal absorption, and from basolateral-to-apical direction representing secretory or efflux transport. Created with Biorender.com.

However, cell-based models can be time-consuming, costly and subject to variability arising from biological factors such as transporter expression, passage number and culture conditions. As a result, non-cell-based methods such as PAMPA are widely used for simpler, higher-throughput screening. Unlike cell-based systems, which use a cellular monolayer supported on a membrane, PAMPA employs an artificial lipid membrane to assess passive diffusion only, thereby eliminating the influence of active transport and metabolic processes³⁵ (Figure 4). PAMPA is therefore commonly used during early drug development for rapid screening of passive permeability. Nevertheless, cell-based models remain essential for evaluating more complex transport mechanisms involving active transport, efflux and metabolic activity.

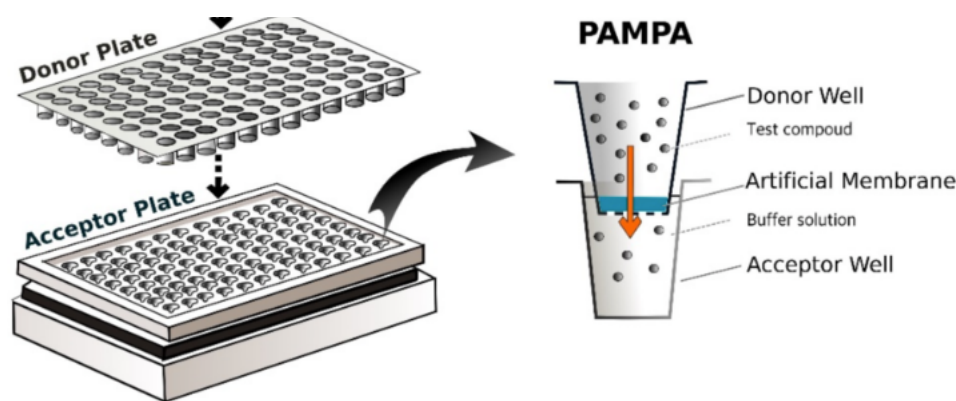


Figure 4 - Representation of the Parallel Artificial Membrane Permeability Assay (PAMPA). Adapted from Ponmozhi et al. 2021³⁹.

The assay consists of a donor plate and acceptor plate separated by an artificial lipid membrane, test compounds are added to the donor well and diffuse passively across the artificial membrane into the acceptor well containing buffer solution, following incubation in the donor and acceptor compartment are measured to determine passive permeability and predict drug absorption characteristics.

Collectively, these approaches enable robust assessment of drug permeability. However, the accuracy and reliability of the resulting data depend on experimental conditions, including buffer composition, cell model, assay configuration and study design.

1.7. Transport buffers in Cellular models

As described above, HBSS and PBS are commonly selected as transport buffers in cell-based permeability models and are typically adjusted to a physiologically relevant pH of approximately 7.4. HBSS is frequently used in the literature because its more complex ionic composition better supports cell function during transport studies⁴⁰. These buffers provide a controlled ionic environment that helps preserve cell viability, tight junction integrity and transporter function⁴¹. Their use also reduces interference from proteins and other components that may bind to the drug or affect permeability measurements. Consequently, HBSS and PBS are often used in both the apical and basolateral compartments of Transwell™ systems⁴¹.

Although this approach provides standardised experimental conditions, it inherently limits the physiological relevance of *in vitro* permeability models. Conventional transport buffers are not biorelevant and therefore may not accurately represent the complex solubility and absorption behaviour of APIs within the gastrointestinal tract. Permeability values generated from cell-based models are often used as input parameters in physiologically based pharmacokinetic (PBPK) models to estimate intestinal absorption. This highlights the need to consider whether integration of biorelevant fluids into permeability assessments could improve the translational value of PBPK modelling⁴².

A critical distinction between HBSS or PBS and biorelevant media such as simulated intestinal fluids is the presence of amphiphilic components that can self-assemble into complex colloidal structures. Formation of these structures can alter the solubilisation capacity of the medium and therefore influence the free fraction of API available for absorption. Recognising these colloidal effects is essential for generating physiologically relevant input parameters for PBPK models and improving the predictive accuracy of *in vitro*-*in vivo* extrapolation⁴³.

A multidisciplinary understanding of API absorption provides essential insight for formulation scientists seeking to optimise drug delivery and maximise bioavailability. Integrating simulated intestinal fluids into *in vitro* models of gastrointestinal absorption may improve the physiological relevance of permeability assessments, enhance predictive capacity and reduce late-stage development failures associated with poor oral bioavailability.

1.8. Integration of SIF in cell-based permeability assessments.

The integration of simulated intestinal fluid (SIF) into cell-based permeability models was first reported in the early 2000s, when the original SIF formulation¹⁵ was applied to the apical compartment of Caco-2 monolayers to investigate its suitability as a more physiologically relevant transport medium⁴⁴. Ingels *et al.* reported that fasted-state simulated intestinal fluid could be used without compromising cell function, whereas fed-state simulated intestinal fluid caused rapid damage to the monolayer and significantly reduced cell viability⁴⁴. The toxicity associated with the fed-state medium was attributed to its lower pH and higher bile

salt concentration. Ingels *et al.* further demonstrated that bile salts exerted a concentration-dependent effect on Caco-2 cells, whereas lecithin, another component of the original SIF formulation, did not show comparable toxicity⁴⁴.

Building on these findings, Ingels *et al.* expanded their work in 2004 by investigating the predictive performance of Caco-2 permeability measurements for 19 model compounds in FaSSIF compared with conventional transport media⁴⁵. The compounds examined had diverse transport characteristics. Although the use of FaSSIF did not substantially improve the absorption prediction for all compounds, the greatest differences were observed for compounds subject to efflux transport, particularly P-glycoprotein substrates such as digoxin and doxorubicin, where bidirectional transport ratios were altered. Improved recovery was also observed for lipophilic compounds, whose solubility and partitioning behaviour may be influenced by the presence of biorelevant fluids and micellar structures⁴⁵. Although the study did not show a marked improvement in the relationship between apparent permeability (P_{app}) and reported human fraction absorbed (F_a), it highlighted the importance of using biorelevant media when assessing poorly soluble and transporter-dependent compounds.

Fossati *et al.* subsequently focused on lipophilic compounds and investigated how the original FaSSIF formulation¹⁵ could be used to improve the poor recovery often observed in conventional Caco-2 assays⁴⁶. Unlike the earlier studies by Ingels *et al.*, which focused primarily on permeability measurements, Fossati *et al.* evaluated 35 compounds with corresponding human absorption data and incorporated bovine serum albumin (BSA) into the basolateral TranswellTM chamber⁴⁶. As lipophilic compounds are exposed to plasma proteins following absorption *in vivo*, the inclusion of BSA provided a more physiologically relevant sink condition intended to mimic the systemic circulation. This approach aimed to maintain the absorptive driving force and reduce non-specific binding losses of lipophilic compounds. These findings emphasised the need for further refinement of Caco-2 transport conditions to improve assessment of lipophilic compound absorption while maintaining physiological relevance.

Although these studies demonstrated the potential value of biorelevant media in Caco-2 permeability assays, they used the original SIF formulations, which only partially reflect the dynamic and complex composition of human intestinal fluid. Patel and colleagues therefore investigated how modifications to SIF composition influenced Caco-2 monolayer behaviour rather than focusing solely on compound absorption⁴⁷. Their aim was to identify formulation boundaries tolerated by Caco-2 epithelial cells, using MTT assays as an initial viability screen. Caco-2 cells were exposed to different bile salt concentrations to determine which conditions were least detrimental to cell viability⁴⁷. Modification of the traditional FeSSIF formulation by reducing the bile salt-to-lecithin ratio from 4:1 to 2:1 improved compatibility with Caco-2 cells. Despite retaining a high bile salt concentration of 15 mM, the modified formulation was tolerated by the cells, with transepithelial electrical resistance (TEER) values decreasing by only approximately 20%⁴⁷. In contrast, the traditional FeSSIF formulation with a 4:1 ratio caused complete loss of monolayer integrity⁴⁷. These findings indicate that the biological impact of biorelevant media cannot be predicted solely from the concentration of individual components; rather, the organisation and physicochemical properties of the colloidal structures formed within the matrix are also critical.

These observations were further explored by Frank *et al.*, who investigated the behaviour of a poorly water-soluble compound in biorelevant medium, comparing its performance in traditional FaSSIF with that in standard HBSS buffer⁴⁸. The compound showed markedly higher apparent solubility in biorelevant medium, with an approximately 30-fold increase attributed to bile salt and phospholipid micelles⁴⁸. However, this enhanced solubility did not translate into increased absorption across Caco-2 monolayers. This was attributed to the unbound, or free, fraction of API remaining largely unchanged, suggesting that micelles acted primarily as a solubilising reservoir rather than increasing the thermodynamic activity of free drug in solution⁴⁸. These findings demonstrate that increased solubilisation does not necessarily translate into enhanced permeability, as drug transport depends on both the properties of the compound and its interaction with the biological membrane.

These findings highlight that solubility values alone are insufficient for understanding absorption behaviour; interactions between the API and the medium must also be

considered. It is well documented that amphiphilic components within biological media can self-assemble to form complex colloidal structures⁴⁹⁻⁵¹. A clearer understanding of how these colloidal structures interact with Caco-2 cells is therefore important for advancing *in vitro* permeability assessment. Markopoulos *et al.* investigated the suitability of modified biorelevant media as transport media in Caco-2 permeability studies, recognising that the original biorelevant fluids had been designed primarily for dissolution testing⁵². The authors modified the FaSSIF and FeSSIF formulations reported by Galia *et al.* and evaluated their effects on monolayer integrity and drug permeability (Table 1).

Table 1- Markopolos Media Composition, TM= Transport Media (Markopoulos media)⁵².

Component	FaSSIF- TM _{caco}	FeSSIF- TM _{caco}	FaSSIF V2	FESSIF V2
DMEM base (g/L)	8.62	8.62		
D-glucose(g/L)	4.5	4.5		
L-Glutamine(g/L)	0.876	0.876		
Maleic Acid(mM)	19.12	55.02		
Sodium Taurocholate(mM)	3	6.8	3	15
Phosphatidylcholine (mM)	0.2	6.8	0.2	3.75
Glyceryl monooleate (mM)	-	3.4		
Sodium oleate (mM)	-	0.8		
NaOH	qs	qs		
pH	6.5	5.8	6.5	5.8

The adapted media were designed to improve compatibility with Caco-2 cells while retaining bile salts and lecithin to support colloidal structure formation. In the presence of FaSSIF-TM_{Caco}, Caco-2 monolayer integrity was maintained over a 180-minute exposure period⁵². Monolayer integrity was also maintained in FeSSIF-TM_{Caco} under Transwell™ conditions. However, when FeSSIF-TM_{Caco} was applied to cells cultured in multi-well plates rather than Transwell™ inserts, cell viability decreased by approximately 60%⁵². These findings suggest

that although the modified media can be compatible with epithelial cells, their suitability depends on the experimental configuration.

Markopoulos *et al.* subsequently evaluated drug permeability under these conditions. Because monolayer integrity was maintained, observed changes in permeability could be attributed primarily to drug-colloid interactions rather than epithelial barrier damage. The authors showed that apparent permeability was reduced in biorelevant media compared with aqueous transport media, which was attributed to partitioning of the API into colloidal structures and consequent reduction in the free fraction available for absorption⁵². This reduction was greatest in FeSSIF-TM_{Caco}, consistent with its higher concentration of colloidal components⁵². These observations align with earlier findings by Frank *et al.*, where increased solubilisation in biorelevant media did not necessarily lead to increased absorption⁴⁸.

Later work argued that traditional biorelevant fluids may not be sufficiently representative because they omit cholesterol, which is naturally present in bile⁵³. To address this limitation, Khoshakhlagh *et al.* incorporated cholesterol into FaSSIF and investigated its impact on colloidal structure, solubility and absorption. The addition of cholesterol altered colloidal structures and changed the solubilisation behaviour of lipophilic compounds⁵³. These findings demonstrate that cholesterol is not simply an additional bile component, but an influential factor affecting API partitioning into micellar and colloidal structures.

Collectively, these findings show that the colloidal environment within biorelevant media is complex and sensitive to relatively small changes in composition. The work by Khoshakhlagh *et al.* demonstrated that introducing an additional biorelevant component can alter colloidal structure and behaviour⁵³, leading to API-specific effects on solubilisation and absorption. When considered alongside the findings of Frank *et al.*⁴⁸ and Markopoulos *et al.*,⁵² it is evident that the relationship between solubility and permeability is not straightforward, but depends on interactions between the API, colloidal structures and the epithelial barrier.

Despite these advances, SIF remains a simplified representation of *in vivo* intestinal fluid. Human intestinal fluid (HIF) contains a dynamic and heterogeneous mixture of bile salts,

phospholipids, cholesterol, digestion products, proteins and colloidal structures that can only be partially represented in synthetic media. The use of HIF as a transport medium would provide the most physiologically relevant approach and could improve the predictive value of *in vitro* permeability models. However, routine use of HIF is limited by challenges associated with sample collection, availability and variability. Consequently, biorelevant media remain the most practical alternative for routine experimental assessment.

Nevertheless, fasted-state HIF (FaHIF) has been used in combination with Caco-2 cells in some studies⁵⁴. Wuyts *et al.* provided a direct comparison between FaHIF and conventional buffer systems and demonstrated successful application of FaHIF to Caco-2 monolayers. Permeability values measured in FaHIF differed from those obtained in synthetic SIF and HBSS, with drug-dependent effects particularly evident for lipophilic compounds⁵⁴. Although a correlation was observed between permeability values measured in FaHIF and FaSSIF, FaSSIF reproduced general trends rather than accurately predicting absolute permeability values. This difference may reflect the greater colloidal complexity of HIF compared with traditional FaSSIF formulations, which typically contain only bile salt and lecithin and therefore generate simpler colloidal structures with different drug-micelle interactions^{32,54}.

Overall, previous studies have shown that biorelevant media can be compatible with Caco-2 cell systems and can alter measured permeability values. However, most Caco-2 transport studies using biorelevant media have relied on the traditional dissolution media proposed by Dressman *et al.*¹⁵. Although this work had substantial impact on the field of biopharmaceutics, reliance on a single average media formulation limits physiological relevance as a model of the gastrointestinal tract. The restricted compositional variability inherent in standard SIF formulations raises concerns about their ability to capture the heterogeneous conditions present *in vivo*^{15,23,55}. This highlights the need for continued refinement and diversification of biorelevant media to improve the predictive accuracy of *in vitro* permeability assessments.

1.9. Relevance of variability in SIF for biopharmaceutics

The relevance of simulated intestinal fluid (SIF) in biopharmaceutics lies in its ability to approximate intestinal conditions and provide a meaningful platform for evaluating drug solubilisation and absorption. SIF formulations designed to capture human physiological variability have been evaluated in studies investigating their impact on drug solubilisation^{9,11,56,57}. These studies demonstrate that increasing media complexity can significantly influence solubility outcomes, indicating that solubility is not a fixed property in biorelevant media but is sensitive to the composition of intestinal fluid.

Collectively, the work of Abuhassan *et al.* and Silva *et al.* provides a coherent framework for understanding solubility in biorelevant fluids, shifting emphasis away from single-point solubility values towards a physiologically informed solubility range^{9,11,56}. Across these studies, a consistent theme emerges intestinal solubility is inherently variable and dependent on physiological composition. The fasted state appears to show more predictable and structured behaviour than the fed state, reinforcing the importance of context-specific biorelevant media assessment.

These studies used SIF formulations generated from the characterisation of human intestinal fluid (HIF) reported by Riethorst *et al.* in 2016²³. The data generated in that study demonstrated not only inter-individual variability within a cohort of 20 healthy adults, but also intra-individual variability arising from repeated sampling of the same individuals at different time points²³. Figure 5 shows the distribution of the five selected variables reported by Riethorst *et al.*, highlighting the extent of variability observed across key intestinal fluid components. The greatest variation was observed for free fatty acids, phospholipids and bile salts, with reported ranges of 0.0009-15.03 mM, 0.005-6.32 mM and 0.12-36.18 mM, respectively²³. This wide distribution of key intestinal components emphasises the extent of physiological diversity and supports the development of SIF formulations that better capture human variability.

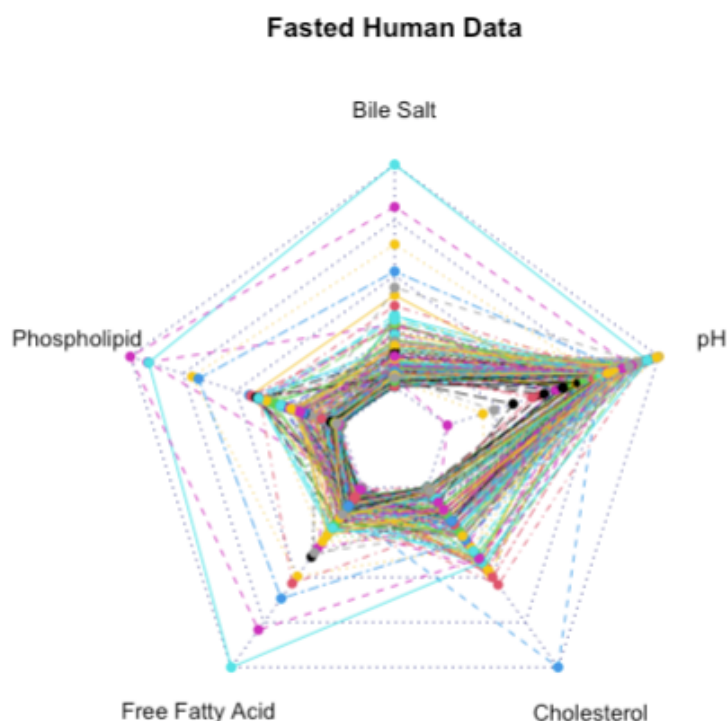


Figure 5 - Radar plot illustrating the variability in fasted human intestinal fluid.

Each axis represents a key biorelevant medium components measured by Riethorst et al. 2015. Individual coloured traces correspond to different fasted state compositions measured from an individual. Axis values are set as Maximum Values are Bile Salt value= 36.18mM, lecithin=6.32 mM, Free Fatty Acid= 15.03 mM Cholesterol= 0.48 mM, pH= 8.3. Axis. Minimum Values are Bile Salt= 0.12mM, lecithin=0.005 mM, Free Fatty Acid=0.0009 mM Cholesterol= 0.07 mM, pH= 2.41. Plot representing the dynamic nature of the human intestinal lumen under fasted conditions.

Current standard formulations which are used when including SIF in permeability assessments are based on traditional SIF medias, which demonstrate a limited range of the physiological distribution of these components^{54,58-63}. The media which have been used in permeability assessment often only have bile salt and phospholipids present within the media set to a pH of ~6.4 (Figure 6) which, when compared against the broader distributions seen in Figure 6 demonstrates the narrow compositional scope of these *in vitro* models. As a result, such current Caco-2 models biorelevant media (Figure 6) are unable to capture the diversity of the intestinal environment (Figure 5) or its influence on compound behaviour.

Literature media values in Caco-2

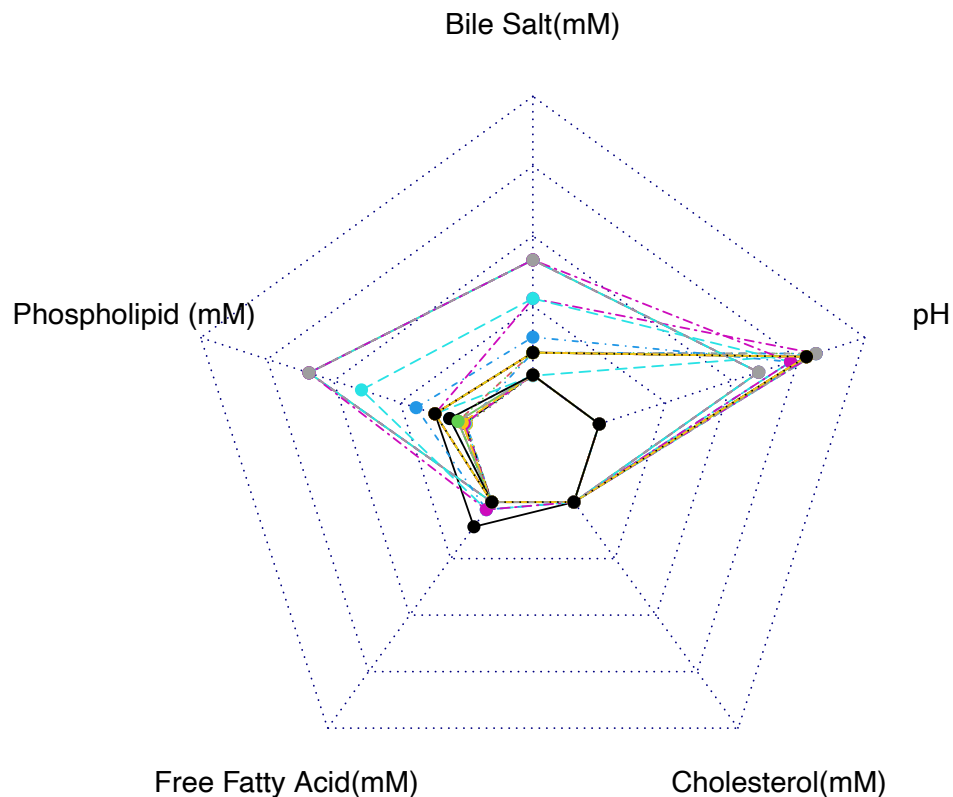


Figure 6 - Comparison of biorelevant media composition used in Caco-2 permeability studies reported in the literature.

Composition of the biorelevant media used within Caco-2 studies from the literature. The area of the radar plot is based on the HIF composition from Riethorst et.al, 2016²³. Serving as a physiological reference. Each coloured line represents a different media formulation from studies. Axis values are set as Maximum Values are Bile Salt value= 36.18mM, lecithin=6.32 mM, Free Fatty Acid= 15.03 mM Cholesterol= 0.48 mM, pH= 8.3. Axis. Minimum Values are Bile Salt= 0.12mM, lecithin=0.005 mM, Free Fatty Acid=0.0009 mM Cholesterol= 0.07 mM, pH= 2.41.

Published studies ^{43-46,51,53,59} indicate that SIF formulations used in Caco-2 permeability assessments predominantly contain bile salts and phospholipids, with only limited adjustment of pH, as illustrated in Figure 6. However, the SIF compositions used in these studies account for only approximately 25.8% of the variability observed in the Riethorst et al. dataset. This suggests that conventional formulations capture only a fraction of the

physiological diversity present in human intestinal fluid. Such limited representation may reduce physiological relevance and overlook variability in compound behaviour arising from differences in individual intestinal fluid composition.

It is important to note that the variability reported by Riethorst *et al.* was derived from a relatively small cohort of healthy adults with a limited age range. Although substantial variability was observed, this dataset may not capture the full extent of variability present across the wider population. Furthermore, intestinal fluid composition is influenced by additional factors beyond inter-individual differences, including diet, fed or fasted state and time of day. These considerations further support the need for SIF formulations that better reflect physiological variability when used in biopharmaceutical testing.

1.10. *Further modifications to enhance the biorelevance of the Caco-2 permeability assessment.*

Improvements to the biorelevance of the Caco-2 model extend beyond the composition of SIF transport media. *In vivo*, the intestinal epithelium is covered by a protective mucus layer composed primarily of mucins, which plays a critical role in regulating drug diffusion and interactions with the epithelial surface^{64,65}. However, this physiological barrier is often absent from *in vitro* permeability models. Consequently, incorporation of mucin into cell-based assays has the potential to increase the physiological relevance of the Caco-2 model by more closely replicating the intestinal environment and providing a more representative assessment of drug permeability.

Several approaches have been used to incorporate a mucus barrier into Caco-2 models, ranging from the direct addition of purified mucin to more complex co-culture systems with mucus-secreting cells and engineered biomimetic mucus hydrogels. Each approach offers specific advantages and limitations in reproducing the *in vivo* intestinal environment.

The simplest approach involves topical application of synthetic or purified mucin to Caco-2 monolayers before incubation with the compound of interest. This allows a mucin layer of

defined and controllable thickness to be applied⁶⁶. However, variability in mucin overlay models may arise from differences in mucin source, extraction and purification methods, batch-to-batch variability and application protocols across studies⁶⁷. Boegh *et al.* explored biosimilar mucus as a means of advancing Caco-2 models⁶⁷, whereas later studies, including Ye *et al.*, used porcine mucin⁶⁸.

Ye *et al.* incorporated mucin by dispersing it in simple transport buffers before applying it to the apical compartment of Transwell™ plates, where it was left to equilibrate and self-assemble into a gel-like coating across the epithelial surface⁶⁸. Following mucin application, transport studies were conducted using biorelevant media, further enhancing the physiological relevance of the experimental conditions. The authors reported that mucin improved monolayer stability in the presence of biorelevant media, reducing media-induced disruption often observed with FeSSIF and dynamic intestinal simulated fluids⁶⁸. When assessing a panel of 15 compounds, the effects of both mucin and media were drug-dependent: lipophilic compounds exhibited reduced permeability in the presence of mucin, whereas hydrophilic compounds showed comparatively minor changes⁶⁸. These findings demonstrate that mucin can substantially alter the permeability environment. However, this method of mucin application remains inherently limited. The resulting coating is structurally oversimplified compared with *in vivo* intestinal mucus, forming a relatively uniform layer that lacks layered organisation, regional heterogeneity and continuous turnover. The model also omits key biochemical components, including lipids, enzymes and antimicrobial peptides, and relies on purified mucin applied in an artificial manner. This can introduce inconsistencies in layer thickness and may exaggerate barrier effects, leading to non-physiological reductions in permeability for some compounds.

To overcome these limitations, co-culture models combining Caco-2 cells with mucus-secreting cell lines, such as HT29-MTX cells, have been explored to generate a more physiologically relevant and self-renewing mucus layer that better mimics the *in vivo* intestinal environment⁶⁶. Similar to the mucin overlay model developed by Ye *et al.*, co-culture models have demonstrated compatibility with both FaSSIF and FeSSIF media, further improving the physiological relevance of *in vitro* permeability assessment⁶⁹. Antoine *et al.*

demonstrated that incorporation of HT29-MTX cells resulted in formation of a stable mucus layer across the epithelial surface while maintaining monolayer integrity in the presence of biorelevant media⁶⁹. The resulting mucus barrier influenced drug permeability in a compound-dependent manner. Highly permeable lipophilic compounds, such as propranolol, were only marginally affected and retained efficient transport across the mucus layer, whereas hydrophilic compounds, such as mannitol, exhibited substantially reduced permeability compared with standard Caco-2 monocultures⁶⁹. These findings demonstrate that co-culture models can provide a more robust and physiologically relevant intestinal barrier than conventional Caco-2 models, yielding permeability data that may be more consistent with human intestinal behaviour.

Although co-culture models improve the physiological relevance of conventional Caco-2 monocultures, several considerations must be acknowledged. Caco-2 monocultures represent only the absorptive enterocyte population of the intestinal epithelium, whereas the native intestinal lining is composed of multiple specialised cell types that collectively contribute to barrier function and intestinal physiology⁷⁰. Despite their enhanced biorelevance, co-culture models remain subject to limitations. Mucin coverage may vary with passage number, culture conditions and the ratio of Caco-2 to HT29-MTX cells, which can limit reproducibility. Experimental optimisation is therefore essential, as excessive numbers of HT29-MTX cells can reduce transepithelial electrical resistance (TEER), compromise barrier integrity and lead to inaccurate permeability values. Hoffmann *et al.* demonstrated this in 2021, reporting a significant reduction in TEER in co-culture models compared with Caco-2 monoculture⁷¹. Zonula occludens-1 (ZO-1) is a key scaffolding protein that anchors transmembrane tight junction proteins to the intracellular cytoskeleton. In Caco-2 monolayers, ZO-1 staining was more intense at intercellular junctions, indicating strong localisation at cell-cell boundaries where it contributes to tight junction structure and barrier function⁷¹. By contrast, HT29-MTX cells displayed predominantly cytoplasmic ZO-1 staining at day 7, suggesting that the protein had not yet been fully recruited to junctional regions and remained more diffusely distributed within the cell⁷¹. With longer incubation, ZO-1 staining became more prominent at cell-cell junctions, indicating progressive recruitment of tight junction proteins and strengthening of barrier function. These observations suggest that ZO-

1 localisation changes over time in HT29-MTX cells, whereas Caco-2 cells show stronger junctional staining earlier in culture. This is relevant for understanding how different intestinal cell types regulate barrier properties. Finally, the mucus produced by HT29-MTX cells is predominantly associated with MUC5AC rather than MUC2, meaning that the resulting mucus layer is not fully representative of the *in vivo* intestinal environment⁷². Consequently, although co-culture models provide a more representative intestinal barrier than Caco-2 monocultures, they do not fully reproduce the composition and function of the native intestinal mucus layer.

Overall, the Caco-2/HT29-MTX co-culture model represents a significant advancement in replicating selected physiological characteristics of the small intestinal epithelium, offering improved relevance over conventional Caco-2 monocultures through incorporation of a mucus-producing cell population. However, limitations relating to reproducibility, optimisation of culture conditions, barrier maturation and differences in mucus composition mean that further mechanistic validation is required before these systems can be accepted as reliable tools for routine permeability assessment. Refinement of the Caco-2 monoculture model through incorporation of mucus-based adaptations alongside biorelevant fluids may therefore represent a practical and readily implemented strategy for improving the physiological relevance of *in vitro* permeability studies.

1.11. *Transporter expression in Caco-2 cells*

Beyond the incorporation of physiologically relevant factors, such as representative intestinal fluids and mucus, transporter expression in Caco-2 cells is a critical determinant of the model's ability to predict *in vivo* drug absorption. The intestinal epithelium acts as a semi-permeable barrier, with transcellular movement regulated in part by membrane transporter proteins. These proteins control both substrate uptake and efflux and therefore play an important role in determining oral drug absorption⁷³. Influx transporters, which are primarily located on the apical membrane, facilitate drug entry into epithelial cells and may increase systemic exposure⁷³. In contrast, efflux transporters actively transport APIs from epithelial cells back into the intestinal lumen, thereby reducing intracellular drug concentrations and limiting net absorption⁷³.

Following differentiation, Caco-2 cells exhibit many of the morphological and functional characteristics of mature small intestinal enterocytes. They express a range of membrane-bound transporter proteins involved in drug uptake and efflux. These transporters belong to two major superfamilies: ATP-binding cassette (ABC) transporters, which predominantly mediate drug efflux, and solute carrier (SLC) transporters, which facilitate uptake of a variety of endogenous compounds and xenobiotics^{74,75}. However, Caco-2 cells are inherently heterogeneous, and transporter expression can vary depending on passage number, culture conditions, differentiation state and laboratory-specific protocols. This variability complicates comparisons between studies because differences in transporter expression and activity may influence measured permeability and lead to inconsistent prediction of *in vivo* drug absorption.

Understanding the influence of passage number on transporter expression before seeding monolayers is important for determining the most appropriate cell age for experimental use. As passage number increases, transporter expression profiles may change as a consequence of repeated subculturing⁷⁶. Under standard Caco-2 culture protocols, differentiated monolayers are generally assumed to reflect the transporter phenotype of the seeded cell population, although this remains subject to intrinsic biological and experimental variability.

Drug transporters represent a complex determinant of intestinal permeability because many exhibit broad substrate specificity and can interact with structurally diverse compounds⁷⁷. Efflux transporters are particularly important because they can limit drug absorption by reducing intracellular drug concentrations and, consequently, systemic exposure. Accurate characterisation of transporter expression in Caco-2 models is therefore essential for improving the extrapolation of *in vitro* permeability data to *in vivo* absorption behaviour⁷⁸.

Ye *et al.* demonstrated in 1997 that increasing the passage number of Caco-2 monolayers was associated with changes in functional transporter expression and altered permeability characteristics, suggesting that high-passage cells may provide less physiologically relevant models⁷⁶. Differences were also observed in transepithelial electrical resistance (TEER), with higher-passage cells showing values consistent with tighter junction formation. Cell density after seven days of culture was also greater in high-passage cells, suggesting differences in growth rate and/or initial adherence. Passively transported mannitol was detected at higher concentrations in the basolateral compartment of lower-passage monolayers, consistent with the TEER data. By contrast, compounds transported transcellularly showed different permeability patterns, indicating that passage number may affect drug transport through changes in membrane transporter activity, tight junction integrity or cellular differentiation⁷⁶.

The authors suggested that these differences may be related to passage-dependent changes in transporter expression, with transporter expression decreasing as passage number increased. However, the study did not quantify transporter protein expression or directly compare Caco-2 transporter expression with human intestinal tissue. Aligning transporter expression in Caco-2 cells with human intestinal biopsies, and identifying which proteins change with increasing passage number, would strengthen the physiological relevance of the bench-side model.

A direct comparison of efflux transporter expression between Caco-2 cells and human intestinal biopsies was reported in 2001 using quantitative PCR⁷⁸. It was found that transcript levels of 90% of the ABC transporters correlated well between jejunum and Caco-2 cells ($r^2 = 0.90$). The expression of BCRP was 100-fold lower in Caco-2 cells compared with human

jejunum. The authors tentatively concluded that the Caco-2 cell line is a useful model of jejunal drug efflux, if the low expression of BCRP is taken into account⁷⁸.

Subsequent work by Englund and colleagues in 2006 investigated expression of selected ABC transporters, including P-glycoprotein, BCRP, MRP2 and MRP3, and SLC transporters, including PEPT1, MCT1, OATPB, OCTN2 and OCT1, along the human intestinal tract and compared these profiles with Caco-2 cells. The findings were consistent with the earlier Taipalensuu *et al.* study⁷⁸, with BCRP identified as one of the most abundantly expressed transporters across human intestinal biopsy samples. Importantly, transporter expression in Caco-2 monolayers showed a stronger relationship with small intestinal biopsies than with colonic tissue, supporting the use of Caco-2 cells as a model for pharmaceutical absorption despite their colonic origin⁷⁹. Given the difficulty of obtaining human small intestinal biopsies, this strengthens the value of Caco-2 cells as an *in vitro* model.

Further work on ABC and SLC transporter expression was reported by Seithel *et al.* in 2006. This study quantified mRNA levels for 15 uptake and efflux transporters in Caco-2 cells, human jejunum and human colon². Gene expression levels in Caco-2 cells were generally consistent across passages 29-43 and across different batches. However, expression of selected transporters, including PEPT1, MDR1, MRP2, OATP-B and BCRP, increased with culture time over one to five weeks⁷³. Eleven expressed genes showed a significant correlation between human jejunum and Caco-2 cells, although some transporters present in human tissue were not expressed in Caco-2 cells⁷³. The three most abundant jejunal transporters were, on average, expressed at levels 6.3-fold lower in Caco-2 cells than in jejunal biopsies. Despite these quantitative differences, the rank correlation between Caco-2 cells and jejunum was 0.699, compared with only 0.079 between Caco-2 cells and colon⁷³. These findings support the suitability of Caco-2 cells for investigating transporter-mediated drug permeation across the small intestinal lining, while emphasising the need to consider quantitative differences in transporter expression when interpreting permeability data.

In 2007, Maubon and colleagues investigated a broader panel of transporters by quantifying mRNA expression for 19 influx and efflux transporters in two independent Caco-2 cell clones

obtained from the European Collection of Authenticated Cell Cultures and the American Type Culture Collection⁸⁰. Characterising transporter expression across different Caco-2 sources provided insight into the interchangeability of these cell lines. Qualitative similarities were observed between the two cell sources, with a correlation coefficient of 0.94. However, 21.6% of transporters differed by more than two-fold between the two clones. When each Caco-2 source was compared with human small intestine, correlation coefficients were similar, with values of 0.69 and 0.65 for the European and American cell sources, respectively⁸⁰. Although this supports broad qualitative similarity, several transporters were quantitatively overexpressed or under expressed in Caco-2 cells compared with human small intestine, including higher expression of MRP2, MRP3, MRP4, MRP5, MRP6, OATP-A, OATP-B, OCT1 and MCT1, and lower expression of BCRP⁸⁰.

A multicentre comparison published in 2008 examined expression of 72 transporter genes, including 14 ABC transporters and 58 SLC transporters, in Caco-2 cells across ten laboratories⁸¹. Cells were cultured using each laboratory's in-house conditions and seeded for permeability experiments according to local protocols. Culture conditions varied between laboratories, including passage number, cell source, culture medium, seeding density, monolayer age and incubation conditions⁸¹. Most transporters were expressed at low levels, and, in many cases, expression did not vary by more than two orders of magnitude across samples. Eleven of the 14 ABC transporters were detected in all cell samples, and five of the eight OATP transporters were detected in all laboratories. Although absolute expression values were variable, overall expression patterns were more consistent, as shown by rank correlation analysis⁸¹.

When transporter expression was compared between laboratories, some genes showed substantial variation, suggesting that experimental protocol may influence transporter expression. Although culture conditions were broadly similar in terms of medium composition and passaging procedures, passage number varied considerably, ranging from 24 to 98⁸¹. Higher passage numbers may increase the risk of cellular senescence and altered expression profiles, including potential changes in inflammatory markers. The influence of passage number appears to be transporter-dependent: some transporters, such as PEPT1,

showed little variation, whereas others, including MRP2, increased between passages 25 and 98⁸¹. BCRP expression was generally similar across most samples but differed markedly at the extremes of the passage range, with lower expression observed at higher passage numbers. These findings suggest that variation in transporter expression may reflect both inter-laboratory variability and differences in cell passage, although earlier work across passages 29-43 reported no statistically significant passage-dependent differences^{73,80,81}.

In addition to passage number, monolayer age, defined as the time after seeding before experimentation, varied between laboratories. These differences can influence transporter expression. For example, MRP2 expression was lower in younger monolayers, such as those cultured for five days, than in older monolayers cultured for up to 27 days, while the greatest variation was reported for the IBAT transporter⁸¹. Understanding how these culture-related variables affect transporter expression and permeability of standard compounds is important for improving the reliability, reproducibility and interpretation of Caco-2 permeability assessments.

Evidence across studies indicates that culture conditions can influence transporter expression. For example, one study reported similar BCRP mRNA levels in Caco-2 cells and human samples, contradicting other studies in which BCRP showed one of the largest differences between *in vitro* and human tissue expression^{78,79,82}. These discrepancies highlight the importance of characterising transporter expression within the specific Caco-2 model being used, particularly when interpreting the role of transporters in oral drug permeation. Taipalensuu *et al.*⁷⁷ and Englund *et al.*⁷⁸ used high-passage cells, whereas Gutmann *et al.*⁸¹ used passage 42. Such differences in experimental set-up may account for the variation observed in transporter expression.

Collectively, these studies demonstrate the value of Caco-2 cells as a model of the small intestine, while also showing that transporter expression does not fully replicate human intestinal tissue. Figure 7 summarises reported differences in transporter expression between Caco-2 cells and human biopsies across published studies^{73,78-80}. Where data are available, higher expression in Caco-2 cells is shown in green, lower expression in Caco-2

cells is shown in red and comparable expression is shown in amber. It is important to note that these studies primarily used qPCR-based methods to quantify gene expression. Although useful, mRNA expression does not necessarily reflect functional protein abundance because post-transcriptional and post-translational processes, including alternative splicing, trafficking and protein turnover, can regulate transporter abundance and activity. Direct protein-level analysis therefore provides a more complete representation of the transporter landscape at the Caco-2 membrane and may offer a stronger basis for comparison with human intestinal tissue.

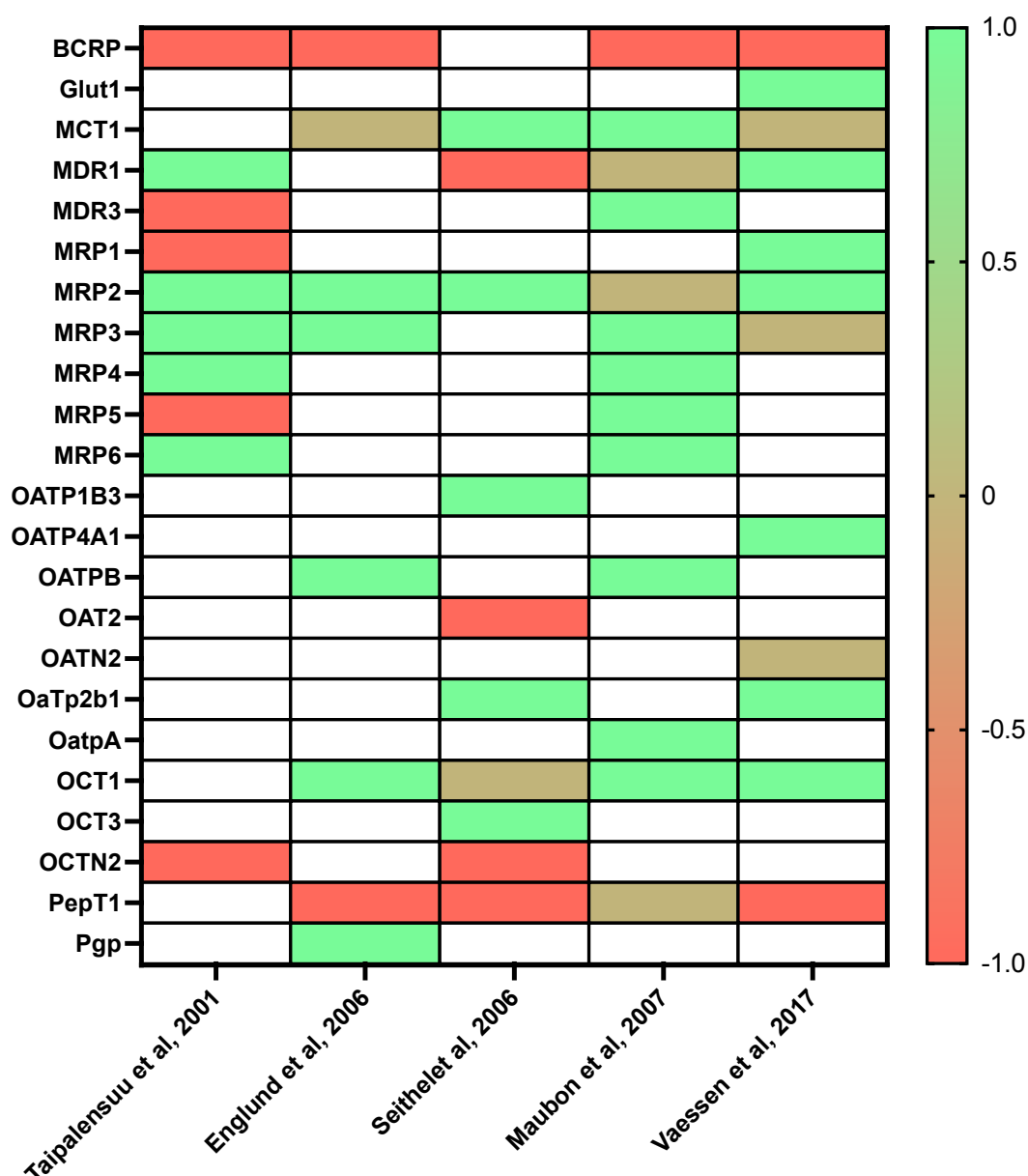


Figure 7 - Heat map summarising reported expression of intestinal transporter protein across in vitro models within the literature.

Rows represent individual transporter proteins and columns represent the different experimental studies. Transporter expression levels are coloured according to their relative abundance correlating in vitro to in vivo. Red indicating low expression in Caco-2 cells when compared to human, amber similar both in vitro and in vivo and green indicating higher expression in vitro than in vivo. Areas of no coloured means no data reported or available for this transporter.

1.12. Incorporation of Caco-2 permeability data into PBPK models

Outputs from Caco-2 permeability studies are frequently incorporated into physiologically based pharmacokinetic (PBPK) models to support prediction of absorption, distribution, metabolism and excretion (ADME) properties in humans and other species. PBPK modelling is a mechanistic mathematical approach in which the body is represented as a series of compartments corresponding to specific organs or tissues. Each compartment is assigned defined physiological characteristics, such as volume, blood flow and tissue composition, and mass-balance equations are used to describe drug movement into and out of each compartment⁸³.

PBPK modelling typically follows a bottom-up approach, in which drug-specific parameters, including solubility, permeability, metabolism and plasma protein binding, are integrated with physiological data to simulate drug disposition. For orally administered compounds, the absorption component of the model is commonly informed by *in vitro* measurements, including apparent permeability coefficient (P_{app}) values obtained from Caco-2 monolayer studies.

Caco-2-derived P_{app} values provide an estimate of how readily a compound crosses an epithelial barrier. Within PBPK software, these values can be scaled to estimate effective human intestinal permeability, which is a key input for predicting oral drug absorption. Once incorporated, the PBPK model can simulate the fraction of drug absorbed and generate predictions of oral bioavailability before progression to animal or human studies. This provides valuable early-stage insight into drug performance and supports decision-making during development⁸⁴.

Mathematical modelling is increasingly central to drug discovery and development because it enables prediction of human exposure following oral and intravenous dosing. PBPK models provide a mechanistic framework that integrates anatomical and physiological parameters, such as organ volumes, blood flow and enzyme or transporter expression, with drug-specific properties to describe ADME processes and support *in vitro-in vivo* extrapolation (IVIVE). These models are usually multi-compartmental, with compartments parameterised

according to organ or tissue volume and perfusion. Depending on drug properties and biological constraints, tissue distribution may be described as perfusion-rate limited or permeability-rate limited. For permeability-rate-limited tissues, intracellular and extracellular spaces are separated by a diffusional membrane barrier, making effective intestinal permeability a critical input for absorption modelling⁸⁵.

Therefore, inclusion of well-characterised Caco-2 permeability measurements in PBPK models can be highly informative, provided that the input data reflect human physiological conditions as closely as possible. Improving bench-side permeability assessments through incorporation of biorelevant elements, such as simulated intestinal fluids, mucus layers and co-culture systems, may generate more representative permeability values for integration into PBPK models⁸⁶. The use of PBPK modelling to predict drug bioavailability offers a higher-throughput alternative to traditional experimental approaches and can streamline early-stage evaluation. Furthermore, understanding how biorelevant components influence both solubility and permeability is essential for guiding formulation strategies, enabling more targeted optimisation of drug delivery and therapeutic performance.

1.13. Hypothesis

The central hypothesis of this thesis is that enhancing the biorelevance of media used in *in vitro* intestinal permeability testing, including modulation of the apical transport medium, will improve the relationship between *in vitro* experimental outputs and *in vivo* drug performance. By better reflecting the composition and physicochemical complexity of the gastrointestinal environment, biorelevant media may generate permeability and solubility data that are more representative of physiological conditions. This, in turn, may support more accurate prediction of active pharmaceutical ingredient (API) behaviour and help to bridge the translational gap between *in vitro* testing and *in vivo* outcomes.

1.13.1. Aims and Objectives

The overall aim of this thesis is to evaluate the behaviour of a selected group of APIs using fasted-state biorelevant media as transport media in permeability testing. Specifically, this research aims to develop an analytical method capable of quantifying multiple components within a design-of-experiments-derived simulated intestinal fluid suite, assess the compatibility of these media with the current gold-standard Caco-2 permeability model, and determine how API behaviour in fasted-state media compares with behaviour in conventional aqueous transport solutions. In addition, this thesis investigates the effect of Caco-2 passage number on transporter protein expression and compares these profiles with paediatric intestinal tissue samples to support evaluation of model relevance for paediatric drug absorption prediction.

The main aim is addressed through four experimental chapters, organised as follows:

Chapter 2 aims to develop a gas chromatography-mass spectrometry (GC-MS) analytical method for the simultaneous quantification of four selected components of the design-of-experiments-derived Strathclyde suite of fasted-state simulated intestinal fluids.

Chapter 3 evaluates the compatibility of Caco-2 cells with Strathclyde biorelevant media to determine their suitability for use in permeability studies. This chapter enables screening and assessment of the media within an *in vitro* intestinal absorption model. The most compatible

media are then selected for subsequent investigation of their impact on API absorption behaviour compared with conventional transport media.

Chapter 4 investigates the impact of changing the transport medium on API behaviour and compares these findings with conventional Hanks' Balanced Salt Solution (HBSS) transport media. This chapter provides a detailed evaluation of how fasted-state biorelevant intestinal conditions influence drug absorption.

Chapter 5 examines changes in transporter protein expression across different Caco-2 passage numbers. As altered transporter expression may influence drug absorption behaviour, this chapter aims to identify whether an optimal Caco-2 passage range can be defined for permeability studies. Comparison of transporter expression profiles with paediatric intestinal tissue samples will also provide greater confidence in the suitability of these *in vitro* models for predicting paediatric drug absorption behaviour.

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**Chapter 2- Development and validation of a
method for the quantitative analysis of
components in Simulated Intestinal Fluid.**

2.1. Introduction

Simulated intestinal fluids are widely used in a range of *in vitro* methods to predict the performance of drugs and drug products. As intestinal fluid composition is inherently dynamic, these media often undergo changes in factors such as pH, composition, and viscosity. Consequently, they represent a critical component of biopharmaceutics research and drug product evaluation. However, the complexity and variable nature of SIF presents analytical challenges requiring a robust and reliable method due to the multiple organic and inorganic constituents present.

Given that bile components are known to undergo reabsorption during intestinal transit and that drug solubilisation is facilitated through interactions with colloidal structures with intestinal fluids, evaluating the permeability behaviour of SIF constituents alongside pharmaceutical compounds may provide a more physiological relevant assessment of drug absorption processes¹. Despite the widespread application of simulated intestinal fluids, analytical investigations have primarily centred on confirming media composition and evaluating drug behaviour within the media. Comparatively few studies have sought to quantitatively characterise the individual constituents of the media themselves. Developing a robust analytical method capable of accurately measuring SIF components could be critical for improving the understanding of media stability, compositional changes during experimental producers and interactions between the media components and pharmaceutical compounds.

The heterogenous composition of SIF presents several analytical challenges², its constituents vary considerably in molecular weights, polarity, concentration and ionisation, behaviour, while the presence of salts lipids and surfactant like molecules can contribute to matrix effect which may compromise quantitative performance^{3,4}. Conventional analytical approaches typically require separate assays for different classes of compounds, increasing analytical complexity, extending analysis time, and introducing additional sources of variability. In recent years, mass spectrometry (MS) has become increasingly prevalent as an analytical technique across a wide range of disciplines. MS provides an opportunity to

develop a unified platform capable of simultaneously identifying and quantifying multiple components with high selectivity and specificity.

2.1.1. Analytical Processes

MS has evolved from a technique primarily used for the analysis of small volatile molecules to one of the most powerful analytical techniques in pharmaceutical and biological sciences. Advances in instrumentations, particularly development of soft ionisation techniques and improvement of accessibility, have enabled the analysis of complex biomolecules including proteins, lipids and peptides⁵.

Chromatography is commonly coupled with MS to separate analytes prior to ionisation, improving both sensitivity and specificity. Separation is achieved through differential interaction between analytes, stationary phase and mobile phase⁶. Two principal techniques of chromatography are gas chromatography (GC) and liquid chromatography (LC), which differ according to the mobile phase employed. GC is best suited for volatile, thermally stable compounds, whereas LC is more appropriate for polar non-volatile and thermally labile molecules^{7,8}. The mobile phase moves across the stationary phase encouraging separation, since analytes have different affinities for the mobile or stationary phase⁹. LC mobile phase is typically composed of aqueous and organic solvents, where the composition of each is optimised to achieve efficient analyte separation⁹. Factors such as solvent composition, pH and gradient profile all influence the retention time of the analytes on the stationary phase¹⁰. Thus influence the separation of the analytes and peak shape, making mobile phase selection a critical step in method development to ensure effective quantitative performance^{9,10}.

Contrastingly, GC uses a gaseous mobile phase typically an inert gas such as helium or nitrogen, to transport analytes across the stationary phase¹¹. Separation across GC is effective and is governed by differences in analyte volatility along with interactions with the stationary phase. GC is often used for flavour and aromatic analysis.

The stationary phase in GC consists of particles coated either with a liquid or solid phase. Solid phase columns are primarily impacted by such as surface area and pore size, where smaller pores and increased surface area generally result in greater retention times due to enhanced interactions¹². These stationary phases offer added flexibility as they can be modified with functional groups which then can promote specific interactions to increase elution¹². Selection of an appropriate stationary phase is critical consideration in chromatography. Understanding the compatibility between the stationary phase and target analytes is optimal for achieving chromatographic performance.

Biological matrices are inherently complex and often require sample preparation to ensure compatibility with both the chromatographic system and the analytical method employed¹³. Sample preparation is particularly important within GC because analytes must be sufficiently volatile and thermally stable to undergo vaporisation without degradation. Derivatisation is therefore commonly employed to chemically modify analytes, improving their stability and suitability for GC analysis¹⁴. Derivatisation is particularly relevant for simulated intestinal fluids (SIFs), which contain numerous highly polar and thermally labile compounds that exhibit poor volatility and may interact strongly with the stationary phase. By chemically modifying reactive functional groups, derivatisation enhances thermal stability, reduces polarity, improves chromatographic separation, and increases mass spectrometric detectability. Silylation is the most widely used derivatisation strategy in GC-MS, with reagents such as BSTFA and MSTFA converting polar functional groups into trimethylsilyl derivatives that are more amenable to GC separation and detection separation^{14,15}. The differing reactivities of these reagents towards specific functional groups enable the selective modification of biomolecules, supporting tailored analytical workflows for complex biological and biorelevant matrices¹⁵.

Following chromatographic separation, analytes are transferred onto the MS where they are identified and quantified on their mass-to-charge ratio (m/z)¹⁶. Prior to detection, analytes are ionised in either positive or negative mode as only charged molecules are detected within the MS^{17,18}. These charged ions may undergo fragmentation, generating characteristic product

ions that aid compound identification¹⁷. MS can only detect charged molecules since it applies an electric field which focuses the particles onto mass analysers neutral molecules can't be detected since they carry no net charge so can be separated on their mass-to-charge ratio and therefore are not quantifiable¹⁸.

2.1.2. Challenges around GC-MS

Current analytical approaches remain limited in their ability to comprehensively characterise simulated intestinal fluids (SIFs). Although validated methods exist for the quantification of pharmaceutical compounds within intestinal media, no unified methodology has been reported that enables the simultaneous analysis of both drug substances and the diverse constituents of SIF. Mass spectrometry-based methods have been applied to selected intestinal components, such as bile acids and gut microbial metabolites; however, a holistic analytical platform capable of quantifying the major physicochemical constituents of intestinal fluid remains unavailable. This represents a significant gap in the analytical characterisation of biorelevant media¹⁹⁻²¹.

The development of a unified analytical method capable of simultaneously quantifying both pharmaceutical compounds and the major constituents of simulated intestinal fluids would significantly enhance understanding of media composition and its influence on transport across Caco-2 cell monolayers. Such an approach would enable the concurrent assessment of the permeability of the drug and individual SIF components, providing mechanistic insight into differences in material transport across the epithelial barrier and the potential interactions between media constituents and drug absorption. Furthermore, increased analytical throughput would facilitate larger and more comprehensive studies, accelerating progress in this area of research.

2.2. Aims and Objectives

A gas chromatography-mass spectrometry (GC-MS) method was developed for the simultaneous quantification of bile salts, phospholipids, free fatty acids and cholesterol, as GC-MS offers several advantages over LC-MS for this application. Following derivatisation while higher operating temperatures reduce the likelihood of column carry over. In additions this will increase chromatographic performance making it a suitable platform for quantification of the complex biomatrices.

Preliminary investigations explored the use of liquid chromatography-mass spectrometry (LC-MS) for the separation and quantification of simulated intestinal fluid (SIF) components. However, method development was hindered by the strong affinity of several analytes for the stationary phase, resulting in excessive retention, incomplete analyte recovery, and prolonged wash-out periods. In addition, the accumulation of retained material compromised column performance, leading to carryover effects and increasing the frequency of column cleaning and maintenance. Owing to these challenges, LC-MS was deemed unsuitable for the simultaneous analysis of all target SIF constituents, prompting the exploration of GC-MS as an alternative analytical platform⁵.

This research describes the development and optimisation of a GC-MS method for the simultaneous analysis of key components of simulated intestinal fluid (SIF). A series of optimisation studies were undertaken to establish suitable chromatographic and sample preparation conditions, including temperature programmes, injection parameters, and derivatisation protocols. The primary objective was to develop a single analytical method capable of detecting and quantifying the four principal SIF constituents, namely bile salt, cholesterol, lecithin, and sodium oleate, within a single injection. A secondary objective was to ensure sufficient sensitivity for the quantification of these components in samples collected from the receiver compartment of Caco-2 permeability assays, where low analyte concentrations place particular importance on method detection limits.

2.3. Materials and Methods

2.3.1. Materials

The following reagents and chemicals were used throughout this study. Sodium taurocholate, sodium oleate, and cholesterol were procured from Merck Chemicals Ltd. Soybean Lecithin (purity 98%) was supplied by Lipoid® Germany. Chloroform was supplied by Rathburn Chemical®. Biorelevant media (FaSSIF-V2) was purchased from biorelevant.com Ltd. Sodium phosphate monobasic monohydrate and sodium chloride ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) were purchased from Fisher Scientific.

The derivatisation reagents consisted of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and Dithioerythritol (cat no. 10197777001) were purchased from Merck (Gillingham, UK), and ammonium iodide from Thermo Fisher (Hertfordshire, UK). 1,2,4 Butanetriol, used as the internal standard for the instrument, was also purchased from Thermo Fisher (Hertfordshire, UK).

2.3.2. Stock Preparation

Individual stock solutions (1 mg/ml) of sodium taurocholate, sodium oleate, cholesterol and, soybean lecithin was prepared for compound identification. Each compound was dissolved in a 50:50 (v:v) ethanol: chloroform mixture. Following dissolution, the solvent was evaporated under a gentle stream of nitrogen until a thin film remained on the inner surface of the HPLC vial. The dried residue was subsequently reconstituted in 800 μl of MTBE containing 0.5 $\mu\text{g}/\text{ml}$ of internal standard. The resulting solution was used as the working stock for derivatisation and GC-MS analysis.

Stocks were derivatised using MSTFA, 200 μl of MSTFA was added directly to each HPLC vials, samples were incubated at 60 °C for 30 minutes before being allowed to cool at room temperature. Samples were then filtered through a 0.22 μm PTFE filter (50 mm). The entire filtrate was retained for analysis. Where 1 μl of derivatised sample was diluted into 1000 μl of MTBE to produce final concentration of 1 $\mu\text{g}/\text{ml}$ sample.

2.3.3. Simulated intestinal Fluid

Commercial FaSSIF-V1 was prepared according to the manufacturer's instructions (Biorelevant.com, UK), composition of the commercial medium is shown in Table 2.

Table 2 - Composition of biorelevant.com media.

Component	Substance	Concentration (mM)
Bile Salt	Sodium Taurocholate	3
Phospholipid	Lecithin (Soybean)	0.75
pH	Adjusted with acid/base	6.64

Media 1 from the suite of Strathclyde intestinal fluids was selected as a more complex SIF variant compared to commercial media²²⁻²⁵. Media 1 was selected from the suite as this had the lowest total amphiphile concentration and would minimise potential saturation of GC-MS detectors^{26,27}. Media 1 simulated intestinal fluid contains the concentrations as shown in Table 3.

Table 3- Concentrations of the Media 1 Simulated intestinal fluid from^{26,27}.

Component	Substance	Concentration(mM)
Bile Salt	Sodium Taurocholate	1.06
Phospholipid	Lecithin (Soybean)	0.16
Free Fatty Acid	Sodium Oleate	1.04
Cholesterol	Cholesterol	0.01
pH	Adjusted with acid/base	6.64
Sodium Chloride		
Monophosphate Buffer		

Stock solutions of the three primary lipid components — sodium taurocholate (bile salt), soybean lecithin (phospholipid), and sodium oleate (free fatty acid) — were prepared at 15× the target concentration by weighing out each component and dissolving them together in 3 mL of chloroform.

A separate cholesterol stock solution was prepared at 1500× the target concentration by dissolving cholesterol in 10 mL of chloroform. From this stock, 100 µL was transferred and combined with the bile salt/phospholipid/free fatty acid chloroform solution, and the combined solution was mixed thoroughly to ensure homogeneity.

The combined lipid solution was then dried under a gentle stream of dry nitrogen gas until all chloroform had evaporated and a thin, uniform lipid film remained on the vessel wall.

The resulting lipid film was reconstituted in 4 mL of deionised water and mixed thoroughly to fully resuspend the film. The reconstituted solution was then quantitatively transferred to a 5 mL volumetric flask, and the volume was adjusted to 5 mL with deionised water to give the final working media.

Prior to addition of sample to GC-MS the sample was diluted in 1:1000 in MTBE spiked with internal standard. This diluent is then treated with the derivatisation agent.

2.3.4. GC-MS Conditions

Chromatographic analysis was performed using a Shimadzu GC-MS system comprising of a Nexis GC-2039 gas chromatograph, coupled to a GC-MS-TQ8040 NX mass spectrometer (Shimadzu, Kyoto, Japan).

Chromatographic separation was achieved on a SH-I-5MS fused silica capillary column (30m x 0.23mm i.d, 0.25 μ m film thickness). Samples were introduced by splitless injection, with helium employed as the carrier gas at a constant flow rate of 1.58 mL/min. The injection needle and detector temperatures were maintained at 260 °C and 250 °C respectively.

The oven temperatures program was as follows: an initial temperature of 50 °C was held for 2 min, then increased to 140 °C at a rate of 40 °C/min and held for 1 min; the temperature was subsequently raised to 290 °C at 20 °C/min and held for 1 min, followed by a final ramp to 320 °C at 15 °C/min. The total chromatographic run time was 20 min.

Mass spectrometric detection was performed with ion source temperature at 220 °C and interface temperature at 300 °C. Initial analyte detection was conducted in scan mode over a mass range of m/z 70–700. A full-scan spectrum (m/z 50–800) was subsequently acquired for each compound, and the three most abundant fragment ions were selected for analysis: the most intense ion was used for quantification, while the remaining two ions served for qualitative confirmation.

2.3.5. Method Validation

2.3.5.1. *Linearity and Calibration Curve*

Method linearity was assessed over a concentration range of 0.5–50 µg/mL, using seven calibration levels (0.5, 0.8, 1, 10, 20, 30, and 50 µg/mL). Calibration curves were constructed across this range with the internal standard maintained at a fixed concentration of 0.5 µg/mL. Linearity was evaluated based on the correlation coefficient (R^2) of the calibration curve, with an acceptance criterion of $R^2 \geq 0.95$.

2.3.5.2. *Specificity*

Method specificity was evaluated by analysing blank matrix samples (matrix without analyte) alongside samples spiked with the analyte and potential interfering substances. Specificity was confirmed when analyte peaks were well resolved and free of significant interference at the corresponding retention times.

2.3.5.3. *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The LOD and LOQ were determined by analysing progressively decreasing analyte concentrations, with quality control (QC) samples prepared at 0.15, 0.2, and 0.3 µg/mL. The LOD was defined as the lowest concentration at which the analyte could be reliably detected, while the LOQ was defined as the lowest concentration at which the analyte could be quantified with acceptable precision and accuracy.

Detection and quantification limits were established in terms of analyte concentration rather than as a signal-to-noise ratio. This approach provides a defined value for the lowest analyte level that can be reliably measured on the instrument and improves comparability of LOD and LOQ values across laboratories, as it is less susceptible to variability in instrument conditions such as baseline noise and detector sensitivity, which more directly influence signal-to-noise-based estimates.

2.3.5.4. *Accuracy and Precision*

Accuracy and precision were assessed in accordance with the ICH M10 guideline on bioanalytical method validation. QC samples were prepared at low, medium, and high concentrations and analysed in six replicates. The acceptance criterion for accuracy was $\pm 15\%$ of the nominal concentration, except at the lower limit of quantification (LLOQ), where $\pm 20\%$ was accepted. Precision was assessed as intra-day and inter-day variability across three separate days, with an acceptance criterion of $\leq 15\%$ coefficient of variation (CV%) at all concentrations except the LLOQ, where $\leq 20\%$ CV was accepted.

Precision was determined by calculating the percentage relative standard deviation (%RSD) of the peak area ratio between each analyte and the internal standard, obtained from six replicate injections of a mixed standard solution. This assessment confirmed the reproducibility of the method across repeated measurements and verified system suitability for precision testing.

2.4. Results

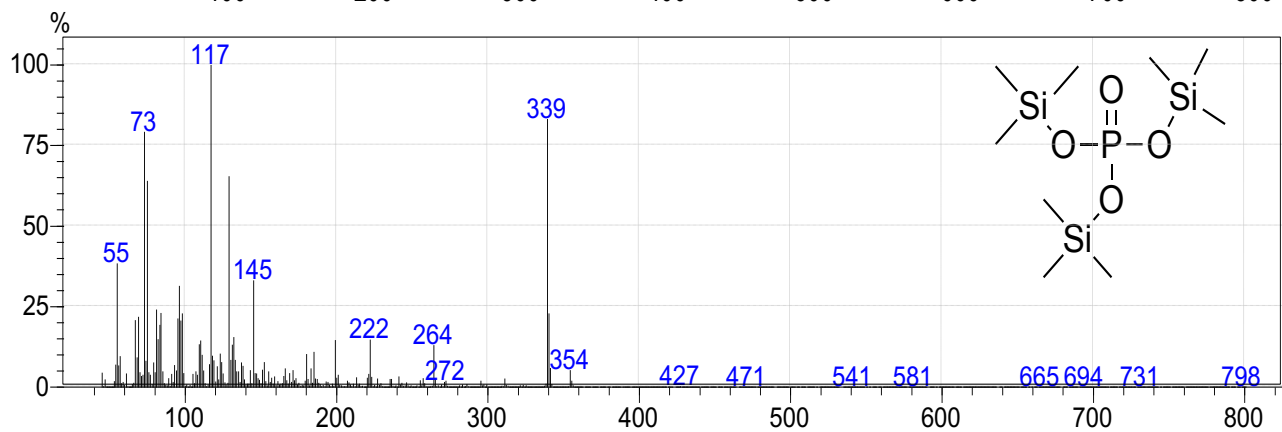
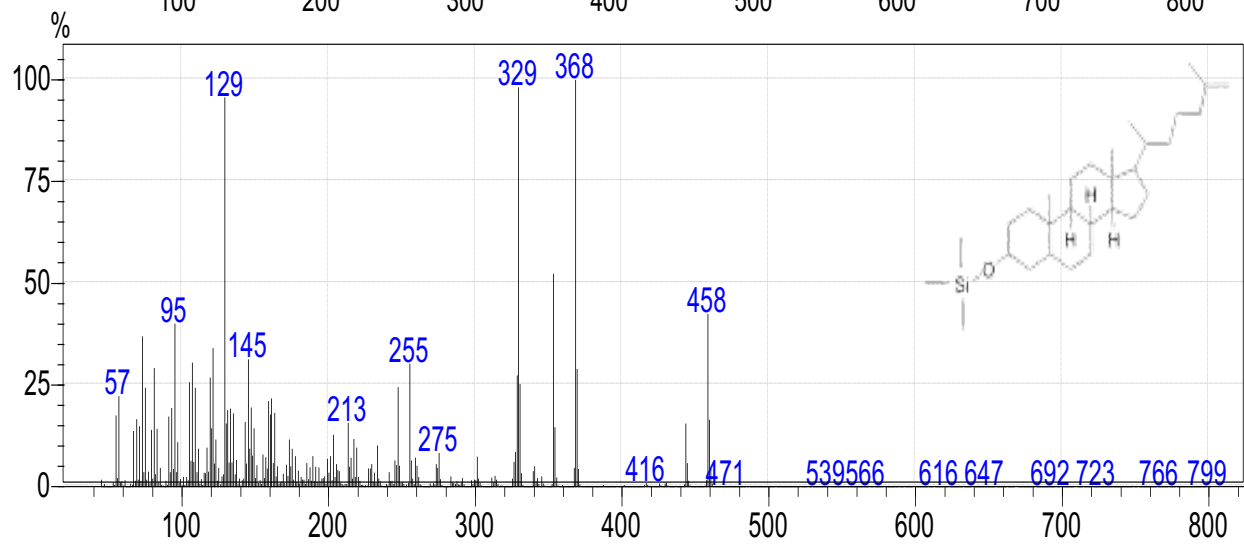
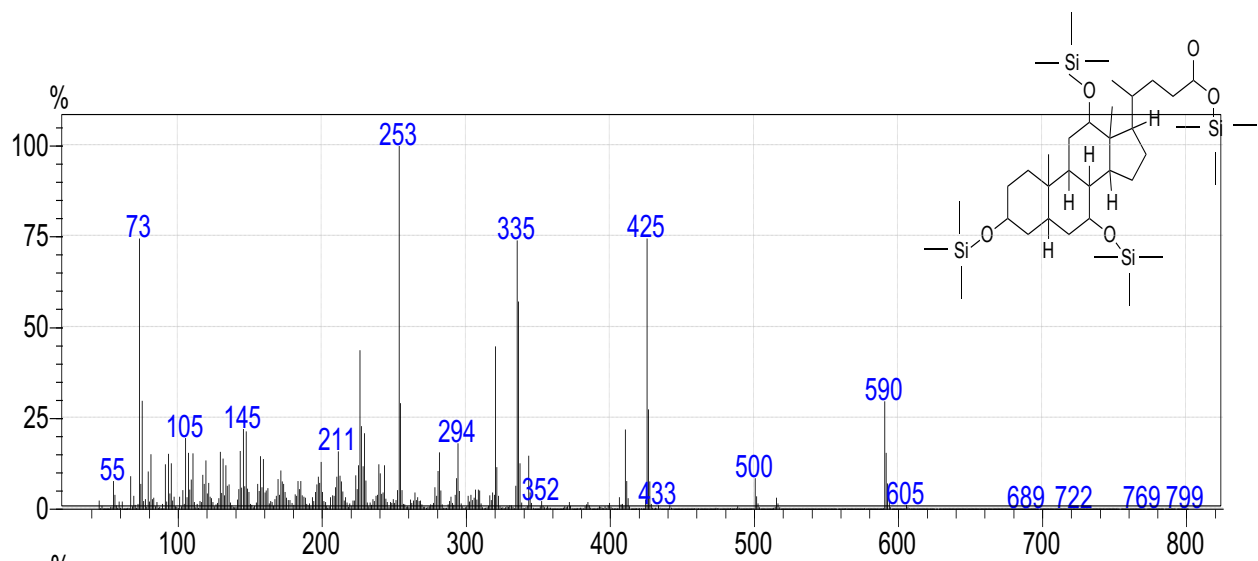
Method development and validation were guided by chromatographic outcomes, adopting a bottom-up approach to achieve optimal resolution of analytical compounds while maintaining an acceptable run time, target <45 minutes given the intended high-throughput nature of this method.

2.4.1. Optimisation of GC-MS parameters

2.4.1.1. *MS detection optimisation*

The quantification (m/z) of the selected peaks of interest is as follows: m/z of 425 for sodium taurocholate, 368 for cholesterol, 339 for sodium oleate, 299 for lecithin and 322 for 1,2,4 butanetriol (IS).

Selected ion monitoring (SIM) mode was used because it can perform a simultaneous determination with limited interferences. The full mass spectra of compounds are in Figure 8.



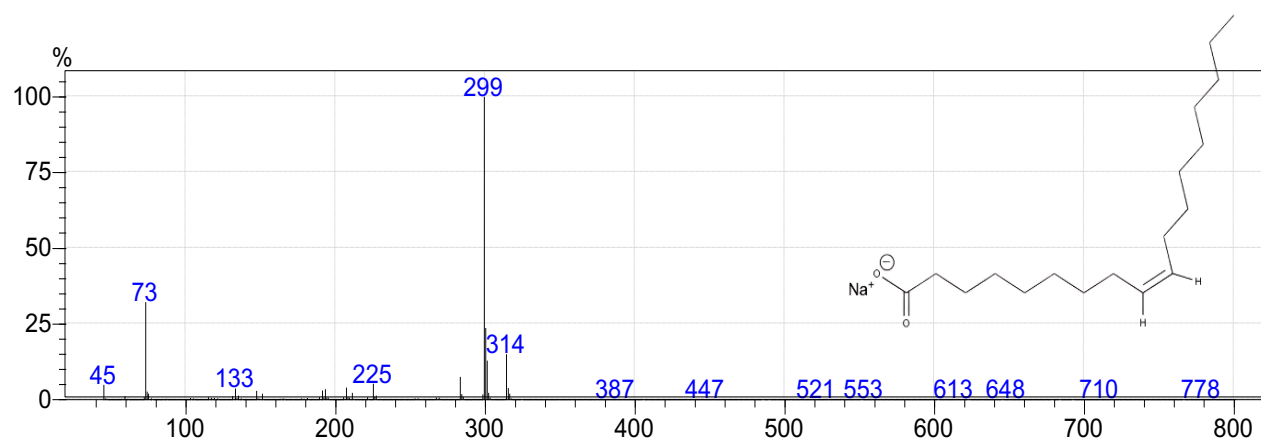


Figure 8 - Respective mass spectra of four compounds of interest.

Top panel: Sodium taurocholate — The spectrum shows the characteristic deprotonated molecular ion $[M-H]^-$ and prominent fragment ions arising from cleavage of the taurine side chain and steroid nucleus. **Second panel: Cholesterol** — The spectrum displays the expected low-intensity molecular ion and dominant fragment ions corresponding to dehydration and steroid-ring fragmentation, consistent with its known EI/MS behaviour. **Third panel: Lecithin (phosphatidylcholine)** — The spectrum is dominated by the phosphocholine headgroup fragment (m/z 184) alongside neutral-loss peaks typical of phospholipid fatty-acyl cleavage. **Bottom panel: Oleic acid** — The spectrum shows a clear $[M-H]^-$ ion and characteristic fragments associated with allylic cleavage along the C18:1 chain.

Table 4- Selected ion monitoring (SIM) parameters and transition quantifications for each of the compounds.

Compound	MW (g/mol)	Product Mass (m/z)				Retention Time (minutes)
Lecithin	758	314	299	133		6.31
Sodium Oleate	304.44	354	339	224		11.80
Cholesterol	386.65	458	369	329		16.59
Sodium Taurocholate	537.68	690	426	343	253	17.74
1,2,4 Butanetriol (IS)	106.12	322	219	107	72	6.92

For the quantification and qualitative confirmation of lecithin, sodium oleate, cholesterol, sodium taurocholate and 1,2,4 Butanetriol. Each analyte, the precursor ion was selected based on the most abundant fragment observed in full-scan mode (m/z 50–800), and the corresponding quantifier and qualifier product ions were subsequently monitored in SIM mode to enable selective and sensitive detection.

2.4.2. Optimisation of sample preparation

2.4.2.1. Optimisation of derivatisation conditions

Initial development focused on selection of an appropriate derivatisation reagent. A literature search indicated MSTFA was likely to be suitable, as it is more volatile than BSTFA²⁸. Although BSTFA was used by Riethorst *et al.*, that method was optimised for cholesterol alone; given the primary aim of the present work was to resolve and quantify all components of the media, a derivatisation agent with broader applicability was considered more appropriate²⁰. The reduced retention time typically associated with MSTFA provided further justification for its selection as the derivatisation agent of choice for this method²⁹.

Initially, 100 µL of MSTFA mix was combined with 900 µL of MTBE diluent and incubated for 60 minutes at 100 °C. However, the resulting MS peak shapes were suboptimal, with poor detection and peak shape observed for sodium taurocholate in particular. These extreme

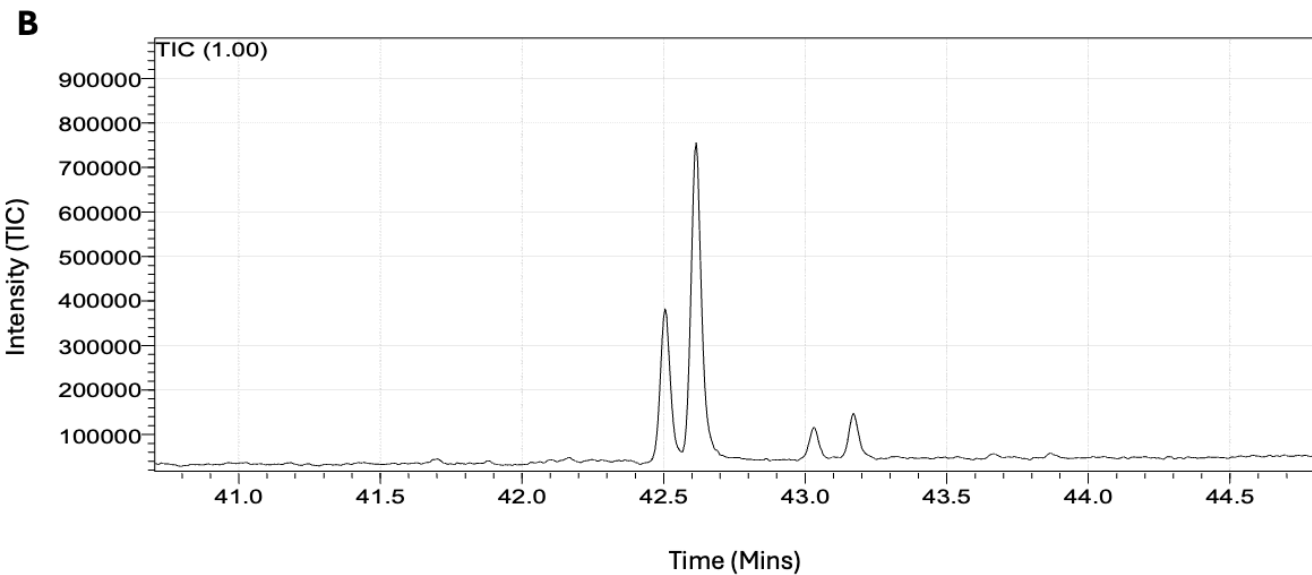
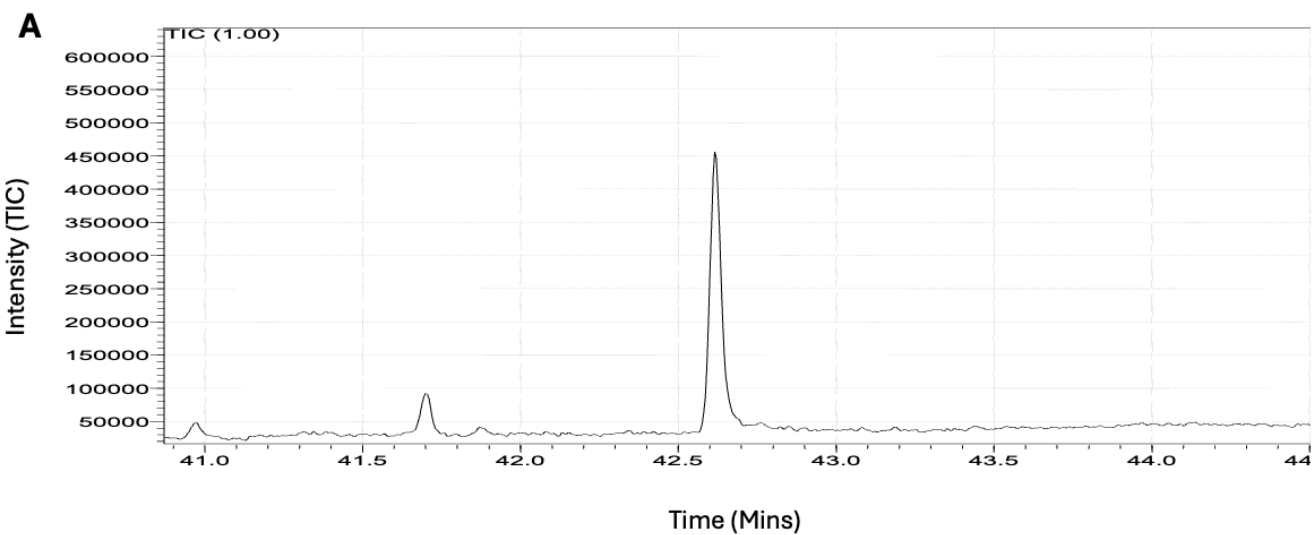
conditions may have caused over-derivatisation of sodium taurocholate, with the elevated incubation time and temperature potentially contributing to analyte loss and the resulting reduction in detection. A review of the literature indicated that, when analysing sodium taurocholate or structurally similar compounds, incubation conditions — both temperature and time — were typically lower than those used in the initial protocol³⁰. Based on these findings, the derivatisation protocol was revised, and incubation conditions were varied to explore their influence on analyte identification. As all other components were successfully resolved under the initial conditions, sodium taurocholate was isolated for further investigation to assess the impact of varying conditions on chromatography performance and to minimise analyte loss.

Various conditions were systematically tested, including different volumes of derivatisation agent and varying incubation times and temperatures. Each condition was tested in triplicate to ensure reproducibility. The optimised derivatisation conditions which produced the best peak separation and detection in the QC were selected as method conditions.

Initially temperature of derivatisation was reduced from 100 °C to 60 °C across all conditions (Figure 9-Figure 12). Across all trials, the reduced temperature resulted in increased detection of sodium taurocholate, suggesting the higher temperature had shifted the reaction away from equilibrium, preventing optimal derivatisation.

Assessment across two incubation times; 30 minutes (Figure 9 and Figure 10) and 60 minutes (Figure 11 and Figure 12) to determine whether a longer incubation period provided an improved yield by allowing a more complete reaction, or conversely reduced peak intensity through decomposition of products or reactants.

200µl of MSTFA, 30-minute incubation



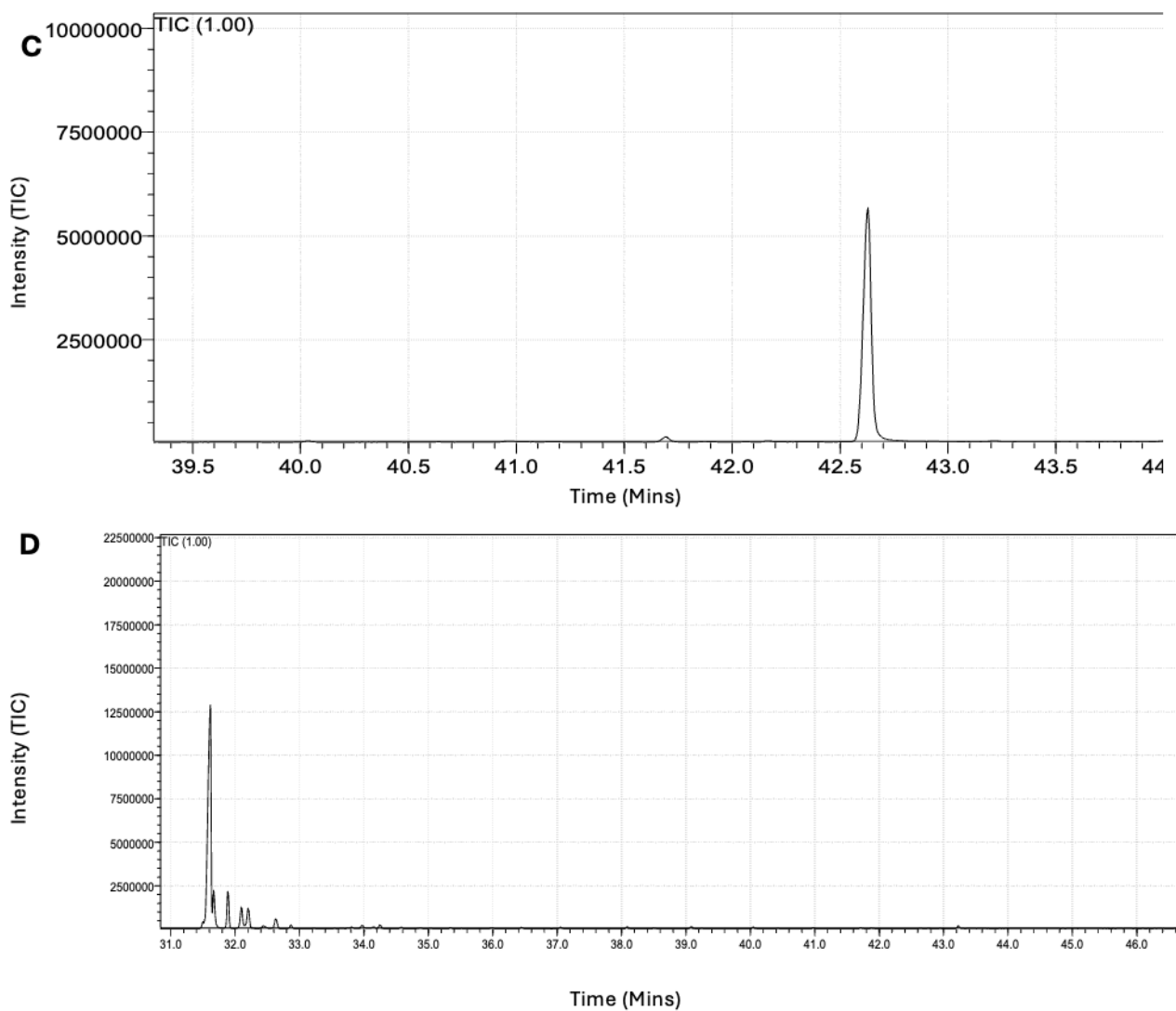
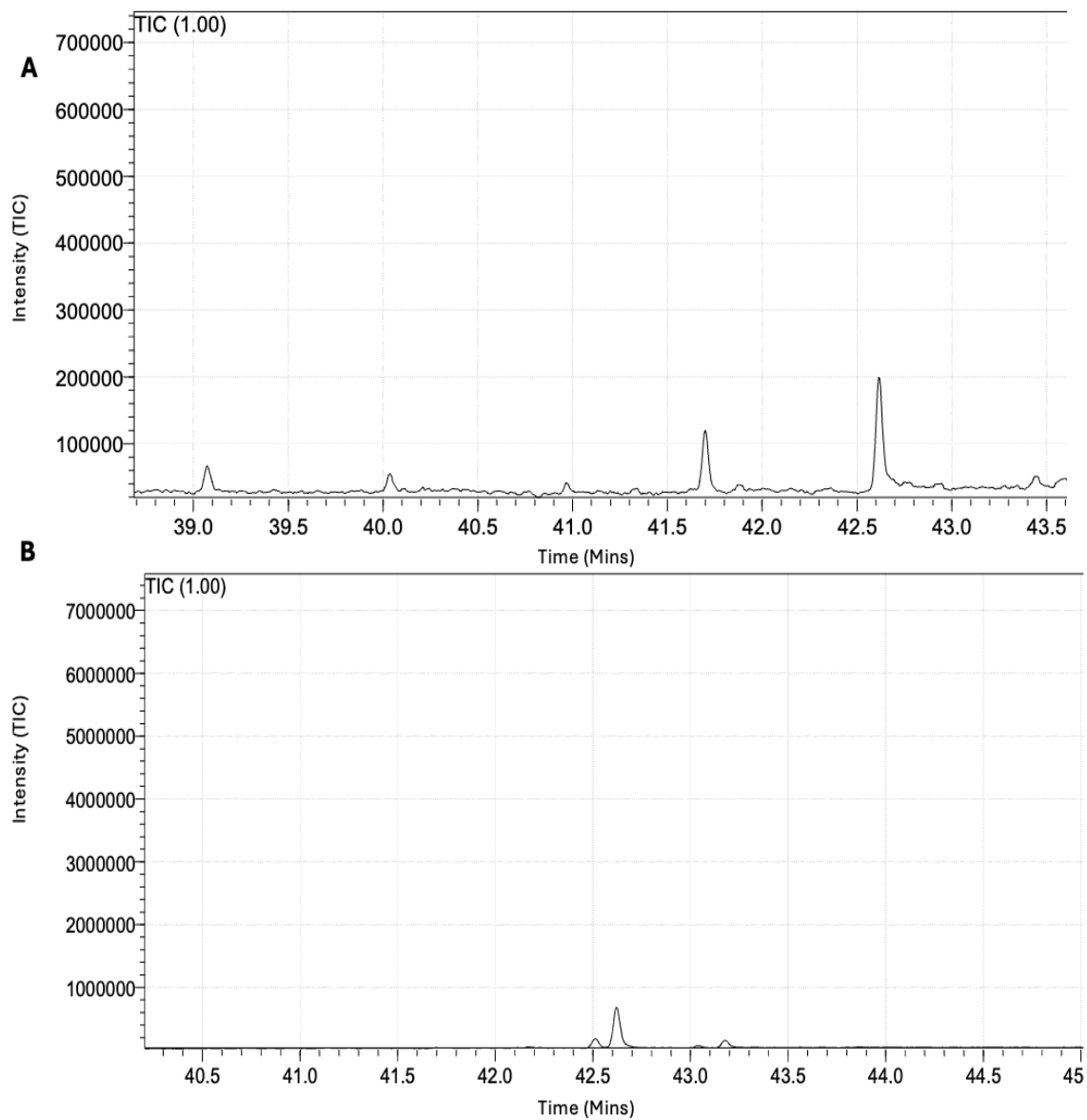


Figure 9- Chromatographic trace of quality control (QC) after derivatisation.

The conditions were as follows: 200 μ L derivatisation agent added to QC and incubated at 60 $^{\circ}$ C for 30 minutes. The diluents used were: (a) 50:50 methanol: methyl tert-butyl ether (MTBE), (b) methanol, (c) MTBE, and (d) a blank control ($n=4$). The trace shows the total ion count (TIC) of the analyte Sodium taurocholate.

100µl of MSTFA, 30-minute incubation

Reduced volume in derivatisation agent in Figure 9 with 100µl of derivatisation agent being used and incubated for 30 minutes at 60 °C.



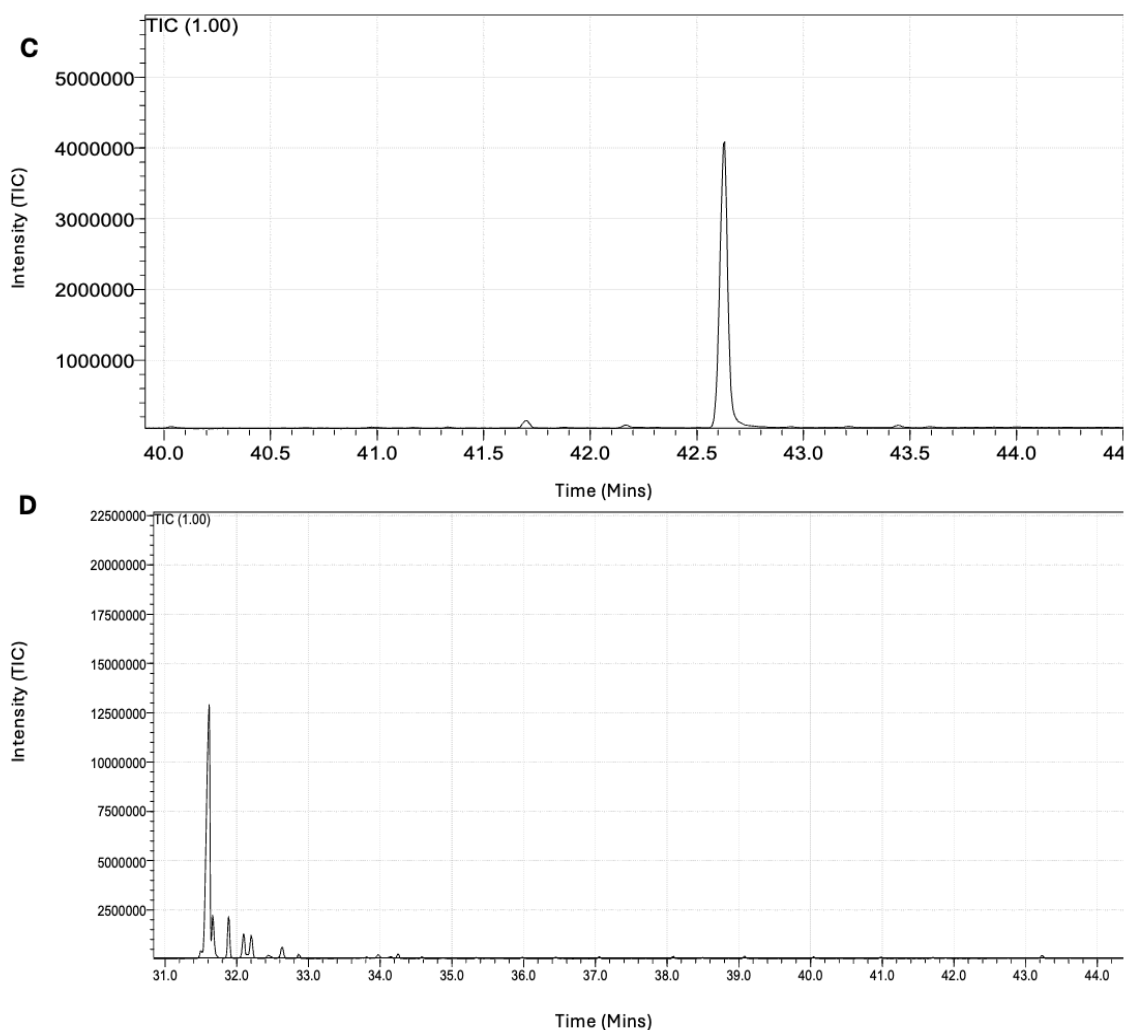


Figure 10-Chromatographic traces for 100ul of derivatisation MSTFA added to QC and incubated for 30 minutes at 60 °C.

The QC was dissolved in a variety of diluents, to observed which gave the greatest peak intensity and best peak shape. a) 50:50 methanol: methyl tert-butyl ether (MTBE), (b) methanol, (c) MTBE, and (d) a blank control (n=4). Sodium taurocholate at a m/z of 425, Peaks in the Chromatogram are representative of the analytes TIC.

200µl of MSTFA, 60-minute incubation

To investigate the split peaks observed in Figures 9a and 10a, two changes were made to the method. The derivatisation incubation time was extended to 60 minutes at 60 °C while keeping the MSTFA volume the same as in Figure 9 (200 µL), and three different diluents were evaluated (Figure 11).

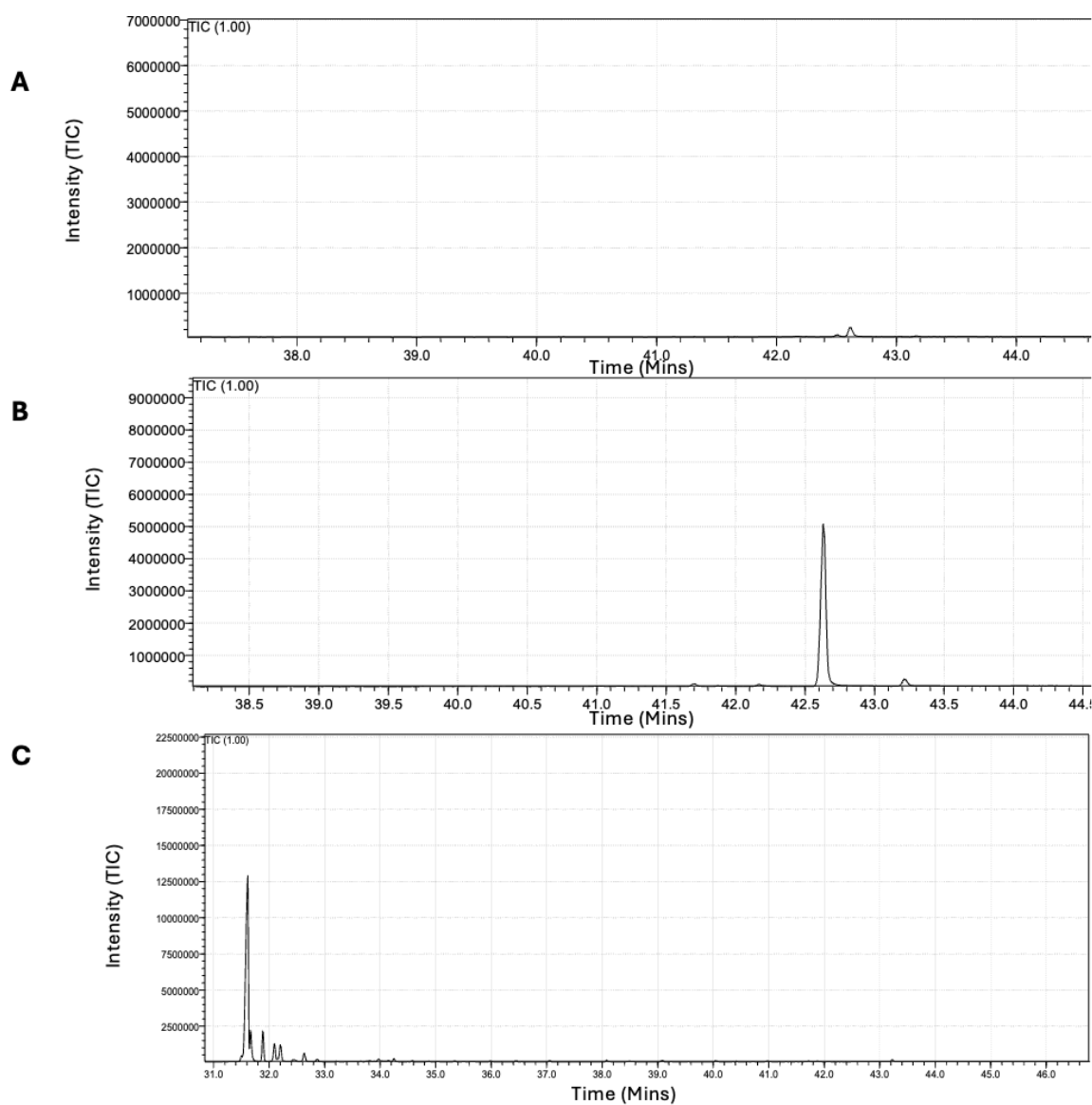


Figure 11 - Chromatographic traces for 200 μL of derivatisation agent MSTFA added to QC and incubated for 60 minutes at 60 $^{\circ}\text{C}$.

Chromatographs demonstrate the level of the analyte under these conditions linking the analyte concentration to the favourable conditions, with the components within the QC being dissolved in variety of diluents (a) methanol, (b) MTBE, (c) Blank ($n=3$). Sodium taurocholate at a m/z of 425.

100 μ L of MSTFA, 60-minute incubation

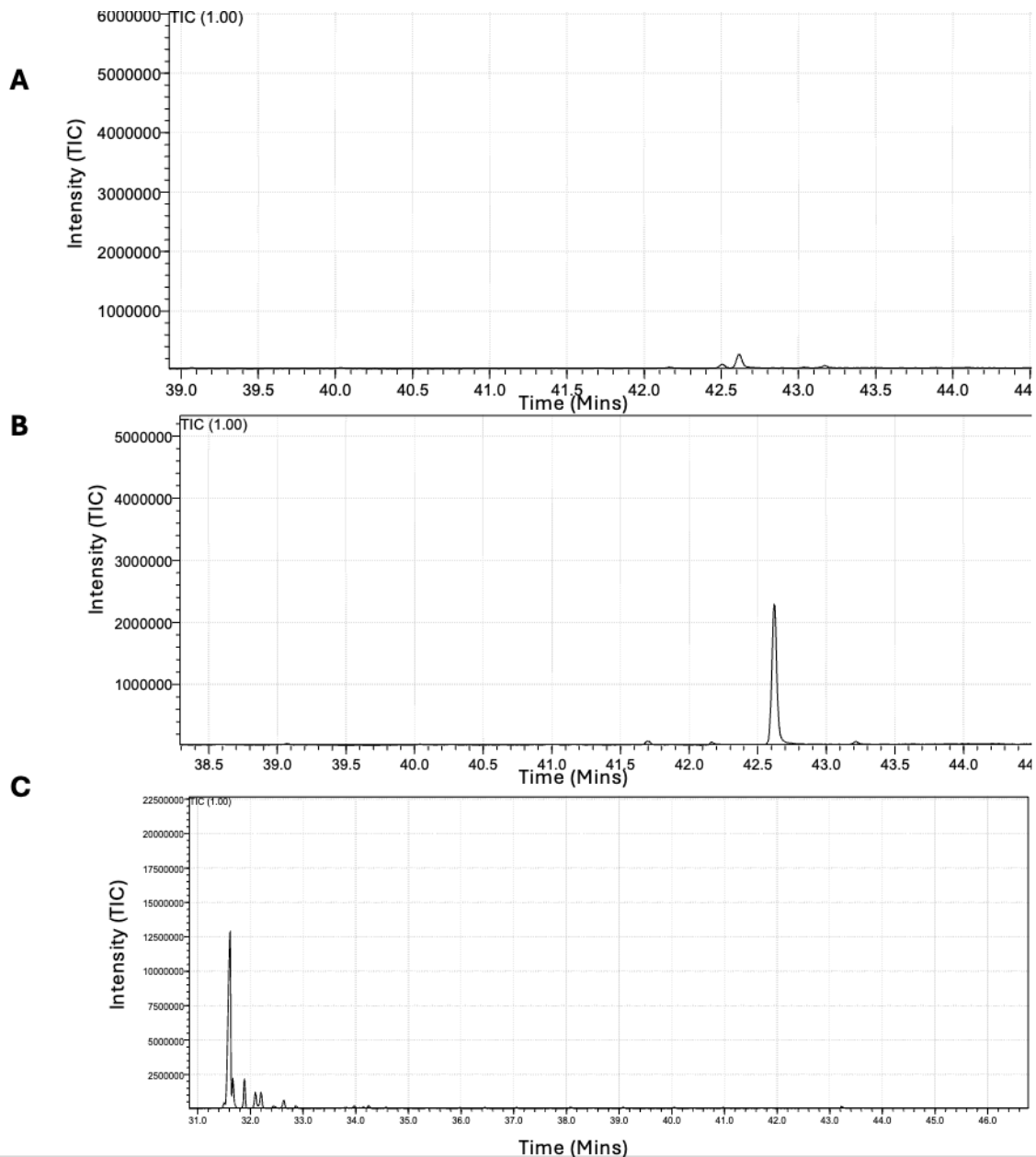


Figure 12 - Chromatographic traces for 100 μ L of derivatisation agent MSTFA added to QC and incubated for 60 minutes and 60 $^{\circ}$ C.

Chromatographs demonstrate the level of the analyte under these conditions linking the analyte concentration to the favourable conditions, with the components within the QC being dissolved in variety of diluents (a) methanol, (b) MTBE, (c) Blank ($n=3$). Sodium taurocholate at a m/z of 425.

2.4.2.2. *Incubation time optimisation*

Comparison between the 30-minute (Figure 9 and 10) and 60 minutes (Figure 11 and 12) data sets showed no time-dependant difference in peak intensity, indicating that the reaction reach completion within the first 30 minutes of incubation. A 30-minute incubation was therefore selected as sufficient to drive the reaction to completion. This shorter incubation time is also favourable for the experimental protocol, as it reduced sample preparation time, enables higher sample throughput, and offers sustainability benefits through reduced energy consumption.

2.4.2.3. *Solvent selection for derivatisation*

Derivatisation agents are often combined with organic solvents for several reasons; to enhance solubility, improve reaction efficiency, reduce side reactions ensure compatibility with gas chromatography, and prevent contamination from water. Solvent choice can also influence the effective mixing of MSTFA with the target compounds, which is critical for achieving a more complete reaction. Two solvents, methanol and MTBE, were compared to determine which most effectively facilitated the derivatisation reaction.

Using methanol alone (Figure 9b, Figure 10b, Figure 11 and Figure12) resulted in splitting of the sodium taurocholate peak indicating inefficient chromatographic separation and reduced resolution-suggesting methanol alone does not efficiently support the derivatisation reaction, or that the compound was ionised differently, interfering with the process. Introducing a second organic solvent — MTBE, in a 50:50 methanol: MTBE mix — resolved the peak splitting observed with methanol alone. When MTBE was used alone (Figure 9c, Figure 10c, Figure 11b and Figure 12c) a single, well-resolved peak was also obtained, indicating that MTBE may better facilitate the MSTFA reaction and support a more effective and reliable derivatisation process. Comparison between methanol and MTBE also showed lower peak intensity when methanol was used alone, providing further evidence that MTBE is the preferable solvent for use with MSTFA. Comparing the 50:50 methanol: MTBE mix Figure 9a and Figure 10a) with MTBE alone likewise showed greater peak intensity when MTBE was used alone, further highlighting its effectiveness as a solvent — suggesting the derivatisation reaction proceeds more effectively in MTBE alone than in the 50:50 mix.

2.4.2.4. *Derivatisation agent volume*

The final factor assessed for the impact of derivatisation reaction was the volume of derivatisation agent used. Given that the initial volume used was 100 μ L, this was compared against 200 μ L of MSTFA to determine whether increasing the volume improved the detection of the sodium taurocholate. The volume of derivatisation agent must be sufficient to react completely with all the available analyte molecules; if the volume is too low not all the analytes will be derivatised resulting in lower signal intensity on the MS, only the derivatised (and therefore detectable) fraction of the analyte is measured. Use of an excess of derivatisation agent can help ensure that all reactive sites are utilised, and the reaction proceeds to completion.

Comparison between Figure 9, Figure 10, Figure 11 and showed that use of a higher concentration of derivatisation agent resulted in greater peak intensity: comparison between Figure 9 and Figure 10 showed a reduction in peak intensity from 5,400,000 with 200 μ L of MSTFA to 4,200,000 with 100 μ L. This higher peak intensity at the 200 μ L volume may be attributable to a more complete reaction between MSTFA and sodium taurocholate, along with enhanced mixing of MSTFA with the analyte, both of which would contribute to improved detection by MS.

The optimised derivatisation conditions selected on this basis were: 200 μ L of MSTFA, 30-minute incubation, 60 °C, with MTBE as the organic solvent.

2.4.3. *Refinement of other factors within the media mix*

Despite focusing on sodium taurocholate throughout the derivatisation method optimisation, it was important to ensure the conditions identified for this analyte also allowed identification and quantification of the remaining components within the media. Under the original conditions, lecithin, cholesterol and free fatty acids were still identified; however, it was critical to confirm the revised conditions did not adversely affect their separation.

Chromatographic trace under the conditions picked from figures 4-7 was used to assess the impact on chromatographic separation on the remaining compounds within the media.

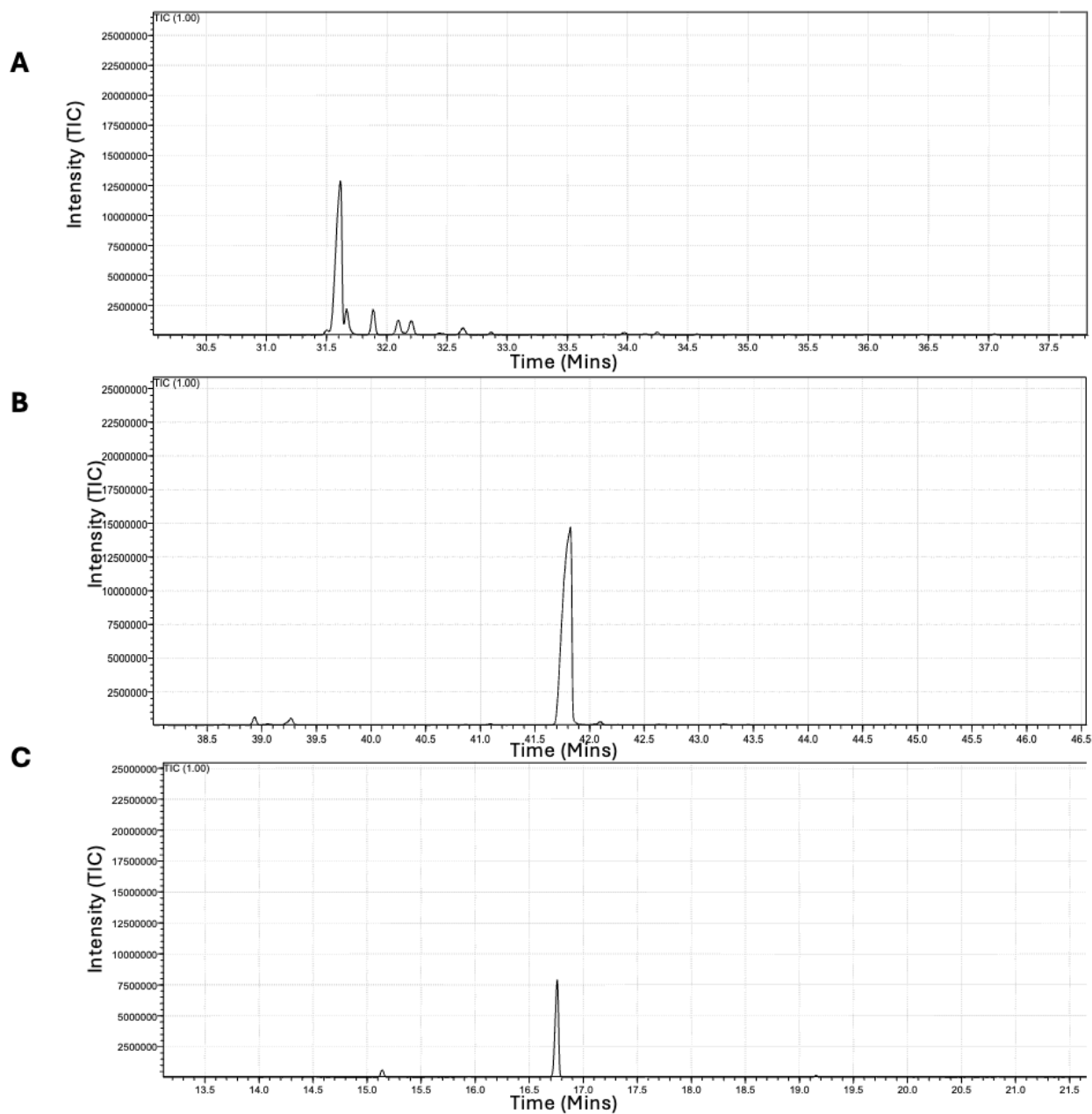


Figure 13-Chromatographic traces under the chosen derivatisation conditions (200 μ L of MSTFA for 30 minutes at 60 $^{\circ}$ C.)

Traces show the remaining components within the media in the optimised conditions chosen based on peak shape and intensity from the Sodium taurocholate traces. For the remaining three components within the simulated intestinal media. a) Sodium Oleate (oleic acid) b) Cholesterol c) lecithin. Chromatographs demonstrate the level of the analyte under these conditions linking the analyte concentration to the favourable conditions of sodium taurocholate ($n=3$), with M/z of the compounds being 339, 368 and, 299 respectively.

As shown in Figure 13, sodium oleate had a peak intensity of 12,500,000, cholesterol a peak of 14,600,000, and lecithin a peak of 4,120,000. These peaks confirmed that the method remained effective in separating the components of the simulated intestinal fluid under the optimised conditions.

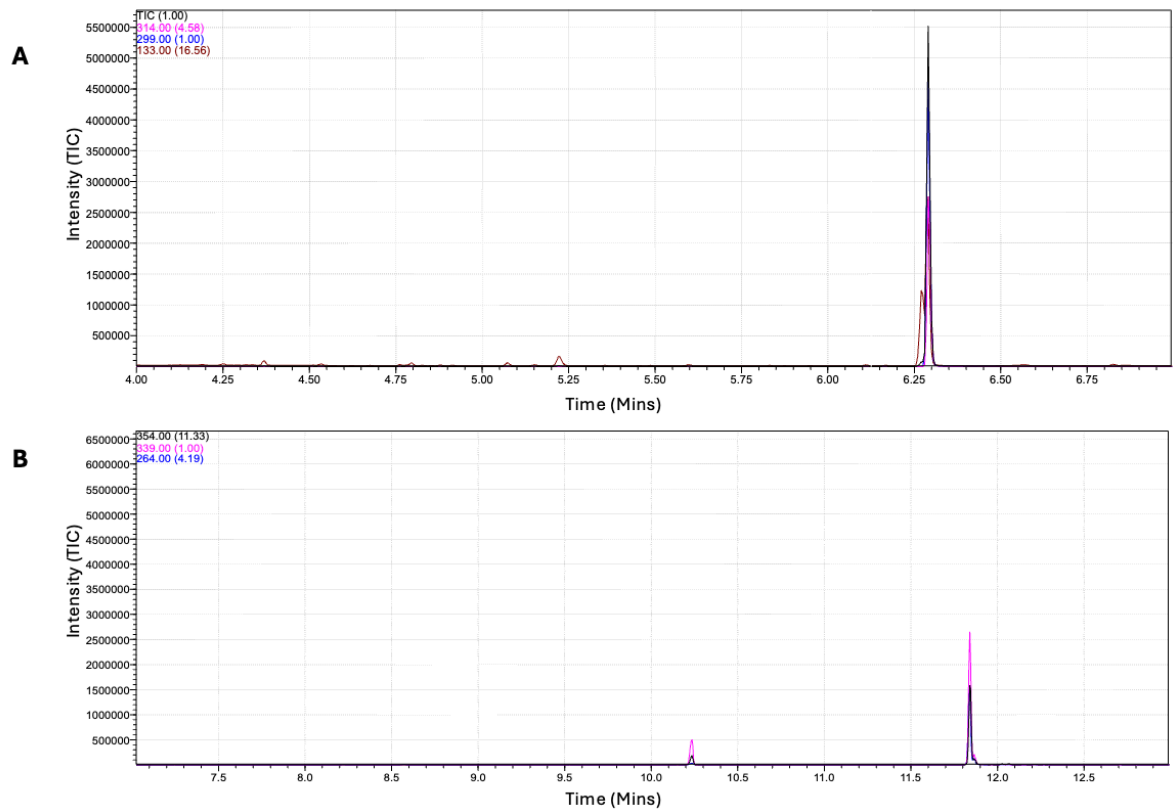
It should be noted that the peak intensity and peak resolution are distinct measurements and should not be conflated. Peak intensity reflects the abundance or concentration of an analyte detected by the MS, whereas peak resolution reflects on how well two adjacent peaks are separated from each other, generally determined by retention time difference and peak width. A component can therefore produce high-peak intensity that is poorly resolved from neighbouring peaks, just as a low-intensity peak can be fully resolved with baseline separation from its neighbours. The peak intensities reported above confirm that each component was detected in sufficient abundance; separation and resolution of all four components were instead confirmed through retention time and chromatographic peak shape (Figure 13-Chromatographic traces under the chosen derivatisation conditions (200 µL of MSTFA for 30 minutes at 60 °C), ensuring no overlap or co-elution occurred between analytes despite differences in their relative peak intensities.

2.4.4. Validation

Validation of the optimised method was carried out through establishment of specificity linearity, precision, accuracy, LOD and LOQ.

2.4.4.1. Specificity

The fragment ion of each of the five compounds was used for quantification: lecithin (m/z 299), sodium oleate (m/z 339), cholesterol (m/z 368), and sodium taurocholate (m/z 425). The retention times of the components used within the simulated intestinal fluid were 6.31, 14.35, 16.59, and 17.74 minutes, respectively.



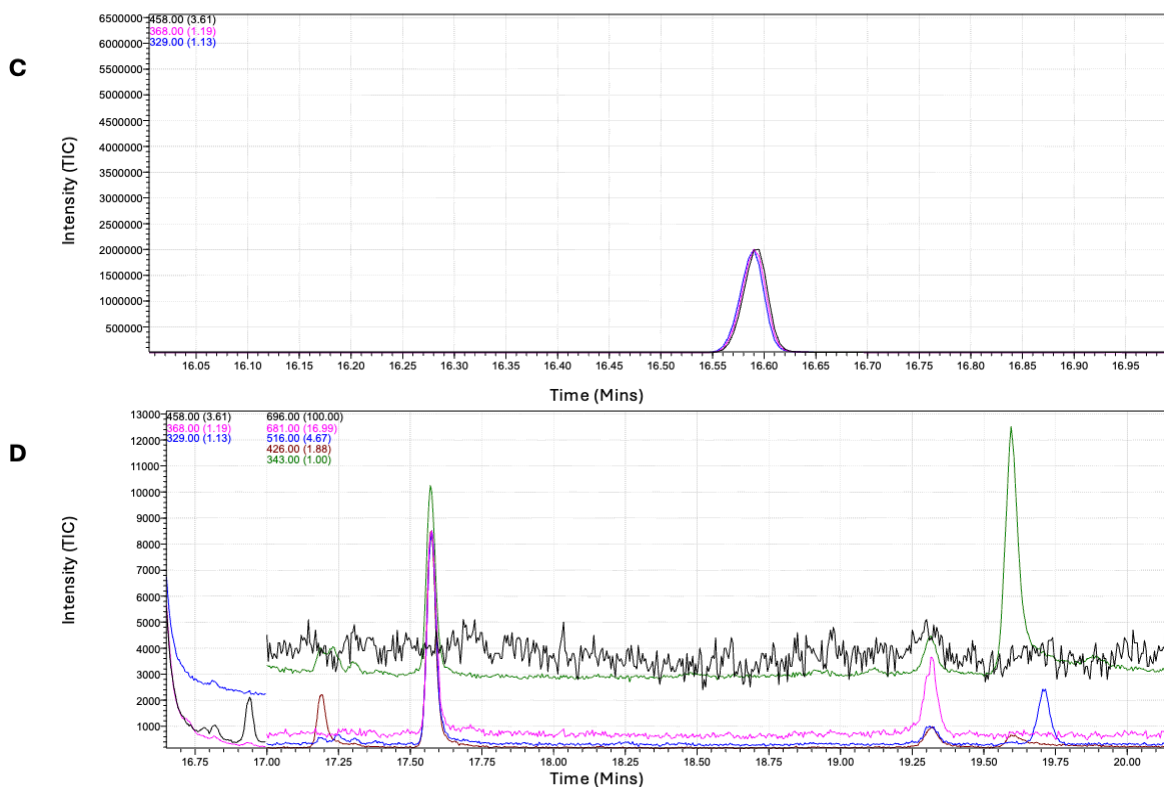


Figure 14 - Chromatographic trace of QC highlighting ability of method to selectively isolate the component from a complex mix.

Time frame in each of the results is snapshot of the full 20-minute run time. a) lecithin, b) sodium oleate, c) cholesterol, and d) sodium taurocholate. Black number on the chromatogram representative of the total ion count from the MS, pink trace is the highest MW fragment of analyte, blue trace is the second highest MW fragment, brown is the third highest, and green is the lowest fragment MW of the analyte.

Determining method specificity is essential for the accurate identification and quantification of target analytes from complex matrices. Some compounds co-eluted from the column with identical retention times Figure 14, so detection of the m/z was required to distinguish them unambiguously. Fragment ions for each compound were first identified via scanning mode, which detects the most abundant fragments generated during MS analysis; these transitions were subsequently incorporated into the method file. When applied within the experimental protocol, the co-elution of compounds alongside consistent detection of their characteristic

fragment ions, without interference from other matrix components, confirmed the specificity of the method.

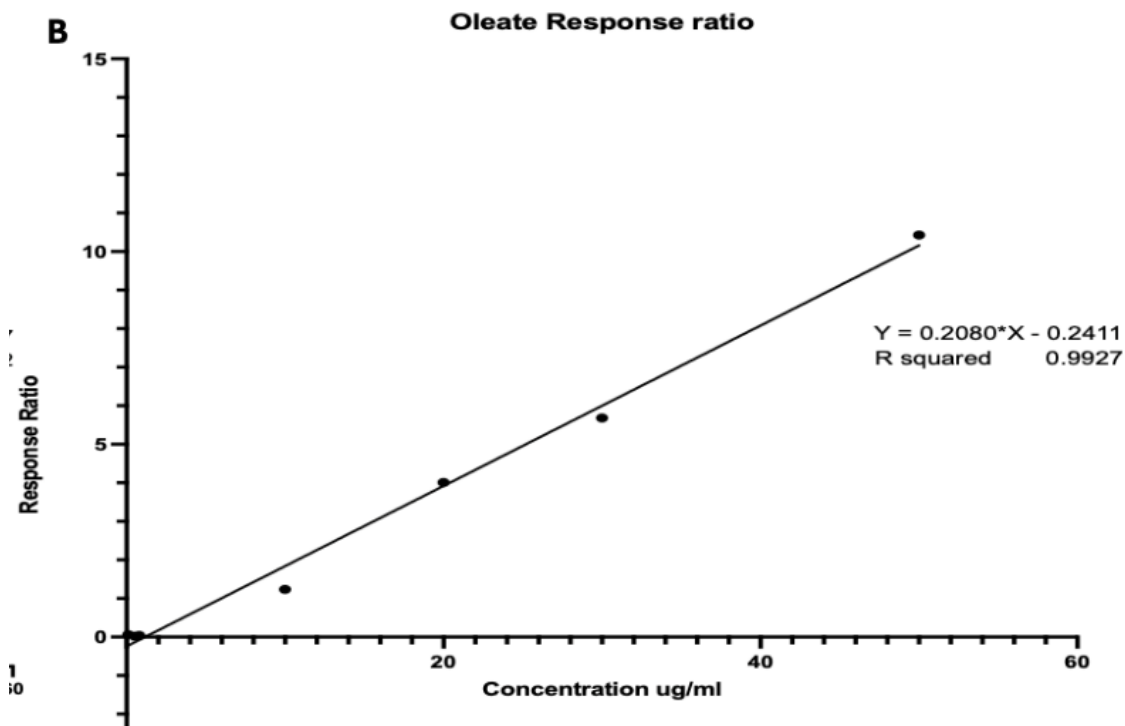
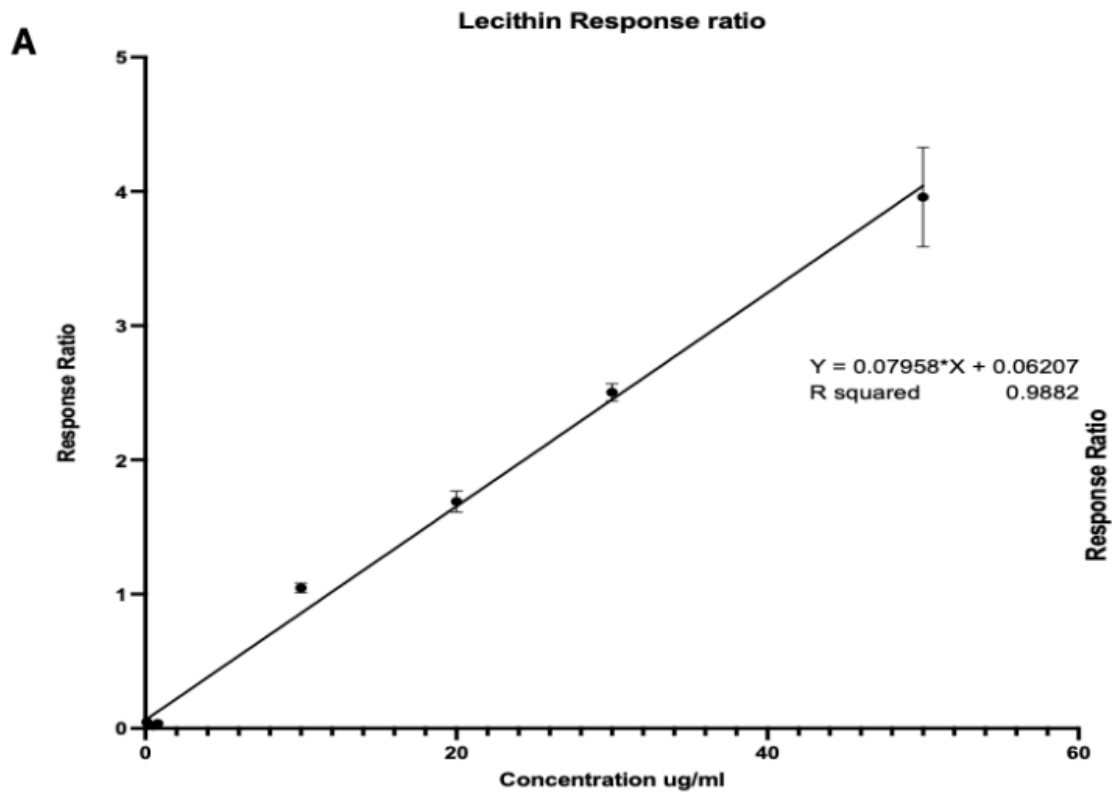
2.4.4.2. Linearity

Calibration curves were generated using an ascending seven-point concentration range of the QC (50, 30, 20, 10, 1, 0.8, and 0.5 µg/ml) in triplicate, with constant concentration of IS (0.5 µg/mL) across all samples. For each of the individual components standard curves were generated where the y-axis represents the peak intensity representative of the ratio between the analyte of interest and the internal standard, with the x-axis reading the concentration set of the analytes. The r^2 values and equations are in Table 5.

Table 5 - Calibration results for the GC-MS analysis across 50, 30, 20, 10, 1, 0.8, and 0.5 µg/ml.

Each row represents a singular component in the media, with columns showing the range of concentrations examined. R^2 value determined the fit of the calibration points to the data, with acceptable $R^2 \geq 0.98$. The final column presents the equation of the line to allow application of the model to unknow concentrations ($n=4$).

Analyte	Concentration Range(µg/ml)	R^2	Equation
Lecithin	0.5-50	0.9882	$y=0.07958*x+0.06207$
Sodium Oleate	0.5-50	0.9927	$y=0.2080*x-0.2411$
Cholesterol	0.5-50	0.9970	$y=0.1407*x+0.02856$
Sodium Taurocholate	0.5-50	0.9960	$y=0.3879*x-0.3488$



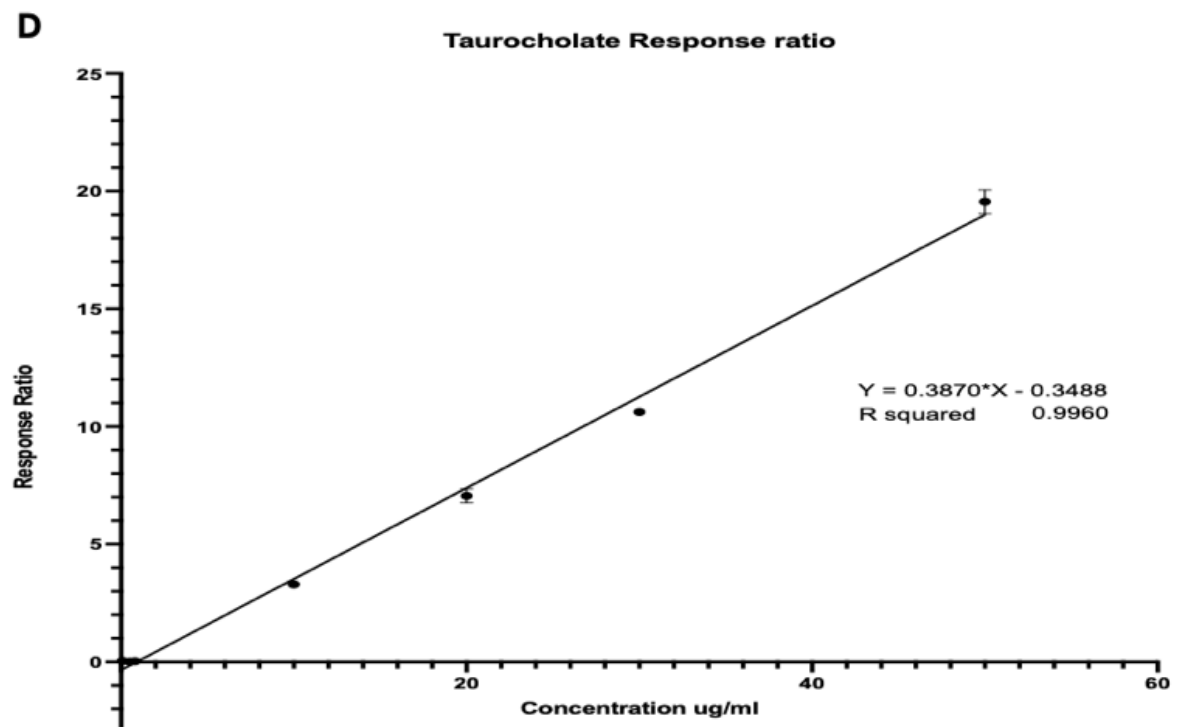
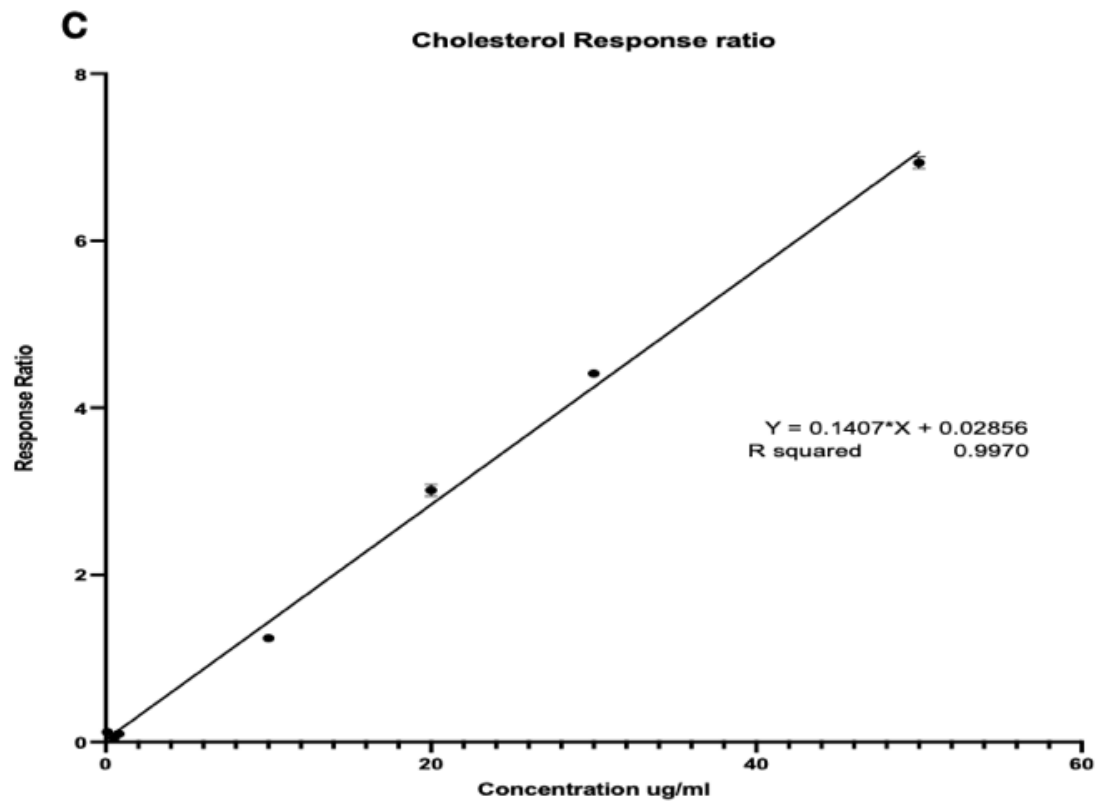


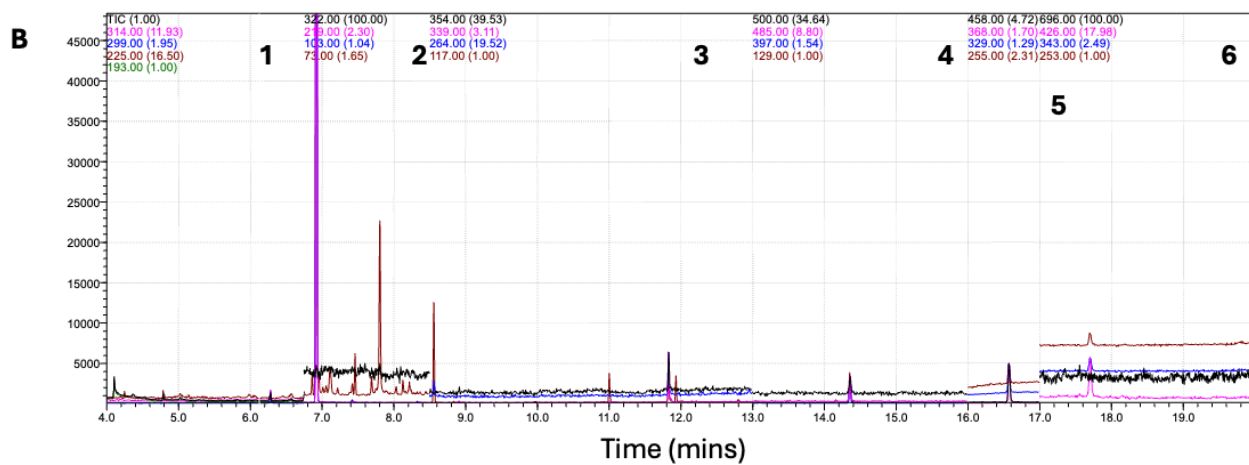
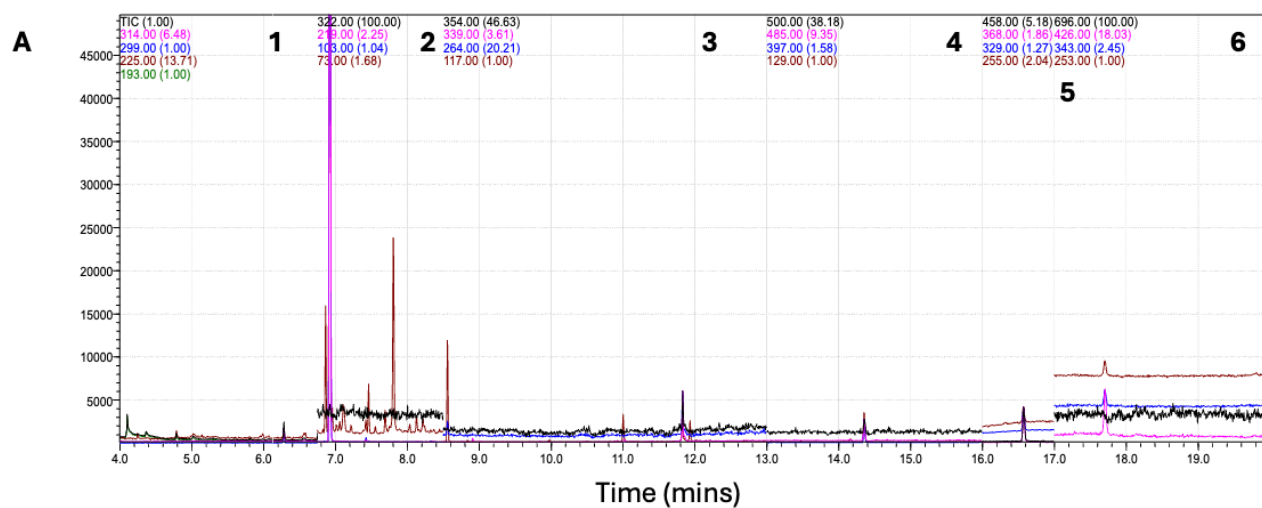
Figure 15 - Calibration curves for the four analytes of interest.

Calibration curves generated in GraphPad Prism for (A) **sodium taurocholate**, (B) **cholesterol**, (C) **lecithin (phosphatidylcholine)**, and (D) **oleic acid**. Each curve displays the fitted regression line with 95% confidence bands and individual calibration standards plotted as mean $\pm$ SD. Linear regression parameters (slope, intercept, and coefficient of determination, R^2) were obtained from $n=3$ replicates per concentration. The curves demonstrate the expected concentration–response behaviour for each analyte across the validated calibration range.

Linearity of final sample preparation and method conditions was assessed across a concentration range of 0.5-50 $\mu\text{g/ml}$ for sodium taurocholate, phospholipids, free fatty acid and cholesterol. Each analyte exhibited good linearity across this range (Table 5), supporting the accuracy and reliability of quantification within the tested concentrations. The consistency and predictability of the calibration response for each compound further indicated that the method is fit for purpose.

2.4.4.3. LOD and LOQ

The LOD/LOQ of the QC sample was determined to be 0.2 µg/mL.



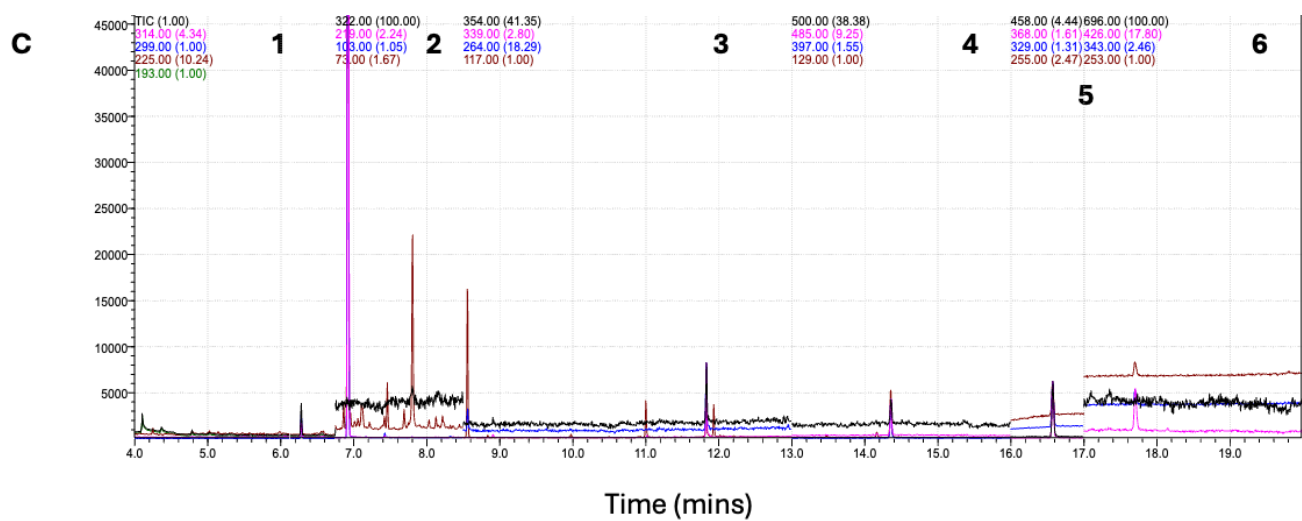


Figure 16 - Chromatographic traces of descending concentration to analyse the LOQ.

Representative chromatographic trace showing quantification of the six components within a single injection: (1) lecithin, (2) internal standard (IS), (3) glyceryl monooleate (GMO), (4) sodium oleate, (5) cholesterol, and (6) sodium taurocholate. Traces are shown for ascending sample concentrations in diluent: (a) 0.15 $\mu\text{g/mL}$, (b) 0.2 $\mu\text{g/mL}$, (c) 0.3 $\mu\text{g/mL}$.

2.4.4.4. Precision

Precision reflects the capability of the method to determine the reproducibility and consistency of measurement under specified conditions. Precision was assessed via six repeat injections of QC samples against an acceptance criterion of $RSD \leq 15\%$, at two different concentrations. At 1 $\mu\text{g/mL}$, RSD ranged from 1.8–3.8%, while at 50 $\mu\text{g/mL}$, RSD ranged from 0.8–8.6% (Table 6).

Table 6 - Summary of individual quality control (QC) components.

Precision is reported as percentage of relative standard deviation (%RSD), calculated from repeat injection of each QC sample.

Analyte	RSD (%)	
	1 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$
Lecithin	3.8	8.6
Sodium Oleate	1.8	1.1
GMO	2.1	0.8
Cholesterol	2.1	1.1
Sodium Taurocholate	2.9	2.8

The precision of the GC-MS method is critical for ensuring reliable and reproducible quantitative analysis of complex mixtures in bioanalytical applications. RSD reflects the degree to which individual sample values deviate from the mean, with lower values indicating tighter clustering of data points and consequently greater consistency of the developed method. In accordance with ICH M10 guidance on bioanalytical method validation, precision should not exceed 15%^{4,31,32}. At the upper end of the calibration curve (50 $\mu\text{g/mL}$), the RSD is calculated at 8.1%, which — while higher than the RSD values observed at other concentration levels — remains well within the ICH M10 threshold. This confirms the precision meets regulatory acceptance criteria, although the comparatively elevated RSD indicates a degree of variability from the mean that warrants consideration when applying this method at higher analyte concentrations.

2.4.4.5. Method Application and sample analysis

Following method development, it was critical to evaluate the method's capacity to resolve individual components within a composite media and quantify them with precision, as this assessment underpins the method's validity and effectiveness. To this end, the method was applied to simulated intestinal fluid supplied by the biopharmaceutics research group at the University of Strathclyde, containing all components assessed within this method²⁴.

Table 7 – GC-MS method validation, results shown are only shown for media 1 from the suite of fluids.

Analyte	Theoretical Value	Reported Values	Recovery (%)
	(mM)	(mM)	
Lecithin	0.16	0.116	72.5
Sodium Oleate	1.052	0.0656	6.2
Cholesterol	0.01	<LOD	-
Sodium Taurocholate	1.067	0.0449	4.2

Table 7 shows substantial discrepancies between theoretical and method-measured values, indicating that significant refinement is needed before the method can accurately quantify all components within the mixed media. Recovery varied considerably across analytes: lecithin was recovered at approximately 72.5% of its theoretical value, while sodium oleate and sodium taurocholate were recovered at only 6.2% and 4.2% respectively, indicating substantial underestimation for these components. Cholesterol fell below the limit of detection entirely. Nevertheless, the method successfully generated a detectable signal for each component across the suite of media, demonstrating baseline detection capacity across all analytes of interest, even where quantitative accuracy requires further optimisation.

The lower-than-theoretical values obtained by GC-MS may be attributed to analyte loss during sample preparation, particularly during derivatisation — a step required for GC

separation. Analyte loss and reduced yield associated with derivatisation-based sample preparation are well documented in the literature^{33,34}. This risk is heightened in more complex media such as SIF³⁵, where the presence of multiple chemically distinct analyte classes within a single derivatisation reaction increases the risk of incomplete reactions, as analytes compete for a shared pool of derivatisation reagent and react at different rates according to their differing functional groups. Further matrix effects may also arise from the aqueous nature of the media, which can consume the reagent and physically limit its access to target analytes, thereby reducing efficiency relative to a simple standard solution. While derivatisation improves detection sensitivity in media containing low analyte concentrations, such as fasted-state SIF, it simultaneously introduces variability that compromises recovery. This variability likely accounts for the discrepancies observed between theoretical and actual values in Table 7, with incomplete reactions and competitive interactions among analytes contributing to partial compound loss.

Overall, these findings establish this approach as a viable preparation method for the simultaneous quantification of multiple components within simulated intestinal media. The method proved suitable for the concurrent determination of bile salt, cholesterol, free fatty acid, and phospholipid presence within a single mixture.

2.5. Conclusion and Future work

This study highlights the potential for further development of a single-run method capable of quantifying all components within SIF. This work represents a significant advancement, as it demonstrated the ability to simultaneously detect a range of key simulated intestinal fluid (SIF) constituents within a single analytical run. The next stage of method development should focus on quantitative analysis of these components. In particular, further optimisation of the derivatisation procedure is warranted to ensure that sufficient reagent is available for complete derivatisation of all analytes and to minimise any competition between compounds for the derivatisation agent. Additional work investigating derivatisation conditions, such as reagent volume, incubation time, and temperature, may improve derivatisation efficiency and help determine whether the observed signal losses reflect incomplete derivatisation or genuine analyte losses.

Once robust and reproducible derivatisation conditions have been established, calibration curves could be developed for each analyte to enable accurate quantification within simulated intestinal fluids. This would allow relationships between analyte concentration and analytical response to be established, facilitating not only the detection of SIF components but also the determination of their concentrations.

Furthermore, this method provides the flexibility to incorporate drug quantification into the analysis, allowing for a detailed examination of how intestinal fluid interacts with orally administered dosage forms. This is particularly valuable for studying the mechanism of drug absorption and transport across the Caco-2 monolayer, a widely used *in vitro* model of the human intestinal barrier.

Importantly, this work also lays the groundwork for applying the developed method to animal- or human-derived samples. The ability to quantify intestinal components from human samples could significantly expand the predictive power of drug studies, bridging the gap between *in vitro* simulations and *in vivo* conditions. Ultimately, these findings contribute to the broader goal of optimising oral drug administration by providing a robust analytical tool that enhances the precision and relevance of gastrointestinal research

Appendix

Table 8 - Selected ion monitoring (SIM) variation used to get the best peak shape. Changes in method conditions. 1-8 stands for the variant methods used.

[illegible]

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Chapter 3- Compatibility of Caco-2 cells with Simulated intestinal fluid

3.1. Introduction

Caco-2 assays are widely regarded as the gold standard for assessing compound permeability during drug development, a status reflected in their inclusion in FDA and EMA regulatory guidelines for BCS-based biowaiver applications and transporter-mediated drug-drug interaction studies. Although the assay design is relatively straightforward, the complexity and functional characteristics of the cells make this model highly valuable for investigating intestinal drug absorption. Caco-2 cells, derived from a human colon carcinoma, can be cultured on permeable membrane supports to form differentiated epithelial monolayers that exhibit key features of the intestinal barrier, including tight junctions, membrane transporters, and brush-border enzymes. It is this close structural and functional resemblance to native intestinal epithelium — combined with regulatory acceptance — that underpins the model's gold-standard status. This enables the model to support investigation of multiple absorptive pathways, including paracellular transport across tight junctions and transcellular transport via passive diffusion or carrier-mediated mechanisms¹.

Current Caco-2 assays typically measure apical-to-basolateral flux using buffers such as Hanks' Balanced Salt Solution (HBSS). However, this approach has notable limitations. Drug solubility in HBSS is often poor, which can lead to underestimation of permeability, and the composition of HBSS does not accurately reflect the luminal environment of the gastrointestinal tract, limiting the physiological relevance of the model^{2,3}. Caco-2 permeability assays are primarily used to assess intestinal permeability and absorption potential, for example in BCS classification and early screening. The FDA specifically refers to a "validated and standardized Caco-2 cell assay" for permeability assessment^{4,5}. A measurement of this kind does not account for inter-individual variability in intestinal fluid composition, further constraining the translational value of the assay.

Incorporating biorelevant media into permeability assessment could address these limitations by improving predictive accuracy and providing greater insight into oral bioavailability, without compromising the simplicity of the assay design. Recent literature reports substitution of HBSS with biorelevant alternatives such as simulated intestinal fluid (SIF), including fasted-state (FaSSIF) and fed-state (FeSSIF) variants that more closely replicate luminal bile salt, phospholipid, and pH conditions under different

physiological states, or, in some cases, human plasma^{3,6-10}. However, bile salts and phospholipids, key components of SIF, possess detergent-like, membrane-disrupting properties at sufficiently high concentrations, raising the possibility that exposure to SIF could compromise Caco-2 cell viability or disrupt monolayer integrity. Evaluating the compatibility of these alternative media with Caco-2 cell viability and monolayer function is therefore essential to determine whether replacing HBSS represents a viable option.

An MTT assay is commonly used to determine the metabolic activity of cells, serving as an indirect measure of cell viability following exposure to different experimental conditions. Viable and proliferating cells reduce the yellow tetrazolium dye MTT to insoluble purple formazan crystals via NADPH-dependent cellular oxidoreductase enzymes, with the intensity of the resulting colorimetric signal correlating with the number of viable cells^{11,12}. Consequently, the assay is particularly useful as an initial screening tool for assessing the compatibility of candidate media compositions before cells are seeded onto permeable supports. However, the MTT assay provides information only on metabolic activity and does not assess the integrity or functionality of the epithelial barrier. As a result, metabolically active cells may remain viable despite disruption of tight junctions and compromised barrier function following exposure to biorelevant media. Therefore, assessment of monolayer integrity via transepithelial electrical resistance (TEER) is required alongside viability measure to confirm that tight junctions remain intact, and that the barrier retains relevant barrier properties required for permeability studies.

3.2. Objectives

The aim of this study was to evaluate the compatibility of a suite of biorelevant simulated intestinal media, designed based on the variability inherent to a human intestinal fluid data set, with Caco-2 cells. The primary objective was to identify media compositions that maintained Caco-2 cells viability and monolayer integrity without inducing cellular damage, thereby enabling the development of an experimental protocol for assessing drug permeability under physiologically relevant conditions.

3.3. Materials and Methods

3.3.1. Chemicals and Reagents

Caco-2 from the American Type Culture Collection (Rockville, MD, USA) were donated by Dr Louise Young (University of Strathclyde), Dulbecco's Modified Eagle's Medium (DMEM) high Glucose ($4,500\text{mg L}^{-1}$) with L-Glutamine and without Pyruvate, Foetal Bovine serum, Non-essential Amino acids, Penicillin ($10,000\text{ U/mL}^{-1}$)-Streptomycin ($10,000\text{ U/mL}^{-1}$), Phosphate buffer saline at pH 7.4, and TrypLE were all purchased from Fisher Scientific (Paisley, UK). Sodium taurocholate, ammonium formate, sodium chloride (NaCl), chloroform, formic acid, monosodium phosphate (NaH_2PO_4), fenofibrate, indomethacin and phenytoin were purchased from Sigma Aldrich (Dorset, UK). Lecithin S PC (phosphatidylcholine from Soybean "98%") was purchased from Lipoid GmbH (Ludwigshafen, Germany). Sodium oleate was obtained from BDH Chemical Ltd. (Poole, UK). The solvents methanol, acetonitrile and water were all HPLC grade from VWR (Leicestershire, UK).

Table 9-Composition of the experimental media used throughout Caco-2 monolayer studies. Based on multidimensional analysis of human intestinal fluid data set^{13,14}.

Media	Bile Salt (mM)	Lecithin (mM)	Free Fatty Acid (mM)	Cholesterol (mM)	pH	Total Amphiphile Concentration (mM)
HBSS	-	-	-	-	7.3	
Biorelevant FaSSIF V1	3	0.75	-	-	6.5	3.75
Biorelevant FeSSIF	15	3.75	-	-	5	18.75
Strath FaSSIF 1	1.06	0.16	1.04	0.01	6.64	2.27
Strath FaSSIF 2	11.45	2.48	2.88	0.38	7.12	17.19
Strath FaSSIF 3	3.56	1.18	1.04	0.06	5.72	5.84
Strath FaSSIF 4	3.4	0.330	2.880	0.090	8.04	6.7
Strath FaSSIF 5	3.35	0.31	0.87	0.17	6.62	4.7
Strath FaSSIF 6	3.6	1.25	3.43	0.03	7.14	8.31
Strath FaSSIF 7	2.27	0.96	1.01	0.08	7.34	4.31
Strath FaSSIF 8	5.33	0.40	2.96	0.07	6.42	8.76
Strath FaSSIF 9	3.46	0.52	1.64	0.032	6.54	5.65
Strath FeSSIF 1	4.94	2.02	10.5	0.95	5.97	18.41
Strath FeSSIF 2	19.04	7.94	47.51	0.34	6.59	74.83
Strath FeSSIF 3	5.65	2.43	18.06	0.1	6.13	26.24
Strath FeSSIF 4	16.65	6.59	27.63	3.45	6.42	54.32
Strath FeSSIF 5	15.66	5.1	10.92	0.5	6.24	32.18
Strath FeSSIF 6	6	3.14	45.68	0.65	6.32	55.47
Strath FeSSIF 7	7.34	6.17	21.82	0.57	5.97	35.9
Strath FeSSIF 8	12.81	2.6	22.85	0.58	6.59	38.8
Strath FeSSIF 9	10.94	4.02	23.38	0.32	6.29	38.6
Strath FaSSIF Min	1.06	0.17	0.07	0.04	2.94	1.34
Strath FaSSIF Q1	2.34	0.16	1.18	0.06	7.22	3.74

Strath	FaSSIF	3.10	0.39	1.69	0.08	7.91	5.26
Q2							
Strath	FaSSIF	5.43	0.57	2.59	0.12	7.76	8.71
Q3							
Strath	FaSSIF	36.18	5.78	15.03	0.2	8.0	TAC
Max							
Strath	FeSSIF	0.395	0.160	2.024	0.001	4.66	
Min							
Strath	FeSSIF	1.620	2.541	13.41	0.308	6.00	3.75
Q1				4		75	
Strath	FeSSIF	2.648	4.004	21.92	0.572	6.28	18.75
Q2				0			
Strath	FeSSIF	4.684	5.501	35.01	0.931	6.51	2.27
Q3				6			
Strath	FeSSIF	23.02	14.395	57.25	3.285	7.09	17.19
Max		0		8			
Markopoulos		6.8	6.8	0.8	-	5.8	5.84
Fed							

The table details the concentrations of salts, buffering agents, bile salts, phospholipids, and other constituents present in each medium¹⁵⁻¹⁷. Biorelevant media were formulated to mimic the composition of human intestinal fluid, whereas HBSS served as the standard transport buffer. Total amphiphile concentration was calculated as the sum of bile salt, lecithin, free fatty acid and cholesterol multiplied by the pH of the corresponding media.

The suite of SIF media was designed to capture physiological variability by incorporating the minimum and maximum concentrations of individual media components reported within the HIF dataset, alongside formulations representing the corresponding statistical quartiles. The minimum and maximum conditions were generated using the lowest and highest reported concentrations of each component across the human dataset, thereby representing the extremes of physiological variability observed within the study population. Evaluation of these extreme conditions was limited to fasted-state media,

reflecting the primary focus of the subsequent work. Equivalent maximum and minimum fed-state formulations were not investigated.

3.3.2. Preparation of Simulated Intestinal Fluid

3.3.2.1. Strath Fasted Media Preparation Stock Media Preparations

Bile salts (sodium taurocholate), phospholipids (soybean lecithin), and free fatty acids (sodium oleate) were weighed and combined in a glass flask before dissolution in 4 mL chloroform (Solution A). Cholesterol (250x Concentration) was weighed separately and dissolved in 10 mL chloroform (Solution B). An aliquot (100 μ L) of Solution B was transferred into Solution A, and the mixture was stirred until forming a homogenous mixture. The organic solvent was subsequently removed under a continuous stream of nitrogen gas until a dry lipid film was formed.

The resulting dry film was reconstituted in deionised water and transferred quantitatively into a 5 mL volumetric flask. The volume was adjusted to 5 mL with deionised water to produce the concentrated lipid stock solution. For preparation of the final experimental medium, equal volumes of fasted-state media stock solution (Solution A), with Stock solutions of sodium phosphate monobasic monohydrate buffer (28.4 mM) and sodium chloride (105.9 mM) prepared separately in deionised water (267 μ L each) were combined, and the final volume was adjusted to 4 mL with deionised water.

3.3.2.2. Fed Media Preparation Stock Media Preparations

Bile salts (sodium taurocholate) and phospholipids (soybean lecithin) were weighed and combined in a glass flask before dissolution in 4 mL of chloroform (Solution A). Cholesterol was weighed separately (250× concentration) and dissolved in 10 mL of chloroform (Solution B). An aliquot (100 µL) of Solution B was transferred into Solution A, and the mixture was stirred until all components were completely dissolved. The chloroform was subsequently removed under a continuous stream of nitrogen gas until a dry lipid film was formed. The resulting lipid film was reconstituted in deionised water and transferred quantitatively into a 5 mL volumetric flask. The remaining volume was adjusted to 5 mL with deionised water to produce the lipid stock solution.

A sodium oleate stock solution was prepared by dissolving 913.32 mg sodium oleate in deionised water in a 10 mL volumetric flask. Stirring and elevated temperature were applied to facilitate solubilisation, and the stock solution was maintained at 50 °C to aid dissolution.

Stock solutions of sodium phosphate monobasic monohydrate buffer (28.4 mM) and sodium chloride (105.9 mM) were prepared separately in deionised water. The final fed-state media formulations were prepared by combining fed media stock solution, phosphate buffer stock (1.6 mL) with equal parts, sodium chloride stock, sodium oleate stock solution (267 µL), with varying free fatty acid stock (sodium oleate) volume (Table 10) and made up to 4 mL with deionised water.

Table 10 - Preparation volumes of sodium oleate stock solution for fed-state media formulations. The table details the volume of sodium oleate stock solution required to achieve the specified free fatty acid (FFA) concentrations within the final 4 mL fed media preparations.

Media	FFA Stock(ml)
Strath FeSSIF 1	0.35
Strath FeSSIF 2	1.584
Strath FeSSIF 3	0.602
Strath FeSSIF 4	0.921
Strath FeSSIF 5	0.364
Strath FeSSIF 6	1.523
Strath FeSSIF 7	0.727
Strath FeSSIF 8	0.762
Strath FeSSIF 9	0.776

3.3.2.3. Fasted and Fed Simulated intestinal fluid (FaSSIF v2, FeSSIF v2)

Biorelevant intestinal media were prepared according to the manufacturer's instructions (Biorelevant.com, UK). Where required, media formulations were prepared following previously published methodologies. Using literature, fed-state media formulations were prepared based on the composition described by Markopoulos *et al.* in 2014³.

3.3.3. Cell Culture

Human colon adenocarcinoma Caco-2 cells were obtained from the American Type Culture Collection (ATCC) and were kindly provided by Dr Louise Young (University of Strathclyde). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA), and 1% (v/v) penicillin–streptomycin (PEST). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Following thawing, cells were initially cultured in T-25 flasks until reaching approximately 80–90% confluence. Cells were detached using TrypLE dissociation reagent and transferred into T-75 flasks for routine expansion. Culture medium was replaced every three days, and cells were passaged approximately every five days. Caco-2 cells between

passages 25 and 45 were used for seeding onto permeable membrane inserts for subsequent monolayer and permeability studies.

3.3.4. *Assessment of viability and media compatibility*

An MTT assay was performed to evaluate the cytotoxic effects of simulated intestinal fluid (SIF) formulations on Caco-2 cell viability. Caco-2 cells were seeded into the wells of a 96-well plate at a density of 3×10^5 cells/well and incubated for 3 days under standard culture conditions to allow cell attachment and growth. Following incubation, the culture medium was removed and replaced with 100 μ L of the test media formulations described in Figure 17. Cells were then exposed to the test media for 2 hours at 37 °C and 5% CO₂ under humidified conditions.

Following exposure, 100 μ L of the test medium was removed and replaced with 100 μ L of complete culture medium containing 15 μ L of MTT reagent. The plate was wrapped in aluminium foil to protect the light-sensitive MTT reagent and incubated for 4 hours at 37 °C and 5% CO₂. Following MTT incubation, 75 μ L of the medium/reagent mixture was removed and replaced with 100 μ L of solubilisation solution. Plates were placed on an orbital shaker at 37 °C for 1 hour to facilitate dissolution of the formed formazan crystals.

Absorbance was measured at 570 nm using a Thermo Labsystems Multiskan Ascent microplate reader at room temperature. Cell viability was determined relative to untreated control wells.

3.3.5. Monolayer protocol

Cells were used for monolayer formation between passages 25–45. Passaged cells were seeded onto Corning Costar Transwell permeable supports (polycarbonate membrane, 0.4 μ m pore size, 12 mm diameter, 12-well plate format). Membranes were pre-soaked with 100 μ L of complete culture media prior to cell seeding. Cells were resuspended in 0.5 mL of culture media and seeded onto the apical chamber of the Transwell insert at a density of 3×10^5 cells per well, with 1.5 mL of culture media added to the basolateral chamber. Media were refreshed 6 hours post-seeding for cells seeded in the morning, or 16 hours post-seeding for cells seeded in the evening, ensuring the media change did not exceed a 16-hour post-seeding interval. Monolayers were maintained over a 21-day incubation period, with media changed every second day. TEER measurements were

recorded every second day throughout the incubation period using an EVOM2 (World Precision Instruments) to monitor monolayer development and confirm the formation of tight junctions, ensuring monolayers met the defined acceptance criteria prior to exposure to simulated intestinal fluid (SIF)¹⁸. The acceptance criterion was set at 450 $\Omega\cdot\text{cm}^2$, which is a more stringent threshold than those typically reported to provide greater confidence in monolayer integrity during the exploratory studies conducted in this work.

3.3.6. DLS

The particle size of the colloidal structures within the SIF media was measured via dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK), which employs a helium-neon (HeNe) laser and an avalanche photodiode detector. Following a 20-minute equilibration period to allow laser stabilisation, back-scattered light from a 632.8 nm laser (4 mW output) was measured at an angle of 173° and a temperature of 37 °C to determine particle size. Each sample was prepared as described in Preparation of Simulated Intestinal Fluid; for media prepared without specific components, the same procedure was followed but omitting the relevant material. Samples were transferred into semi-micro disposable cuvettes, and the instrument was equilibrated for two minutes prior to each measurement, with the auto-attenuator function used to identify the optimum position for analysis. Three independent batches were prepared, with three measurements taken per sample. The mean $\pm$ standard deviation of the most prominent peak in the size distribution by intensity was calculated from 12–16 individual measurement runs per sample ($n = 9$).

3.4. Results and Discussion

3.4.1. Compatibility Analysis using the MTT assay

. The impact of each medium on cell survival is expressed as a percentage of survival relative to HBSS, used as a positive control.

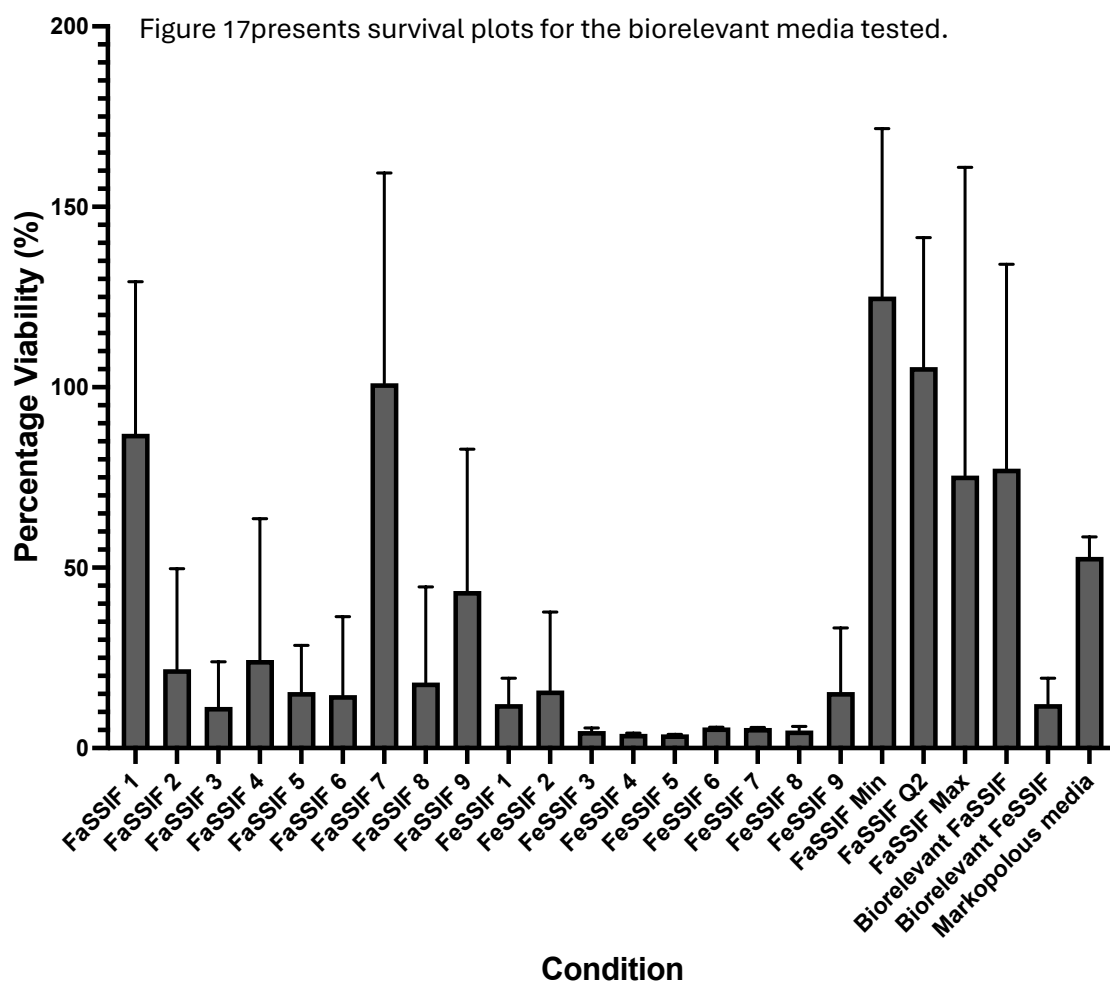


Figure 17 - MTT based cell survival in different culture media shown as the percentage of cell viability in relation to HBSS.

Cell viability was quantified using the MTT assay following 24-hour incubation in the indicated media. Absorbance values (570 nm) were normalised to the control condition and are presented as mean $\pm$ SD from independent experiments. Label indicative of SIF media in Table 9 ($n=12$).

A comparative analysis of FaSSiF media, based on the statistical application of HIF (Human Intestinal Fluid) composition as described by Riethorst et al. (2016), was undertaken to evaluate compatibility with Caco-2 cells¹³. The rationale for this approach was to generate a suite of biorelevant media that more closely reflect the natural variability of human intestinal fluid composition¹⁴, rather than relying on a single fixed formulation, thereby allowing identification of the formulation(s) best suited to support Caco-2 monolayer development. The findings indicate that not all media formulations demonstrate adequate support for cellular viability. For consistency with established practice, acceptance criteria were defined such that a medium would be considered compatible if cell survival was equivalent to or exceeded that observed in biorelevant FaSSiF media, as widely reported in the literature as an alternative medium to HBSS^{7,9,19,20}.

Figure 17 illustrates the survival of Caco-2 cells across the suite of proposed fasted and fed media developed at the University of Strathclyde. For comparison, the figure also includes the current biorelevant FaSSiF and FeSSiF media formulations commonly employed in Caco-2 permeability studies reported in the literature. Of the media generated from the HIF study, two showed promise as a substitute for the current HBSS transport media. Strath FaSSiF 1 and Strath FaSSiF 7 demonstrated the best cell survival, both reporting within approximately 90% of survival observed in HBSS.

Observed survival rates of approximately 90% relative to HBSS indicate that the biorelevant media do not exhibit an increased cytotoxic influence compared to the standard media currently in use, supporting their substitution for further applications.

The behaviour of commercialised FaSSiF (Biorelevant.com) within this study was similar to values reported in the current literature, with monolayers in the literature also demonstrating survival in fasted-state media^{2,3,7}. The survival of cells in Strath FaSSiF Media 1 and Strath FaSSiF Media 7 is of particular interest, given that these formulations contain additional components beyond standard biorelevant media, including the addition of free fatty acids and cholesterol within the SIF media matrix. Demonstrating cell survival in these media broadens the scope for a more biorelevant biological transport medium within the Caco-2 monolayer model. The selection of Strath FaSSiF Media 1 and 7 reflects realistic SIF conditions derived from population variability,

whereas FaSSIF Min, FaSSIF Q2, FaSSIF Max, and Biorelevant FaSSIF are based on statistically averaged compositions that aggregate all values without accounting for their interdependencies^{7,14,16}. A limitation of these findings is that Strath FaSSIF Media 1 and 7 have some of the lowest concentrations of the media components. Therefore, only a snapshot of human variability can be examined within the window of media in which the cells are able to survive, as increased concentrations of bile salt, cholesterol, free fatty acid, and phospholipid have been shown to be toxic to cell survival.

Although Biorelevant FaSSIF media are generally considered less physiologically representative, as they comprise only bile salt and lecithin, lacking additional components such as free fatty acids and cholesterol present in human intestinal fluid and incorporated within the Strath FaSSIF formulations, they nonetheless remain widely used due to their greater accessibility and ease of use. As observed in Figure 17, cell viability in FaSSIF Q2 and FaSSIF Max was similar to, if not greater than, that observed with Biorelevant FaSSIF. This may be attributable to additional media components within FaSSIF Q2 and FaSSIF Max — beyond bile salt and lecithin alone — promoting the formation of mixed micelles, which could contribute to improved cell survival.

High concentrations of bile salts and fatty acids are typically cytotoxic, as bile salts function as detergents once their concentration exceeds the critical micellar concentration^{21,22}. The cytotoxicity of bile salts toward Caco-2 cells was first systematically evaluated by Velardi et al. (1991), using carboxyfluorescein (CF) release as a marker of cell membrane lysis²³. Their data revealed a steep increase in CF release with rising bile salt concentration, consistent with membrane disruption; importantly, the inclusion of phospholipids attenuated this cytotoxic effect²³. Subsequent studies corroborated these findings, demonstrating that media lacking lecithin (phosphatidylcholine) exerted greater toxicity toward cell viability than media lacking bile salts^{7,24}. Collectively, these results suggest that phospholipids mitigate the deleterious effects of bile salts through incorporation of bile salt monomers into mixed micelles, rather than through a reduction in total bile salt concentration. Above the critical micellar concentration, free bile salt monomers partition preferentially into these phospholipid-containing mixed micelles; as phospholipid availability increases, a greater proportion of bile salt becomes sequestered within the micellar core, thereby lowering the concentration of membrane-active free monomer available to interact with, and disrupt,

the Caco-2 cell membrane. It is this reduction in bioavailable monomeric bile salt — rather than any change in total bile salt content — that is proposed to underlie the protective, "anti-toxic" effect observed with phospholipid-containing formulations such as FaSSIF.

Micelle formation has the potential to attenuate the cytotoxicity associated with amphiphilic components such as bile salts²⁵. This effect arises when surfactant species, including bile salts, are present at concentrations exceeding their critical micelle concentration (CMC), provided that prevailing physicochemical conditions — including temperature, pH, and solvent polarity — are favourable for micellar assembly²⁶. Incorporation of bile salts into mixed micellar structures reduces the concentration of freely dissolved monomers available to interact with and disrupt cellular membranes. On this basis, the higher lecithin concentration present in FaSSIF Max may promote the formation of mixed bile salt-phospholipid micelles, thereby reducing bile salt-mediated membrane damage and contributing to the enhanced Caco-2 cell survival observed with this formulation. By contrast, several of the Strath FaSSIF formulations may not maintain a bile salt-to-lecithin ratio sufficient to support equivalent mixed micelle formation and increasing cellular exposure to free bile salts, which may in part account for the reduced survival observed across these media. Work has shown that an imbalance in mixed micelle composition — specifically, elevated bile salt concentrations — exerts detrimental effects on Caco-2 cell viability. Notably, the presence of phospholipids was shown to mitigate this cytotoxicity, underscoring their protective role in maintaining cell survival²⁷.

A more pronounced compositional effect was observed between fasted- and fed-state formulations. While several fasted-state Strath FaSSIF media supported partial or substantial Caco-2 cell survival, this protective effect was not observed in any of the fed-state formulations: all nine fed-state media developed at the University of Strathclyde demonstrated markedly reduced cell survival relative to HBSS^{14,17}. This pronounced loss of viability in the fed-state media is consistent with the substantially higher total amphiphile concentrations characteristic of the fed state relative to the fasted state, which may exceed the buffering capacity of any protective micellar interactions and instead result in net membrane disruption. These findings suggest that, beyond a certain compositional threshold, increasing bile salt, phospholipid, free fatty acid, and

cholesterol concentrations shifts the system from a predominantly protective micellar environment toward one in which cytotoxic monomeric or sub-micellar species predominate, consistent with the concentration-dependent cytotoxicity profile.

3.4.2. *Impact of media on particle size.*

The critical micelle concentration (CMC) and the subsequent formation of mixed micelles appear to play a pivotal role in modulating cell survival. In human bile, micelles form spontaneously once bile salt concentrations reach approximately 14 mM with phospholipids constituting the other principal component²⁸. The interaction between bile salts and phospholipids facilitates the formation of these mixed micelles²². Mixed micelles of this kind have been widely exploited to enhance the solubility of orally administered drugs, with several studies demonstrating that the co-presence of bile salts and lipids substantially increases drug solubilisation²⁹.

As described in Section 3.3.1, the literature-derived fed-state medium reported by Markopoulos and colleagues (2014) demonstrated cell survival exceeding 50%, comparable to that observed in HBSS, despite its higher bile salt concentration (see Table 9 for details). To elucidate the mechanisms underlying cell survival in the fed-state medium, colloidal particle size was examined across the different media formulations. Figure 18 presents the relationship between media type and colloidal particle size. Visual inspection revealed a progressive shift in particle size distribution within the FaSSIF media as TAC increased, ranging from the lowest value of 1.34 to the third-quartile value of 8.71. In contrast, particle size in the fed-state media did not increase progressively with rising TAC (from 2.58 to 46.13); rather, it remained largely stable until the maximum TAC of 97.95, at which point a marked reduction in hydrodynamic radius was observed. Notably, the fed-state medium derived from the Markopoulos formulation exhibited the largest particle size overall, at 658.22 nm, compared with substantially smaller maximum particle sizes in the fasted- and fed-state human-derived media (205.2 nm and 319.14 nm, respectively). This pronounced increase in particle size within the Markopoulos medium may be attributable to its cell culture backbone: Dulbecco's Modified Eagle Medium (DMEM) contains inorganic salts such as NaCl, CaCl₂, and MgSO₄, which can screen electrostatic surface charges on particles and thereby promote aggregation — potentially explaining the larger particle sizes observed in this medium.

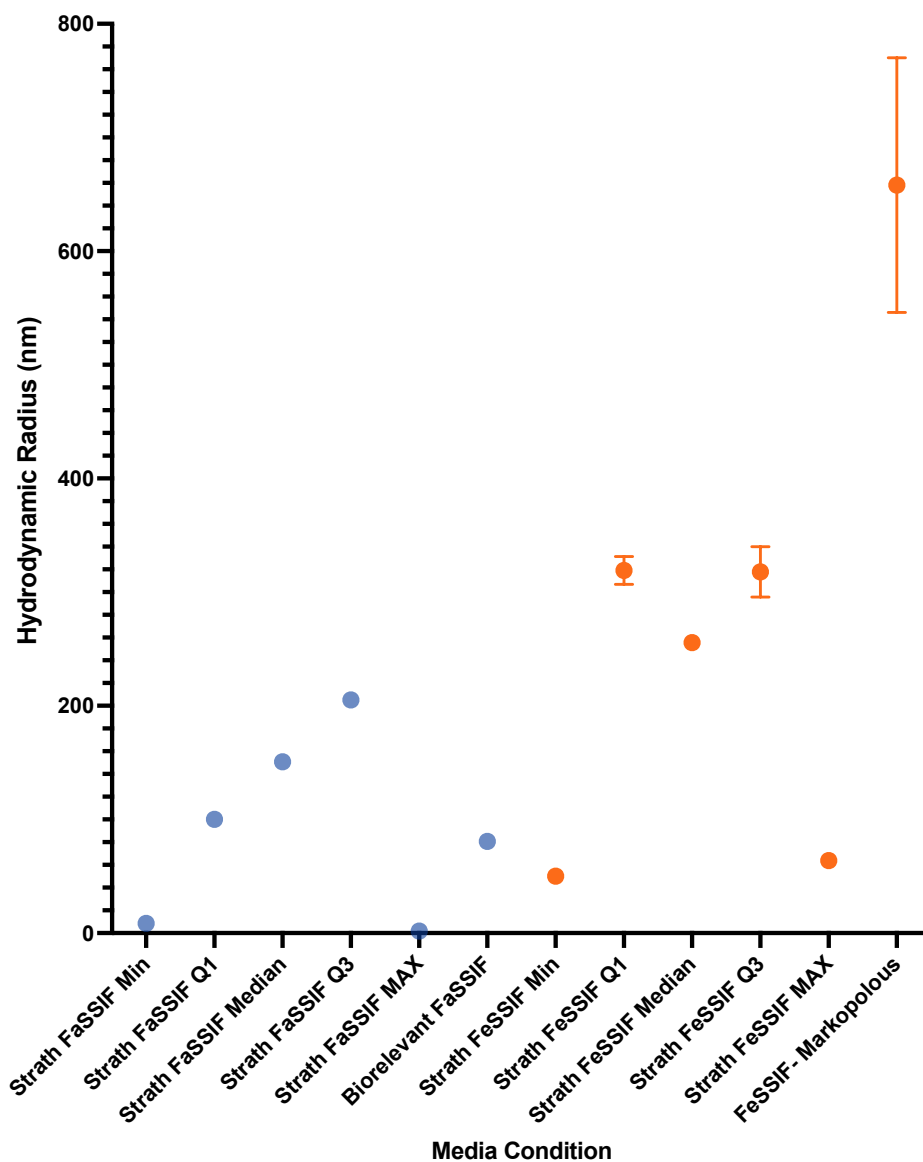


Figure 18 - Particle size distribution of biorelevant media compared with published formulations.

Particle-size distributions of the prepared biorelevant media were measured using dynamic light scattering and are presented alongside distributions reported for the corresponding published media compositions. Each point represents the intensity-weighted hydrodynamic diameter profile for the respective formulation $n=3$. Not all nine media were assessed to align with previously published data, addition of this work was the fed conditions, alongside the literature-published fed-state formulation from Markopolos in 2014^{3,30}.

Examination of mean particle size data presented in Figure 18 for the FaSSIF media reveals a trend of increasing hydrodynamic radius with rising TAC. Specifically, hydrodynamic radius increased from 8.51 nm at the FaSSIF minimum to 205.2 nm at the FaSSIF Q3 medium. However, at the highest TAC tested (Figure 18), a marked reduction in hydrodynamic radius was observed, with values falling below even the minimum medium (1.80 nm). A comparable pattern was evident in the Strathclyde fed-state media, where TAC values approaching the maximum were similarly associated with a sharp decline in hydrodynamic radius, to 63.8 nm. By contrast, the fed media exhibited a less gradual increase in hydrodynamic radius, rising from approximately 50 nm to 316 nm before plateauing despite continued increases in TAC, until reaching FaSSIF maximum conditions in Figure 18, at which point a pronounced reduction in radius was again observed.

Bile salt micelles are self-assembling structures that typically exhibit low polydispersity. Micellar association increases beyond the CMC, at which point spheroidal or globular morphologies are generally adopted³¹. Upon reaching a secondary CMC, micellar architecture may undergo further transformation, giving rise to more elongated structures³¹. The introduction of additional environmental complexity — such as the formation of mixed micelles — further broadens the range of possible micellar morphologies. Nevertheless, it is widely accepted that micellar structure is governed primarily by bile salt concentration. It is therefore plausible that, as TAC increases, higher amphiphile concentrations drive a transition from larger vesicular structures to smaller mixed micelles, a process that may account for the observed reduction in hydrodynamic radius. This is consistent with molecular dynamics simulations, which have similarly shown self-assembly of fed-state bile salt/phospholipid/free fatty acid aggregates as mixed micelles with dimensions in the low nanometre range³².

Although the Markopoulos et al. (2014) medium demonstrated promising cell survival results, its physiological relevance is limited by significant differences in formulation. This medium is based on DMEM, a standard cell culture medium, to which micellar components were added post hoc; DMEM does not, however, replicate the complex physicochemical environment of the gastrointestinal tract^{2,3}.

By contrast, the biorelevant media developed by Pyper *et al.* in 2020 were specifically designed to mimic intestinal conditions, including bile salt content and pH¹⁴. Comparison of particle size profiles reveals that the Markopoulos medium produces larger, less dynamic particles, which may not interact with the intestinal epithelium in a physiologically representative manner.

As shown in Figure 18, particle size generally increases with TAC across the media formulations up to a certain concentration; however, in both FaSSIF and FeSSIF, particle size decreases again at the maximum concentration tested, suggesting a shift in colloidal structure under these high-concentration conditions. Notably, despite its comparatively low TAC (14.4), the Markopoulos medium exhibited larger particle sizes than all fed-state biorelevant media, further calling into question its suitability as a representative simulated intestinal fluid (SIF). This discrepancy may be attributable to the use of DMEM as a formulation backbone, which could promote the formation of larger, less physiologically relevant micelles. Such artefactual micelle formation may misrepresent how compounds interact with Caco-2 cells, thereby reducing the model's predictive value for intestinal absorption and toxicity.

3.4.3. Impact of specific media components on Caco-2 viability

Evaluating the influence of each individual component on Caco-2 cell viability enables a greater understanding of which components of SIF are compatible with the cells. This provides insight into which components could be adjusted in future formulations, and, in particular, the concentration threshold each component may reach before becoming detrimental to cell viability.

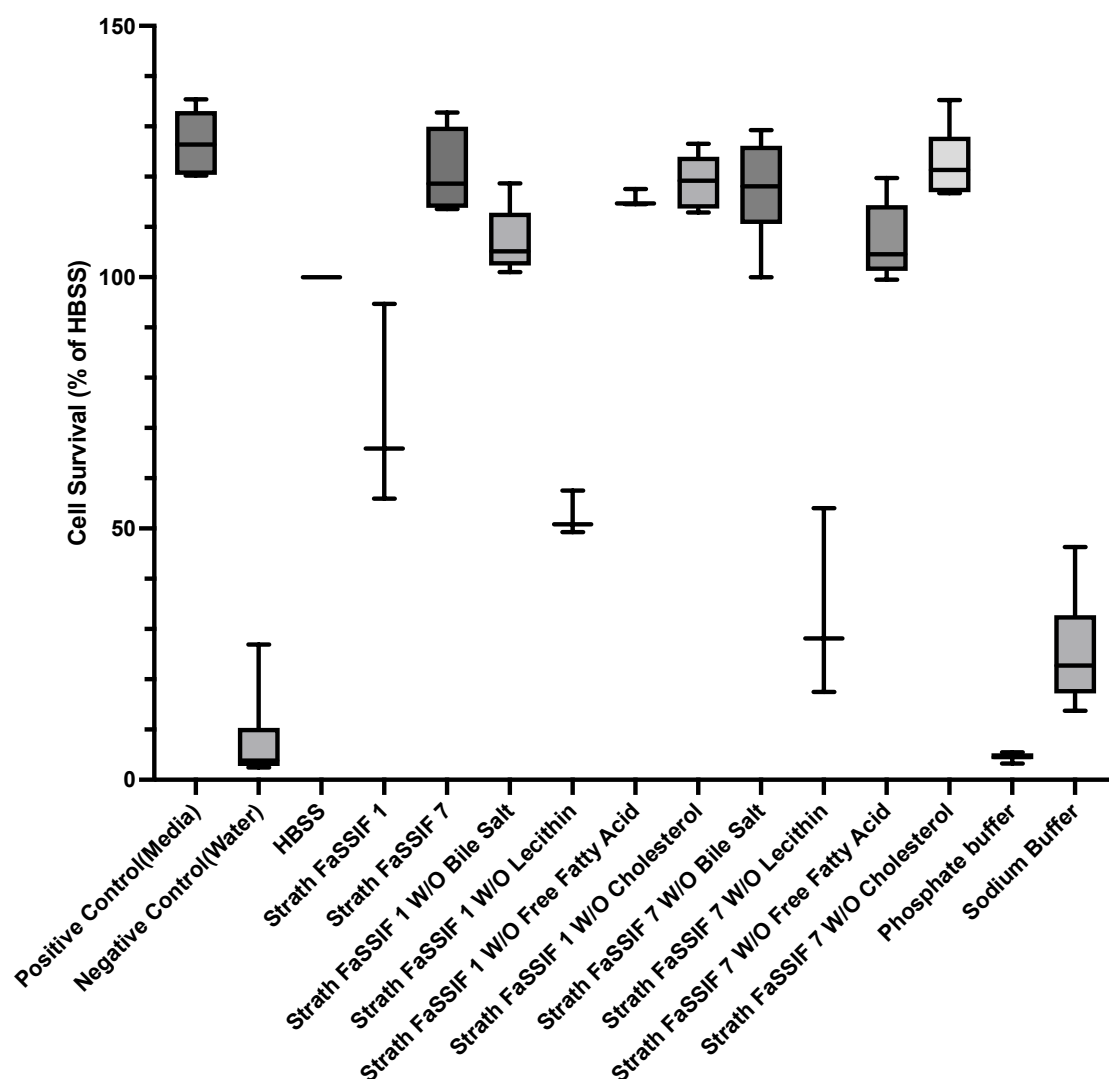


Figure 19 - Comparative assessment of biorelevant media formulations with systematic variation in amphiphile composition.

Cell survival following exposure to biorelevant media with systematically varied amphiphile composition, quantified using the MTT assay. Viability values are expressed as a percentage relative to the HBSS control (N = 9). Data are presented as mean ± SD, illustrating the impact of bile salt, phospholipid, and fatty-acid content on cellular metabolic activity under each media condition.

To evaluate the contribution of individual amphiphile components to media behaviour, cell survival was assessed in SIF media systematically lacking one component, as outlined in Table 9. As shown in Figure 19, Strath media 1 and Strath media 7 both demonstrated cell survival within 10% of that observed in HBSS, supporting their suitability as substitutes for enhancing the biorelevance of bench-side permeability models. Notably, omission of bile salt produced survival levels comparable to HBSS: in Strath media 1, survival remained at approximately 100% of the HBSS value in the absence of bile salt, while Strath media 7 showed a similar pattern, at approximately 120% of the HBSS value. Bile salts are known to act as detergents, capable of injuring cell membranes and disrupting cellular tight junctions, with prior studies demonstrating bile salt-induced injury not only in colon cancer cell lines³³. Subsequent work in 2005 by Zhang *et al.* investigated the mechanism underlying bile salt-induced apoptosis, showing that exposure to bile salts induced membrane blebbing and cellular shrinkage; apoptosis was promoted in 83% of the bile salts tested, in both a time- and dose-dependent manner³³. These findings underscore the established detergent-like behaviour of bile salts and their capacity to compromise cellular integrity and initiate programmed cell death³⁴.

By contrast, removal of lecithin from both Strath media 1 and Strath media 7 produced comparable outcomes, with cell survival reduced to 50% or less relative to HBSS (Figure 19). These findings indicate that lecithin exerts a protective effect, mitigating cellular stress induced by bile salts and free fatty acids; lecithin removal proved the most detrimental intervention to cell survival across the incubation period for both media. Interestingly, cell survival in Strath FaSSIF 7 was lower than that observed in Strath FaSSIF 1 in the absence of lecithin, which may reflect the higher concentrations of other components in Strath FaSSIF 7 exerting a greater cumulative toxic effect on the cells.

The pronounced negative impact of phospholipid depletion may be explained by elevated concentrations of bile salts and fatty acids within mixed micelles, which are capable of triggering apoptotic responses^{35,36}. Lipids within the media appear to play a stabilising role, incorporating bile salts and fatty acids into mixed micelles and thereby reducing the concentration of free, membrane-active monomers³⁵. Their removal results in micellar collapse, increasing the fraction of bile salts and fatty acids present in their unbound state. These unbound species are considerably more cytotoxic, as they readily insert into

epithelial cell membranes, destabilise lipid bilayers, and trigger apoptotic signalling pathways^{35,36}.

These findings underscore the cytotoxic impact of altering media composition, defining a clear constraint on the media formulations suitable for use in permeability studies. The data suggest that there is a threshold level of amphiphile content beyond which cells are no longer able to maintain membrane integrity or withstand biological stressors. At higher concentrations of cytotoxic components, the stabilising effect of other amphiphiles becomes insufficient to support the formation of effective mixed micelles, resulting in an increased proportion of free, membrane-active monomers that act in a detergent-like manner on the cellular membrane surface. Maintaining this balance is essential to ensure that observed transport reflects genuine compound permeability rather than artefacts of membrane damage: where amphiphile load exceeds the protective capacity of the cells, Papp values become confounded by compromised barrier function rather than true transcellular or paracellular transport.

3.4.4. *TEER analysis of biorelevant SIF media on Caco-2 monolayers*

The acceptance criterion for monolayer integrity was set in-house at 450 Ω , representing the minimum value at which a monolayer was considered intact. This threshold was deliberately set above the standard reported in the literature¹⁸, reflecting a more conservative in-house standard for confirming barrier competence at baseline. However, this threshold had been established based on baseline TEER measurements taken prior to compound/media exposure, and did not account for the expected decline in TEER that typically accompanies prolonged exposure to complex biorelevant media, independent of any loss of barrier function. Consequently, applying this stricter baseline-derived criterion at the 120-minute time point risked excluding monolayers that remained functionally intact but had undergone this expected reduction. To address this, the acceptance threshold following the 2-hour experimental window was revised to 300 Ω , a value consistent with literature precedent¹⁸. Because the original in-house threshold had already been set conservatively above the literature standard, this revision did not represent a loosening of the underlying standard for barrier competence, but rather a correction of an overly stringent criterion specifically at the 120-minute time point — allowing monolayers that remained genuinely intact to be appropriately retained.

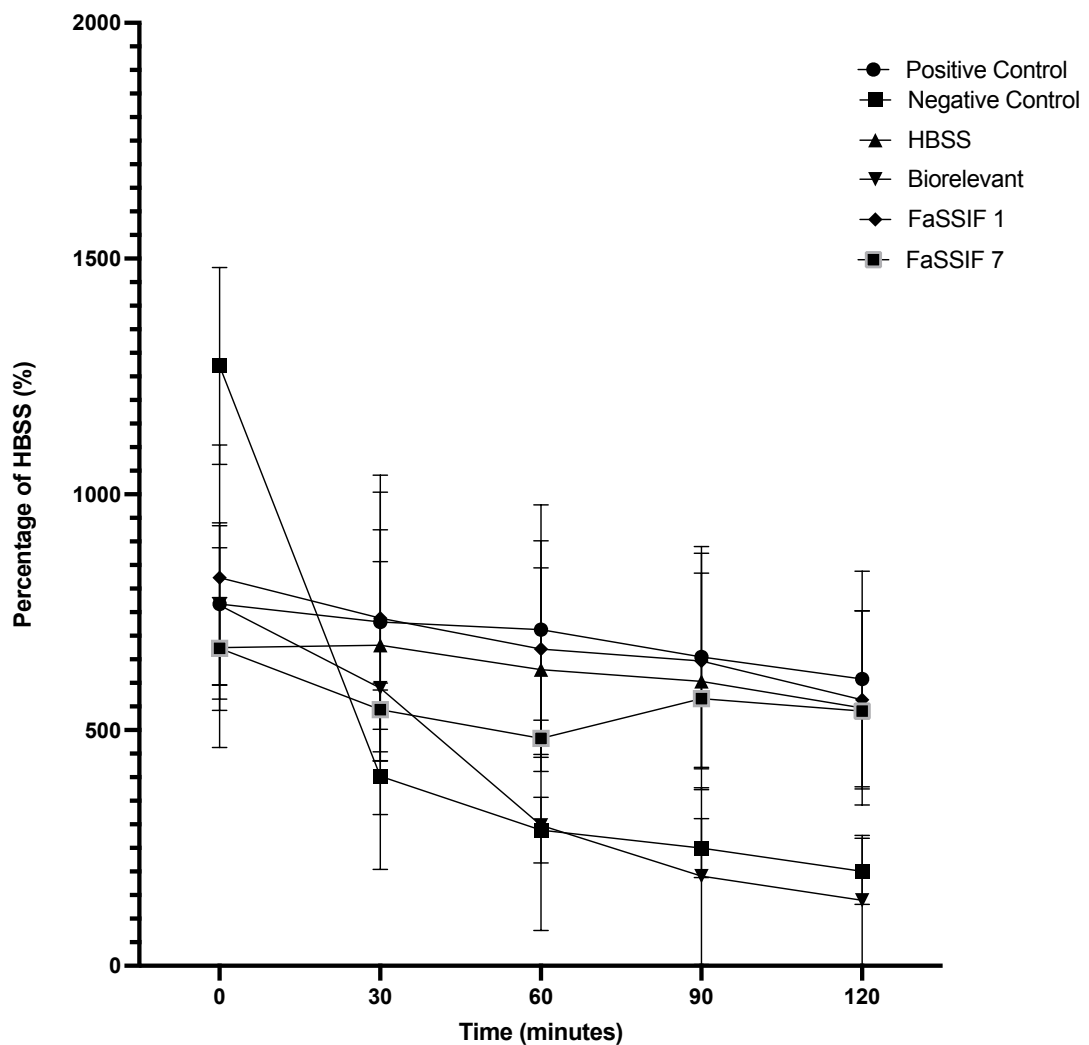


Figure 20 - Impact of biorelevant SIF media on monolayer tight-junction integrity.

Tight-junction integrity of the epithelial monolayer following exposure to biorelevant media formulations was assessed ($n = 12$). The positive control consisted of culture media alone, representing intact barrier function, while the negative control was sterile deionised water, representing maximal junctional disruption. Data are presented as mean $\pm$ SD, illustrating the extent to which each biorelevant formulation perturbs tight-junction structure relative to these defined controls.

The impact of incubation with biorelevant media on epithelial tight junction integrity was assessed by monitoring transepithelial electrical resistance (TEER). As shown in Figure 20, cells maintained under standard culture conditions exhibited stable TEER values, with resistance across the monolayer remaining consistent throughout the experiment. This stability confirms that the culture medium alone does not compromise barrier function. By contrast, incubation with the negative control (sterile deionised water) resulted in a

pronounced decline in TEER, falling below the established acceptance threshold within 60 minutes. This rapid loss of resistance indicates substantial disruption of tight junction integrity and highlights the sensitivity of the monolayer to non-physiological conditions.

Introduction of complex media, including both commercial and in-house FaSSIF formulations, yielded initial TEER values exceeding 500 Ω . Prior to the experimental "0-minute" time point, monolayers were incubated with the respective media for 30 minutes to permit cellular stabilisation following the medium change. During this equilibration period, incubation with the in-house biorelevant formulations Strath FaSSIF 1 and Strath FaSSIF 7 maintained TEER values above the pre-experimental baseline (>500 Ω). Consistent with the favourable cell viability observed in the MTT assay, this sustained epithelial resistance indicates that tight junction integrity was preserved in the presence of these complex media, with no evidence of monolayer destabilisation across the full 120-minute duration of the assay. Furthermore, TEER values obtained with Strath FaSSIF 1 and 7 were comparable to those measured under standard HBSS transport conditions. The absence of significant differences between HBSS and the in-house biorelevant media supports the suitability of these formulations as donor experimental fluids, providing justification for their substitution in transport studies.

In contrast, incubation with the biorelevant.com commercial medium, although initially meeting the experimental acceptance criteria following the equilibration period, demonstrated a sustained decline in transepithelial electrical resistance (TEER) over the course of the study (Figure 20). By 120 minutes, TEER values had fallen below the 300 Ω threshold, indicating a significant compromise in barrier integrity. Notably, there was no statistical difference between the TEER profile of the biorelevant.com medium and that of the negative control (sterile deionised water), underscoring the disruptive effect of this formulation on tight junctions.

Despite this decline in TEER, cell viability remained relatively high (>80% compared to HBSS), suggesting that the observed effect was not due to overt cytotoxicity but rather to perturbation of tight junction function. The divergence between viability and TEER outcomes highlights the importance of assessing both parameters, as survival alone does not guarantee preservation of epithelial barrier properties.

The greater impact of the biorelevant.com medium on tight junction integrity may be explained by its micellar composition. Unlike the in-house Strath FaSSIF formulations, which incorporate cholesterol and free fatty acids to diversify micelle structures and buffer bile salt activity, the biorelevant.com medium relies primarily on bile salts and lecithin. This limited micellar diversity likely results in a higher proportion of pure bile salt micelles, which exert stronger detergent-like effects on cellular membranes and junctional complexes. Consequently, the reduced structural buffering capacity of this medium may facilitate bile salt-driven disruption of tight junctions, accounting for the pronounced decline in TEER observed.

These findings provide assurance that the experimental setup is capable of distinguishing between conditions that preserve barrier function and those that induce junctional breakdown, thereby validating the use of biorelevant media in subsequent analyses.

3.5. Conclusions

These findings highlight the importance of considering both physicochemical properties and media composition when predicting intestinal permeability. The suite of nine biorelevant media developed by the Strathclyde Biopharmaceutics Group was specifically designed to capture the variability observed in intestinal fluid composition, as reported by Riethorst et al. (2016). By encompassing both fasted- and fed-state conditions, these formulations represent best- and worst-case scenarios across the range of total amphiphile concentrations (TAC), thereby providing a robust framework for evaluating compound solubility and transport.

While solubility testing is essential, it is equally important to assess the impact of these complex media on cellular integrity ahead of follow-up permeability studies. Cell culture systems are inherently sensitive, and deviations from optimal culture conditions inevitably impose stress on the monolayer. Consequently, refinement of the nine-media suite was necessary to identify which formulations could serve as viable substitutes for standard protocols without compromising cell survival.

Initial screening suggested that specific media components may be driving cytotoxicity. This observation underscores the need for future work to delineate concentration thresholds for individual amphiphiles, thereby defining the limits within which cellular integrity can be maintained. Evidence from this study indicates that bile acids and free fatty acids are the primary contributors to toxicity, whereas cholesterol and lecithin exert protective effects, stabilising membranes and preserving tight junction integrity. Such insights are critical for guiding the rational design of biorelevant media that balance physiological relevance with experimental feasibility.

Following selection of the media from the screening process and having established that the cells were not apoptotic in their presence, it was necessary to further examine the impact of these formulations on tight junction integrity. Assessing this additional layer of complexity confirmed that there were no barriers to carrying these media forward for use in comparing drug transport across the monolayer under different conditions.

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Chapter 4- The use of Caco-2 Cellular assay as a predictability tool of drug permeability.

4.1. Introduction

The oral route remains the most widely and preferred means of drug administration, owing to its convenience, non-invasive and the high level of patient compliance it affords relative to parenteral routes¹. However, the clinical success of an orally administered drug depends on the complex sequence of physicochemical and physiological events that begin with dissolution in the gastrointestinal (GI) lumen and culminate in absorption across the intestinal epithelium into the systemic circulation. Of these determinants, intestinal permeability — the capacity of a drug molecule to cross the epithelial barrier of the gut wall — is one of the most critical, and it is here that a substantial proportion of drug candidates fail during preclinical and clinical development.

The Caco-2 cell line, derived from a colorectal adenocarcinoma was first proposed as an intestinal permeability model in the late 1980s following observations that under standard culture conditions the cells spontaneously differentiated into a polarised, columnar epithelium exhibiting many of the morphological and functional characteristics of mature enterocytes². Formations of tight junctions, the expression of brush-border hydrolase enzymes, and- critically- the functional expression of a range of influx and efflux transporters implicated in intestinal drug disposition, such as P-glycoprotein (P-gp), the peptide transporter PEPT1, and members of the organic anion and cation transporter families³.

While cultured as a confluent monolayer on a semi permeable insert, Caco-2 cells permit bidirectional transport studies-apical-to-basolateral and basolateral-to-apical-enabling estimation of apparent permeability coefficients and the efflux ratios that have been shown to correspond well with the fractions of dose absorbed (%FA) in humans for a wide range of compounds⁴. Correlation between the two underpins the model's regulatory acceptance and its routine use across pharmaceutical industry as a first-line screen for intestinal permeability, as well as mechanistic tool for probing transport-mediated and paracellular transport pathways⁴.

Despite its widespread adoption, the conventional Caco-2 assay is not without significant limitations. Compounds are typically dosed from simple aqueous buffers — most commonly Hank's Balanced Salt Solution (HBSS) at fixed pH — that bear little

resemblance to the complex, dynamic luminal environment encountered *in vivo*⁵. The human gastrointestinal fluid is not a simple buffer: it contains bile salts, phospholipids, and other endogenous surfactants that solubilise lipophilic compounds, influence the free (unbound, permeable) fraction of drug available at the apical membrane, and can alter apparent permeability independently of any change in the drug's intrinsic membrane-crossing ability⁵.

The disconnect is particularly consequential for poorly soluble, lipophilic compounds—a growing proportion of the modern drug discovery pipeline—where solubilisation by intestinal micelles can dramatically affect the concentration gradient driving passive diffusion, and where standard buffer-based Caco-2 assay may therefore systematically over- or under-predict true *in vivo* permeability^{5,6}. This has driven increasing interest in the use of biorelevant media, such as simulated intestinal fluids formulated to mimic fasted- or fed-state luminal composition, as apical dosing vehicles in the Caco-2 assay, with the aim of generating permeability data that more faithfully reflect the physiological absorption environment and that can serve as more accurate inputs into physiologically based pharmacokinetic (PBPK) models.

4.2. Objectives

The objective of this study is to evaluate the impact of biorelevant media, selected from Chapter 3, on drug permeability across Caco-2 monolayers in the absence of a mucus layer. These media, chosen for compatibility with the Caco-2 cells, were employed to assess the apparent permeability of a range of BCS-classified compounds and compared against the industry-standard HBSS transport medium.

4.3. Materials and Methods

4.3.1. Chemicals and Reagents

Caco-2 from the American Type Culture Collection (Rockville, MD, USA) were donated by Dr Louise Young (University of Strathclyde), Dulbecco's Modified Eagle's Medium (DMEM) high Glucose (4,500mg L⁻¹) with L-Glutamine and without Pyruvate, Foetal Bovine serum, Non-essential Amino acids, Penicillin (10,000 U/ml⁻¹)- Streptomycin (10,000 U/ml⁻¹), Phosphate buffer saline at pH 7.4 and, TrypLE were all purchased from Fisher Scientific, Paisley UK. Sodium taurocholate, ammonium formate, sodium chloride (NaCl), chloroform, formic acid, monosodium phosphate (NaH₂PO₄), fenofibrate, indomethacin and phenytoin were purchased from Sigma Aldrich Poole, Dorset UK. Lecithin S PC (phosphatidylcholine from Soybean "98%") was purchased from Lipoid.com. Sodium oleate was obtained from BDH Chemical Ltd. Poole, England. The solvents methanol, acetonitrile and water were all HPLC grade from VWR, UK.

4.3.2. Preparation of Simulated Intestinal Fluid

4.3.2.1. Media Preparations

Fasted media was prepared according to the methods described in Chapter 3, with pH adjusted using 1M HCl and 1M NaOH solutions. Fasted and fed simulated intestinal fluid (FaSSIF v2 and FeSSIF v2) were prepared according to the instructions provided by the manufacturer (Biorelevant.com), and fed media was additionally prepared according to the method published by Markopoulos et al.⁵.

To reflect conditions at the mucosal surface rather than in the bulk intestinal lumen, the simulated intestinal fluids prepared in the preceding sections were also adjusted to mucosal pH. The bulk intestinal lumen typically has a pH of around 6.5–7, whereas the pH within the mucosal microenvironment is higher, at approximately 7.34. Media were therefore prepared as previously described, but rather than being adjusted to the standard set pH for each fluid, all media were instead adjusted to pH 7.34 to reflect this mucosal microenvironment.

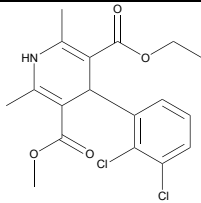
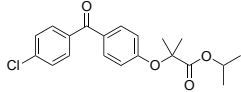
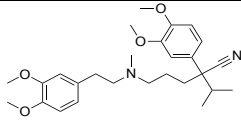
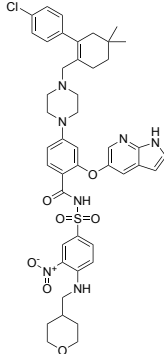
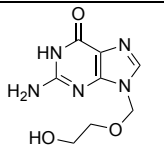
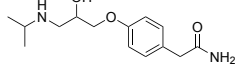
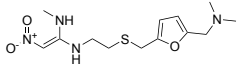
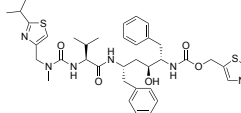
4.3.3. Cell Culture

Human colon cancer cells were gifted from Louise Young at the University of Strathclyde, the cells were cultured and incubated at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS, 1% Non-essential amino acid and 1% PEST giving the complete media which it will be referred to as from now on. Culturing procedure and experimental set up was guided from Press *et al.*⁷. Initially thawed cells were cultured in a T-25 flask until reaching ~80-90% confluence. Cells were trypsinised to remove cells from flask with TrypLE and transferred to a 75cm² flask, with media being changed every third day and trypsinised every fifth day for passage. Cells were passaged and seeded on permeability inserts between passages 25-45. Monolayers were seeded under the same conditions from Chapter 3.

4.3.4. The drugs

The drugs selected were previously used in SIF solubility studies⁸⁻¹⁰, which provided an existing, well-defined dataset of solubility in fasted- and fed-state simulated intestinal fluids. Using these compounds allowed the permeability data generated here to be interpreted alongside established solubility data, so that the combined influence of SIF on both solubility and permeability could be assessed for the same molecules. This pairing is particularly relevant for generating biorelevant inputs for PBPK modelling, where solubility and permeability are key determinants of predicted absorption. In total 8 drugs were studied; the characteristics of these drugs are presented in Table 11.

Table 11 - Physicochemical properties (molecular weight; pKa and log P) and structures of compounds investigated in this study.

Compound	Clinical Application	Molecular Weight (g/mol)	Log P	pKa	Structure
Felodipine	Antihypertensive	384	3.86	-	
Fenofibrate	Antihyperlipidemic	361	5.2	-	
Verapamil	Antihypertensive/ Antiarrhythmic	454.6	2.6	8.6	
Venetoclax	Chemotherapeutic	868.4	5.5	5.5	
Acyclovir	Antiviral Agent	225	1.56	2.5	
Atenolol	Antihypertensive(β-blocker)	266.3	0.23	9.6	
Ranitidine	Antiulcer(H2 Antagonist)	314.4	3.6	2.2	
Ritonavir	Antiretroviral(HIV protease Inhibitor)	720.9	5.6	2.8	

4.3.5. *Initial Experiment*

A stock solution of all tested API was prepared in dimethyl sulfoxide (DMSO) at a high concentration (>10mM). This was then spiked into SIF media at desired concentration (10 μ M); important to ensure final DMSO concentration in experimental set up was low to avoid any damage to cell. Given this the concentration of the DMSO present in 500 μ L of SIF exposed to the donor chamber of the transwell plate being ~0.1% as typical drug concentrations spikes into SIF media was ~0.5 μ L.

4.3.6. *SIF and Caco-2 Incubation*

24 Hours prior to experimental procedure involving SIF; the complete media in both apical (500 μ L) and basolateral (1.5 mL) wells is replaced with fresh pre-warmed cell-culture media. Simulated intestinal media is prepared (Section 2.2), the SIF and HBSS is pre-warmed to 37 °C prior to addition to the apical side. Prior to addition to the monolayer plate, the drug of interest is spiked into 2 mL of SIF at 10 μ M concentration and HBSS is supplemented with 4% Bovine Serum albumin. 500 μ L of SIF + drug is added to the apical compartment while HBSS + BSA is placed in the basolateral compartment. Plates are incubated for 2-hours at 37 °C 5%CO₂. 100 μ L samples are removed from the basolateral side at 30-minute time intervals, with the removed volume being replaced with fresh HBSS+BSA. Samples were placed in the freezer prior to analysis.

4.3.7. *Sample clean-up*

Sample clean-up was performed using 2:1 ratio of acetonitrile: sample. Following addition of acetonitrile samples were vortexed for approximately 5 seconds and centrifuged for 10 minutes at 12,000 RPM in an Eppendorf® Centrifuge MiniSpin G. The supernatant (~300 μ L) from each sample was then placed in HPLC vial inserts for analysis.

4.3.8. *Analytical Methods HPLC*

4.3.8.1. HPLC

Reverse phase HPLC analysis was performed using Shimadzu High-performance Liquid Chromatography Prominence-I LC-2030C (Kyoto, Japan). The HPLC method was previously used to quantify the drugs of interest within SIF⁹⁻¹¹, using a gradient method on

an Xbridge® C18 5 µm (2.1 × 50 mm) at 30 °C. Mobile Phase A was 10 mM of ammonium formate in deionised water adjusted to pH 3 (± 0.1), and mobile phase B was 10 mM of ammonium formate in acetonitrile: water (9:1) (v/v). A gradient between mobile phases A and B was used: start 70:30 (A: B), 3 min 0:100, 4 min 0:100, 4.5 min 70:30; total run time 8 min. Flow rate used was 1 mL/min. This method was only used when no bovine serum albumin (BSA) was used in the acceptor chamber of the monolayer plate. Due to sample clean up not being present so no dilution of the drug. Calibration was conducted for each drug, and linear regression was used to interpolate drug concentration.

Table 12 - HPLC method details gradient start 70:30 (A: B), 3 min 0:100, 4 min 0:100, 4.5 min 70:30; Total run time 8 min. FR-Flow Rate.

Total run time is 8 minutes injection volume was set to 10 µL to ensure there was enough loading of each sample onto the column. Given the analytical method was already reported to accurately quantify the drugs ^{9,10,12-15}. Calibration curves with a minimum of 6 points and 3 replicates were constructed for each drug with the coefficients >0.99, with the equation of the line used to extrapolate the drug concentration. The calibration range from each compound was set at 4 µM-1 nM.

Drug	Mobile Phase	Column	Flow (mL/min)	Injection Volume (µL)	Detection λ (nm)	Retention Time (min)
Felodipine	Mobile Phase A: 10 mM	Bridge C18	1	10	254	2.6
Fenofibrate	Ammonium Formate pH 3 (adjusted with formic acid) in H2O	5 µm 2.1x 50 mm	1	10	291	3.2
Verapamil	Mobile Phase B: 10 mM Ammonium Formate in ACN:H2O (9:1 V/V)		1	10	200	1.1

4.3.9. LC-MS/MS Method

The inclusion of 4% BSA in the acceptor chamber to maintain sink conditions introduced a complex protein matrix, requiring more sensitive analytical methods to quantify drug permeation across the monolayers. Prior to analysis, each BSA-containing sample underwent a clean-up step to prevent interference with quantification. This involved combining the sample with acetonitrile at a 2:1 ratio, followed by centrifugation at 14,000 RPM for 10 minutes; the resulting supernatant was then transferred into a labelled HPLC vial for analysis. Samples were held in an autosampler (Shimadzu Nexera, SIL-40°C X3) at 4 °C, with 1 µL injected onto the column for each run.

LC-MS/MS analysis was carried out on a Shimadzu LC-MS-8060NX operating in MRM mode, coupled to a Shimadzu Nexera HPLC system (LC method described in Section 4.2.8.1). The final LC-MS/MS methods for each compound are presented in Table 12, Table 13 and Table 14. For each drug, a six-point calibration curve was generated over the range 0.06–250 ng/mL, using standards prepared in acetonitrile. Peak area was calculated at each calibration point and plotted against concentration, and the resulting linear equation was used to determine API concentration in subsequent samples. All calibration curves showed good linearity, with R^2 values exceeding 0.99 for every drug, confirming their suitability for quantification; compound-specific calibration curves are provided in Appendix 1.

For each sample, quantification was based on the most abundant MRM transition, with peak area used as the measure of signal intensity. Peak integration and quantification were performed using Shimadzu LabSolutions Insight software. Samples for which the software failed to detect a peak were excluded from the dataset.

Table 13 - Parameters of the LC-MS/MS method, reverse phase method - gradient start 70:30 (A:B), 3 min 0:100, 4 min 0:100, 4.5 min 70:30; Total run time 8 min. FR-Flow Rate, IV-Injection Volume, RT- Retention Time.

Drug	Mobile Phase	Column	Flow (mL/min)	IV (μL)	M/Z Detection (Bold Quantifier)	Retention Time (min)
Venetoclax	Mobile Phase	XBridge	0.4	1	636.1	3.3
	A: C18				321.0	
	10 mM	5 μm			177.2	
Fenofibrate	Ammonium	2.1x	0.2	1	233	1.1
	Formate pH	50 mm			138.95	
	3 (adjusted with formic acid) in LC-MS Grade H ₂ O				111	
	Mobile Phase B:					
	10 mM					
	Ammonium Formate in LC-MS grade acetonitrile: LC-MS grade H ₂ O (9:1 V/V)					

Table 14 - Parameters of LC-MS/MS HILIC method - Gradient starting at 20:80, 5.5 mins 60:40, 7.50 20:80, 10 min 20:80; Total run time 10 min. FR-Flow Rate, IV-Injection Volume, RT- Retention Time.

Drug	Mobile Phase	Column	Flow (mL/min)	IV (μL)	M/Z Detection (Bold Quantifier)	Retention Time (min)
Acyclovir	Mobile Phase A: 10 mM Ammonium Formate pH 3 (adjusted with formic acid) in LC-MS Grade H ₂ O	SeQuant ZIC- pHILIC, 5μm, Polymetric 2.1x100m m	0.2	2	152.05	2.7
					135	
					143.95	
					45	
					110	
Atenolol	Mobile phase B: Acetonitrile		0.2	1	145.05	4.1
					190.1	
					74.15	
					225.1	
					116.10	
Felodipine	Colum oven 40°C		0.2	1	338.0	1.1
					352	
					224.9	
					323.95	
Ranitidine			0.2	1	175.9	3.8
					130.1	
					125	
					102.1	
					224.15	
Ritonavir			0.2	1	296.15	1.1
					268.1	
					140.1	
					197.1	
					171.15	

Troubleshooting to minimise carry-over of highly lipophilic drugs guided a switch from reverse-phase to hydrophilic interaction liquid chromatography (HILIC) separation. In reverse-phase chromatography, highly lipophilic compounds are strongly retained on the non-polar stationary phase, which can lead to incomplete elution and subsequent carry-over into later injections, compromising the accuracy of quantification^{16,17}. HILIC instead employs a polar stationary phase with a predominantly organic (acetonitrile-rich) mobile phase, retaining analytes based on their polarity rather than lipophilicity. This mechanism reduced retention of lipophilic residues on the column, minimising carry-over, while also improving the separation and detection of the more polar drug compounds within the panel, which were poorly retained under reverse-phase conditions¹⁸.

Prior to sample analysis, each drug was optimised for its MRM transitions and collision energy to refine the method; the full list of monitored mass transitions is presented in Table 11.

4.3.10. Data Analysis

4.3.10.1. Apparent permeability Calculation

To determine the permeability profiles of each drug across different media, the apparent permeability coefficient (P_{app}) was calculated. The chromatographic peak area was first substituted into the corresponding calibration curve equation for the respective API, yielding the quantified concentration. This concentration was then normalized to a per-second value by dividing by 7200 seconds (equivalent to 120 minutes), thereby providing the transport rate across the membrane (dB/dt). The obtained rate was subsequently divided by the membrane surface area (1.12 cm^2) and multiplied by the initial donor concentration ($10 \text{ }\mu\text{M}$), in accordance with the apparent permeability equation. The final values were expressed in units of cm/s :

$$P_{app} = \frac{dB/dt}{A \cdot C_0}$$

Where dB/DT is the rate of transport across the membrane, A is the surface area of the membrane and C_0 is the initial concentration in the donor compartment.

4.3.10.2. *Statistical analysis*

To calculate the variability within the sample set the coefficient of variability was used with all data being plotted and further analysis conducted on GraphPad Prism 9 for MacOS.

4.4. Results and Discussion

4.4.1. Method Optimisation

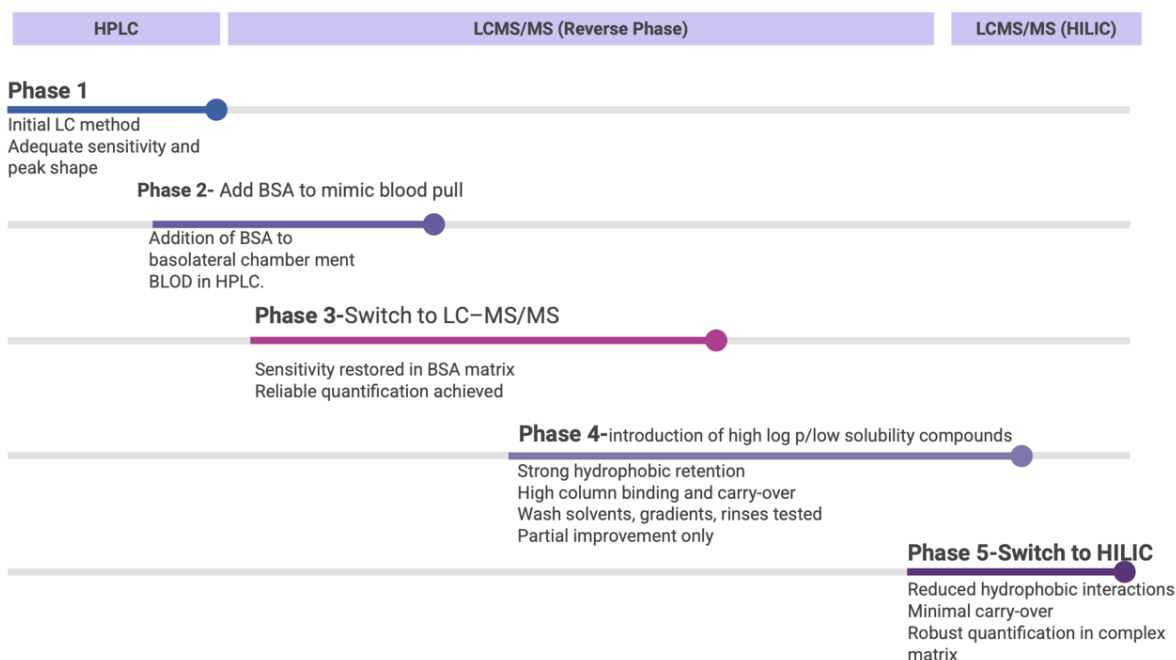


Figure 21 - Workflow of the analytical methods.

Schematic overview of the analytical workflow used throughout the study, outlining the sequential stages from sample preparation through instrumental analysis and data processing Timeline of the five phases of method development, with the analytical platform used at each stage indicated by the bars above.

The progression of method development is illustrated in Figure 21, which outlines the analytical workflow followed to generate the results presented in 4.3.2. Initial screening of compounds was performed using HPLC, based on the method described by Silvia *et al.*¹⁰. The experimental set-up originally used HBSS in the basolateral chamber; however, following initial results, 4% BSA was introduced into the basolateral compartment to better replicate the sink conditions experienced *in vivo*, as described by Hubatsch *et al.*¹⁹, thereby increasing the physiological relevance of the model. *In vivo*, drug reaching the basolateral side of the enterocyte is rapidly removed by blood flow and bound by plasma proteins, principally albumin, maintaining sink conditions and a favourable concentration gradient across the epithelium. Including BSA in the receiver compartment mimics this plasma protein binding, reduces non-specific binding and cellular retention, and therefore improves recovery and provides a more physiologically relevant estimate of permeability, particularly for highly lipophilic drugs. Given the complexity of the

resulting BSA sample matrix, a clean-up step was required prior to analysis (Section 4.3.7); however, this process diluted the compound within the sample, reducing its concentration below the limit of quantification on HPLC. To address this, the method was transitioned to LC-MS/MS, taking advantage of the instrument's greater sensitivity.

Detection was not problematic for the initial drugs analysed. However, the introduction of higher logP, low-solubility compounds led to sample carry-over, driven by the high injection volumes required and subsequent compound build-up within the system. This was initially attributed to retention within the analytical column (XBridge C18, 5 μ m, 2.1 $\times$ 50 mm), as the hydrophobic nature of these compounds favours strong interaction with the silica-based stationary phase via hydrophobic, ionic, and π - π interactions, preventing complete elution under the method conditions in use. Column backwashing was performed to address this; however, carry-over persisted even after thorough cleaning, indicating that the column was not the sole source of contamination.

Attention was subsequently turned to the HPLC system itself: system tubing was cleaned, the needle was manually cleaned, the needle seat was removed, and each inlet valve of the high-pressure pump was individually flushed to eliminate any residual compound trapped within the fluidic pathway. This intervention temporarily resolved the carry-over issue; however, it reappeared after approximately 50 subsequent sample injections, indicating that the underlying cause had not been fully eliminated.

To definitively resolve the carry-over issue, the separation method was transitioned to a HILIC-based approach. The increased polarity of the HILIC stationary phase reduced retention of these highly hydrophobic analytes, eliminating residual binding and preventing carry-over in blank injections. This provided confidence in the reliability and accuracy of subsequent sample quantification.

Table 15 - Calibration curves for the quantified drugs, showing the linear regression equations and corresponding coefficients of determination (R^2).

Each calibration curve was generated from standard solutions prepared across the validated concentration range, and peak area was plotted against nominal concentration. The resulting regression equations and high R^2 values confirm excellent linearity of the analytical method for all compounds.

Drug	Calibration Equation	R^2 Values
Verapamil	$y=38816*x-89369$	0.995
Felodipine	$y=76192*x-256327$	0.994
Fenofibrate	$y=21089*x-395026$	0.995
Acyclovir	$y=46477*x-102753$	0.99
Atenolol	$y=12623*x-132553$	0.992
Ranitidine	$y=47501*x+106078$	0.999
Venetoclax	$y=42234*x-2536$	1
Ritonavir	$y=14345*x+22456$	1

4.4.2. *Permeability Behaviour*

Permeability data are presented as violin plots rather than bar charts of mean $\pm$ SD. Bar charts summarise each condition as a single value, which can mask the spread of the underlying data. Violin plots display the full distribution of replicate measurements, with the median and interquartile range indicated by dashed and dotted lines respectively and individual data points overlaid. This format was chosen because a key finding of this work is the variability in apparent permeability observed between replicates, particularly in the presence of SIF. For example, verapamil Papp in HBSS was relatively consistent, whereas values in FaSSIF 1 spanned approximately a four-fold range. Presenting the complete distribution therefore makes it clear that the biorelevant media did not produce a uniform, reproducible shift in permeability, supporting the argument that no single experimental condition behaves consistently across repeats.

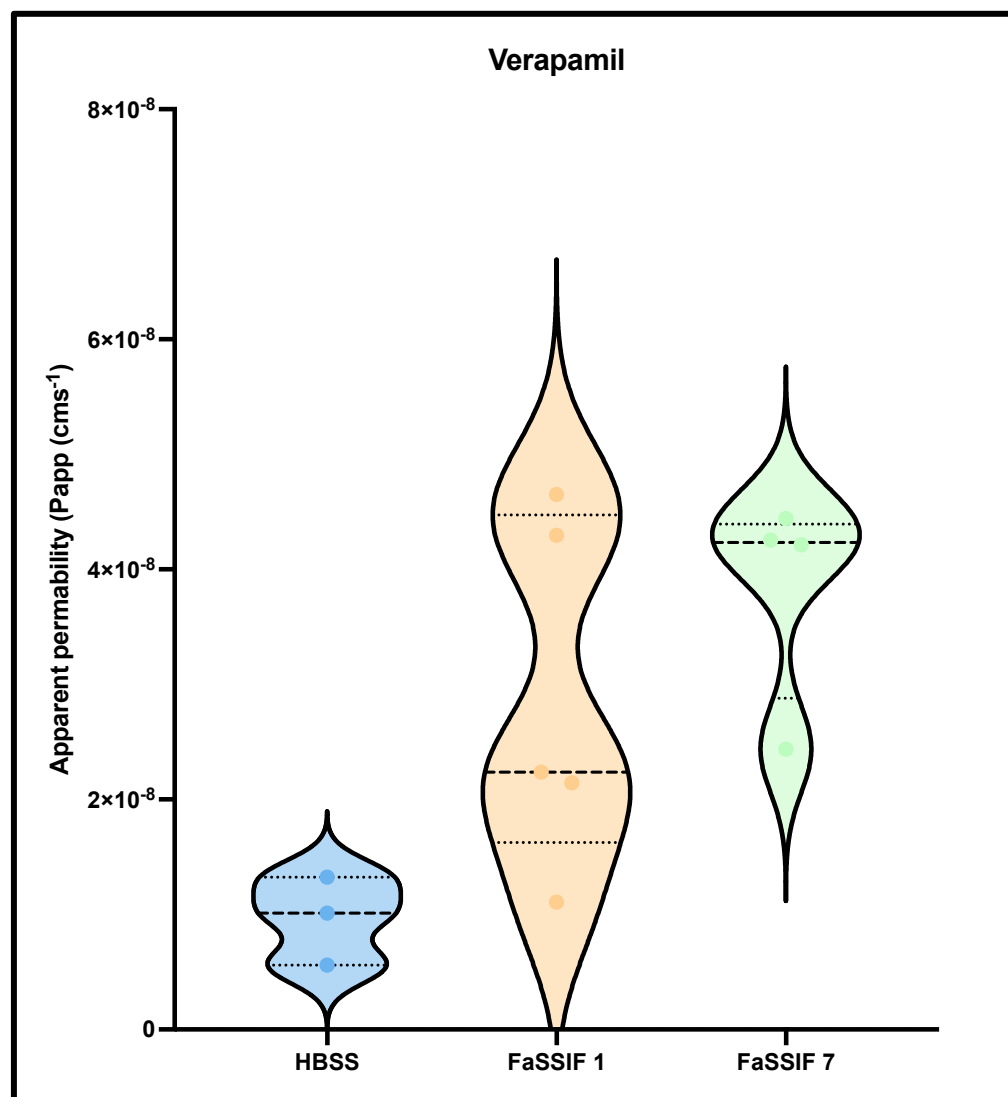


Figure 22 - Violin plots showing the distribution of apparent permeability (P_{app}) values for the BCS Class I compounds across all experimental conditions.

Here experimental pH of each media were set HBSS-7.34, FaSSIF 1-6.3 and FaSSIF 7-7.34. The experiment media was performed with the set media in the Chapter 3.

Each point indicated the measured apparent permeability of the compound; P_{app} is calculated based on: $P_{app} = (dQ/dt) / A \cdot C_0$ where P_{app} = apparent permeability (cm/s), dQ/dt = rate of drug transport across the membrane ($\mu\text{g/s}$ or mol/s), A = surface area of the cell monolayer (cm^2) and C_0 = initial concentration of the drug in the donor compartment ($\mu\text{g/mL}$ or mol/mL). Dashed lines indicate the median P_{app} for each condition, and dotted lines show the full range of the data.

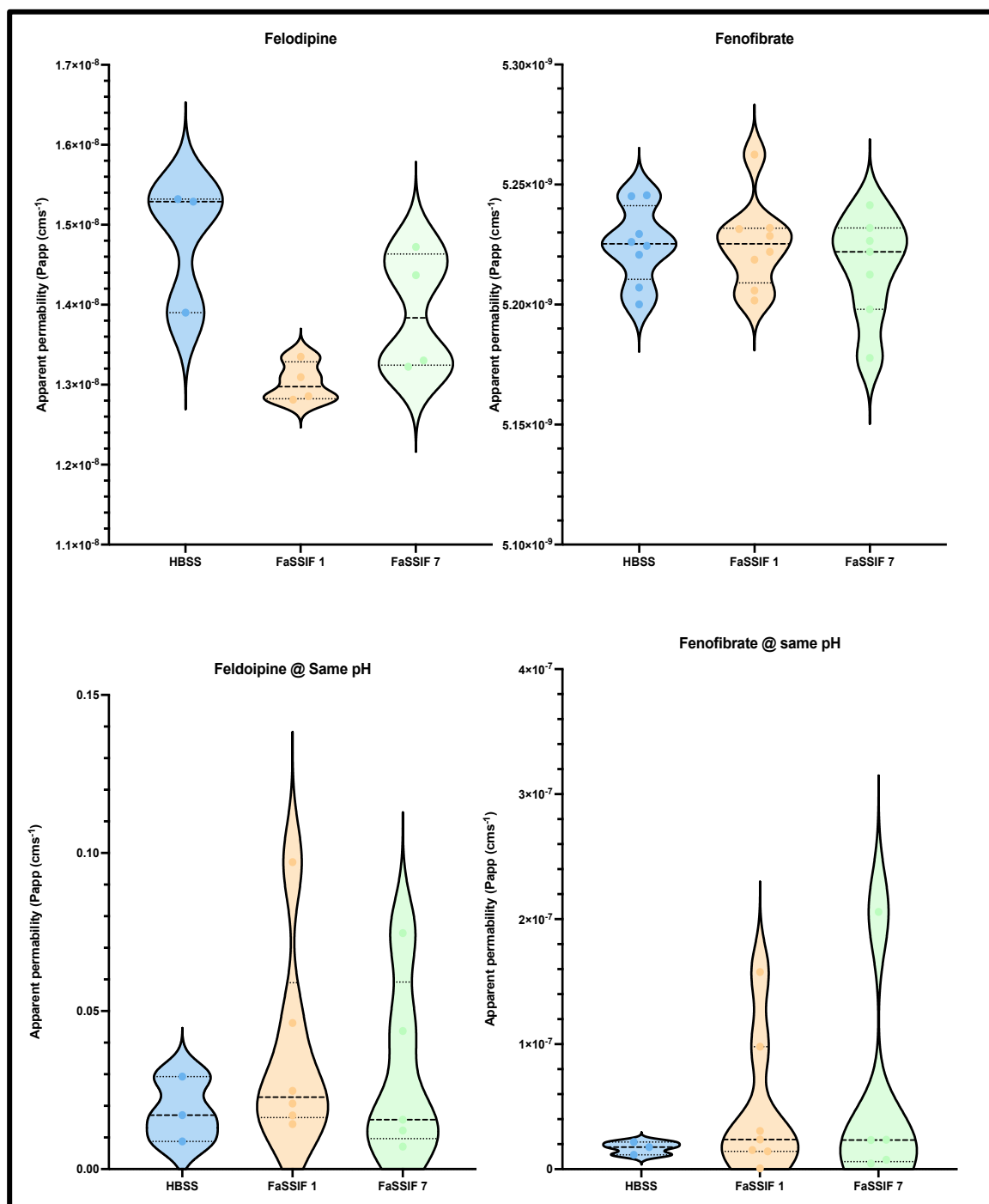


Figure 23 - Violin plots showing the distribution of apparent permeability (P_{app}) values for the BCS Class II compounds across all experimental conditions.

For the upper panels, each medium was tested at its native biorelevant pH to reflect the distinct environments encountered along the gastrointestinal tract. In contrast, the two lower panels present data generated using the same media after adjustment to a unified pH of 7.34, selected to align with the mucosal surface pH.

Each Point indicated the measured apparent permeability of the compound, P_{app} is calculated based on: $P_{app} = (dQ/dt) / A \cdot C_0$ where P_{app} = apparent permeability (cm/s), dQ/dt = rate of drug transport across the membrane ($\mu\text{g/s}$ or mol/s), A = surface area of the cell monolayer (cm^2) and C_0 = initial concentration of the drug in the donor compartment ($\mu\text{g/mL}$ or mol/mL). Dashed lines indicate the median P_{app} for each condition, and dotted lines show the full range of the data.

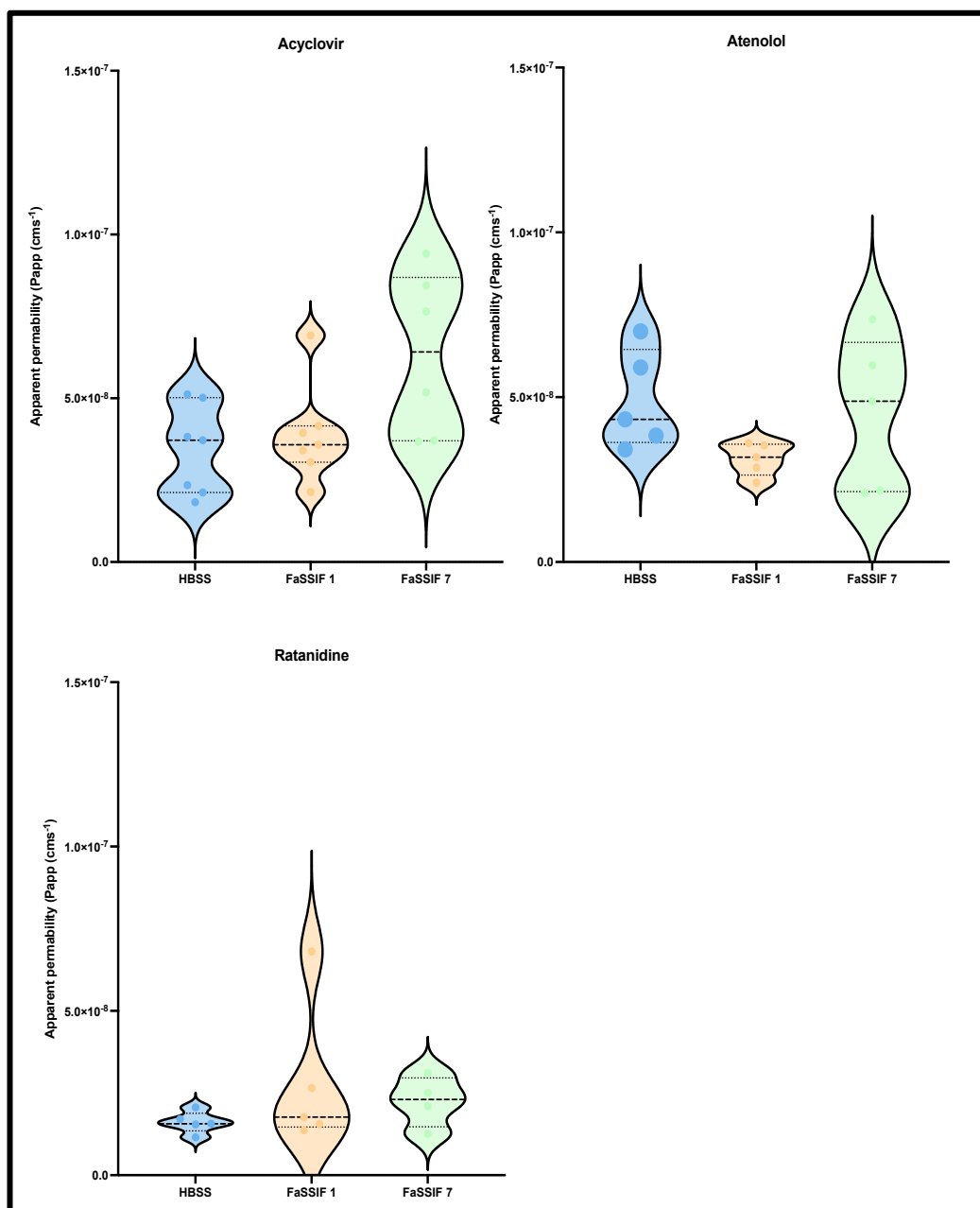


Figure 24 - Violin plots showing the distribution of apparent permeability (P_{app}) values for the BCS Class III compounds across all experimental conditions. All present data generated using the same media with a unified pH of 7.34, selected to align with the mucosal surface pH.

Each point indicated the measured apparent permeability of the compound, P_{app} is calculated based on: $P_{app} = (dQ/dt) / A \cdot C_0$ where P_{app} = apparent permeability (cm/s), dQ/dt = rate of drug transport across the membrane ($\mu\text{g/s}$ or mol/s), A = surface area of the cell monolayer (cm^2) and C_0 = initial concentration of the drug in the donor compartment ($\mu\text{g/mL}$ or mol/mL). Dashed lines indicate the median P_{app} for each condition, and dotted lines show the full range of the data. All measurements were performed at a controlled pH of 7.34 to reflect mucosal conditions; differences between plots at the same pH reflect variations in media composition rather than pH effects.

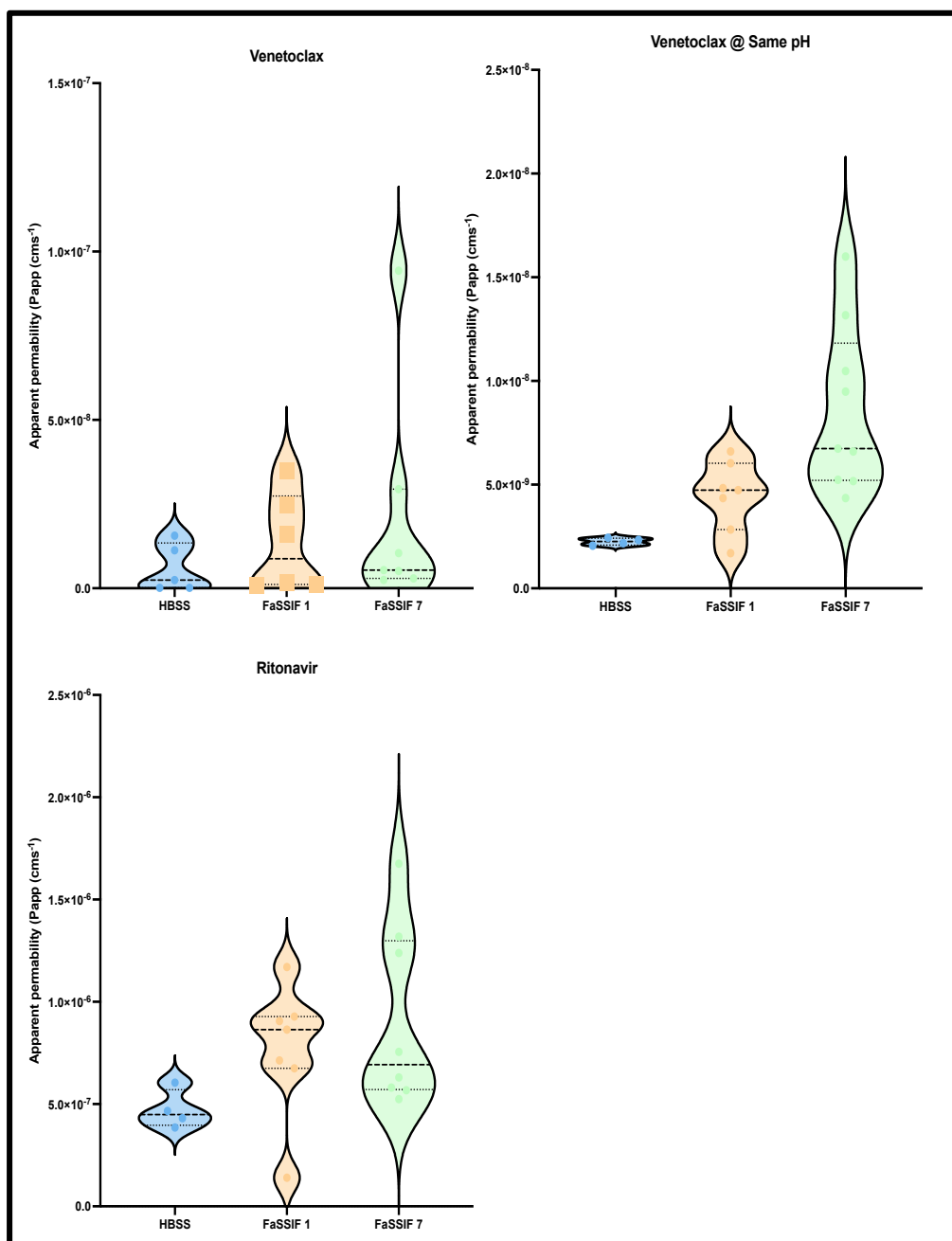


Figure 25 - Violin plots showing the distribution of apparent permeability (Papp) values for the BCS Class IV compounds across all experimental conditions.

For the right panels, each medium was tested at its native biorelevant pH to reflect the distinct environments encountered along the gastrointestinal tract. In contrast, the left panel present data generated using the same media after adjustment to a unified pH of 7.34, selected to align with the mucosal surface pH.

Each Point indicated the measured apparent permeability of the compound, P_{app} is calculated based on: $P_{app} = (dQ/dt) / A \cdot C_0$ where P_{app} = apparent permeability (cm/s), dQ/dt = rate of drug transport across the membrane ($\mu\text{g/s}$ or mol/s), A = surface area of the cell monolayer (cm^2) and C_0 = initial concentration of the drug in the donor compartment ($\mu\text{g/mL}$ or mol/mL). Dashed lines indicate the median P_{app} for each condition, and dotted lines show the full range of the data.

4.4.3. Physicochemical impact on permeability

The permeability of a compound across the Caco-2 monolayer determines its oral absorption potential, and the prevailing assumption in PBPK workflows is that a single apparent permeability value, conventionally generated in a simple transport buffer, adequately represents transport throughout the gastrointestinal tract. The data presented here do not support that assumption uniformly. Apparent permeability varied systematically with the composition of the apical medium, and compounds sharing a BCS classification did not maintain a consistent rank order across media. More significantly, the magnitude of the media effect was not predicted by biopharmaceutical class. This section argues that the determining factor is not solubility but the location of the rate-limiting resistance to transport, and that biorelevant media act principally upon the aqueous boundary layer rather than upon the quantity of drug available for absorption.

For verapamil, a BCS Class I compound ($pK_a = 8.6$) (Figure 22), exhibited a monotonic increase in apparent permeability with increasing biorelevance of the apical medium, from a median of $2.6 \times 10^{-8} \text{ cm s}^{-1}$ in HBSS to $2.8 \times 10^{-8} \text{ cm s}^{-1}$ in Strath FaSSIF 1 and $3.8 \times 10^{-8} \text{ cm s}^{-1}$ in Strath FaSSIF 7. The interpretation of this result is constrained by a feature of the experimental design that excludes the most obvious explanation. Verapamil was fully dissolved at the donor concentration employed in all three media, and the total concentration gradient driving transport was therefore invariant across conditions. Any explanation invoking enhanced dissolution or an increased thermodynamic driving force is consequently untenable, and the effect must originate in the resistance to transport rather than in the quantity of drug presented to the monolayer.

The serial barrier model provides the appropriate framework²⁰. Total resistance to transport comprises contributions from the unstirred water layer and from the epithelial membrane, and these contribute in series, such that the observable permeability is dominated by whichever resistance is larger²¹. For a compound of verapamil's intrinsic membrane permeability, the boundary layer contributes substantially, and in static Transwell systems its effective thickness considerably exceeds that estimated *in vivo* under the hydrodynamic conditions of the small intestine. Amphiphilic components of biorelevant media reduce this effective thickness, both by lowering interfacial tension and by providing a micellar carrier phase that shuttles drug across the boundary layer

more rapidly than free molecular diffusion permits, thereby raising the concentration presented at the membrane surface without altering the bulk concentration^{22,23}. That the greatest enhancement was observed in Strath FaSSIF 7, the medium of highest amphiphile content, is consistent with this mechanism (Figure 22) The corollary carries consequences beyond this chapter: if buffer-based Caco-2 systems systematically overestimate boundary layer resistance relative to the intestine, then permeability values generated in such systems underestimate membrane permeability for precisely those compounds in which the membrane is not rate-limiting, and the bias is compound-dependent rather than a uniform scaling offset.

Verapamil is a well-characterised P-glycoprotein substrate and inhibitor, and bile acids in FaSSIF are reported to improve drug solubility, prevent drug adsorption and suppress P-glycoprotein transporter activity, effects that may be decisive for compounds that are actively transported, poorly soluble, poorly permeable or difficult to recover²⁴. Interestingly, the BCS Class III compounds which displays the similar high solubility of the Class I compounds but differ in that they characteristically display low permeability, also demonstrated apparent permeability profiles that remained largely unaffected by changes in SIF media (Figure 23). Median values for acyclovir ($3.42, 3.88$ and 6.34×10^{-8} cm s^{-1} in HBSS, Strath FaSSIF 1 and Strath FaSSIF 7 respectively), atenolol ($3.42, 3.88$ and 6.34×10^{-8} cm s^{-1} in HBSS, Strath FaSSIF 1 and Strath FaSSIF 7 respectively), ranitidine ($1.6, 2.83, 2.24 \times 10^{-8}$ cm s^{-1} respectively) varied by less than two-fold across all media, with distributions that overlapped substantially in every case. This behaviour in both classes may be explained by their high solubility, which is not substantially altered by media composition; the resulting large concentration gradient across the membrane favours compound movement toward the basolateral chamber regardless of the specific media used.

The direction of the trend is nonetheless mechanistically interpretable and worth examining, since acyclovir is the most hydrophilic compound in the class III dataset, with a reported LogP of approximately -1 (Table 11), considerably lower than the other Class III compounds examined in this study²⁵. The higher amphiphile concentration in Media 7 increases the overall lipophilicity of the system, promoting more rapid exchange of drug between micelles and the surrounding medium. Despite this, acyclovir shows only a marginal increase in solubility, from 2.66 mg/mL in Strath FaSSIF 1 to 2.80 mg/mL in

Strath FaSSIF 7, indicating limited incorporation into micelles. This is consistent with acyclovir's hydrophilic character and suggests that the freely dissolved fraction, the form available for passive diffusion, remains high across both media. Notably, this range represents a substantial increase over the solubility typically reported in purely aqueous systems (~1.6 mg/mL), despite the comparatively small difference between the two SIF media themselves²⁶. The observed increase in permeability across the monolayer may therefore arise not only from this modest solubility enhancement but also from reduced micellar binding, resulting in a larger free fraction of drug available to permeate the cellular membrane.

The compounds considered in (Figure 24) differ from those considered so far in that Class II compounds are poorly soluble but highly permeable. Micellar solubilisation therefore acts simultaneously on the quantity of drug in solution and on its transport, and these consequences oppose one another: incorporation into bile salt–phospholipid micelles raises total dissolved concentration, but only freely dissolved drug permeates the membrane²⁷, while micelle-bound drug continues to diffuse across the aqueous boundary layer and delivers solute to the membrane surface at concentrations above those predicted from free diffusion alone^{28,29}. The most striking feature of these data is not the difference between media but its absence. Median apparent permeability varied by less than 1.5-fold across all three media for felodipine and less than 1.4-fold for fenofibrate with each media at the set pH, this fold change remained for both compounds below 1.2. The dispersion of the data is nonetheless informative: it demonstrates that a single apparent permeability value does not adequately represent the behaviour of these compounds even under nominally identical conditions, and by extension that applying one condition across compounds of differing solubility and lipophilicity, as conventional buffer-based screening does, imposes a uniformity that the underlying transport does not possess.

The final BCS Class Compounds explored in Figure 25 are Class IV, poorly soluble and poorly permeable, and therefore differ from the Class II. Both compounds showed a monotonic increase in apparent permeability with increasing amphiphile content of the medium: venetoclax rose from a median of $2.24 \times 10^{-9} \text{ cm s}^{-1}$ in HBSS to 8.7×10^{-9} in Strath FaSSIF 1 and 5.3×10^{-9} in Strath FaSSIF 7 under varying pH conditions, whereas under unified conditions this apparent permeability was reported with a median of $2.2 \times$

$10^{-9} \text{ cm s}^{-1}$ in HBSS to 4.4×10^{-9} in Strath FaSSIF 1 and 8.5×10^{-9} in Strath FaSSIF 7 and ritonavir from 4.7×10^{-7} to 7.7×10^{-7} and 9.1×10^{-7} . The venetoclax data displays a tightly clustered HBSS replicates and no overlap between HBSS and Strath FaSSIF 7. This is the pattern expected where transport is boundary-layer-limited, micelle-bound drug contributing to diffusion across that layer in proportion to its diffusion coefficient and drug loading^{23,29}. Permeability is therefore governed not by the compound alone but by the relationship between the compound and the medium in which it is presented, and BCS classification, which describes the compound in isolation, does not predict which compounds that relationship will affect.

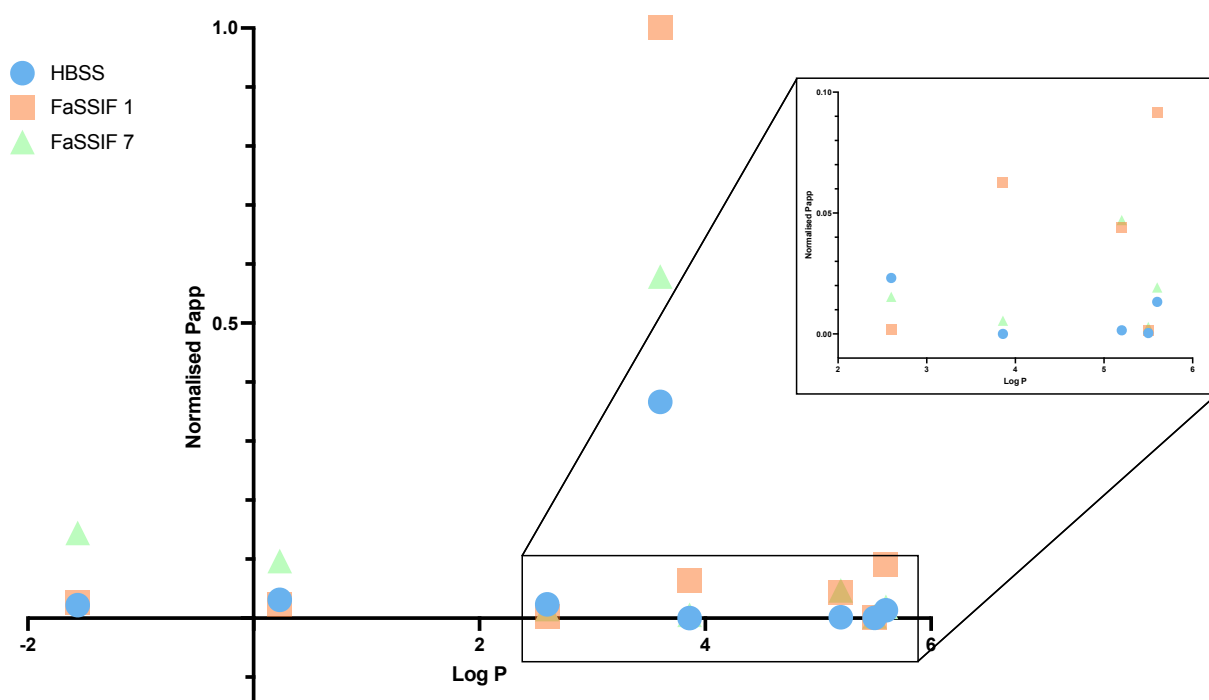


Figure 26 - Comparison of normalised apparent permeability (P_{app}) and lipophilicity ($\log P$) for selected compounds.

Normalised apparent permeability (P_{app}) values are plotted against lipophilicity ($\log P$) for a panel of structurally and biopharmaceutically diverse compounds: Verapamil, Felodipine, Fenofibrate, Acyclovir, Atenolol, Ranitidine, Venetoclax, and Ritonavir.

Class III compounds are characterised by high aqueous solubility, and as a result, variations in transport media exert minimal influence on their permeability, as demonstrated by atenolol and ranitidine. This may be attributed to their high solubility,

which ensures an abundance of freely dissolved drug regardless of media composition, maintaining a concentration gradient that favours apical-to-basolateral movement. Consequently, permeability across the monolayer remains consistent despite changes in the surrounding media³⁰.

In contrast to BCS Classes I and III, compounds in Classes II and IV exhibit low aqueous solubility, which often renders their permeability behaviour more complex and highly dependent on formulation strategy and media composition. Within Class II, comparison of means indicated no statistically significant difference in apparent permeability across the biorelevant media tested at their native pH values.

However, Figure 27 illustrates a further comparison for fenofibrate and felodipine, examining permeability under two conditions: biorelevant media at their respective native pH values, and the same media adjusted to a uniform pH of 7.34 to reflect mucosal conditions. Under this standardised pH, both compounds showed a notable increase in apparent permeability relative to their native-pH conditions (Figure 26). This increase cannot be attributed to changes in drug ionisation. Fenofibrate lacks ionisable groups and is therefore unaffected by pH, while felodipine, a very weak base ($pK_a \approx 5.1$), is already predominantly non-ionised at native pH (approximately 97% at pH 6.64), rising only marginally to >99% at pH 7.34. According to the pH-partition hypothesis, the neutral form of a molecule diffuses more readily across the lipophilic cell membrane than its ionised form^{31,32}. As the neutral fraction of both compounds is essentially unchanged across the pH range studied, this mechanism cannot account for the observed differences. Instead, this suggests that pH-dependent changes in the media themselves, such as alterations in micellar structure or drug-micelle partitioning, are responsible.

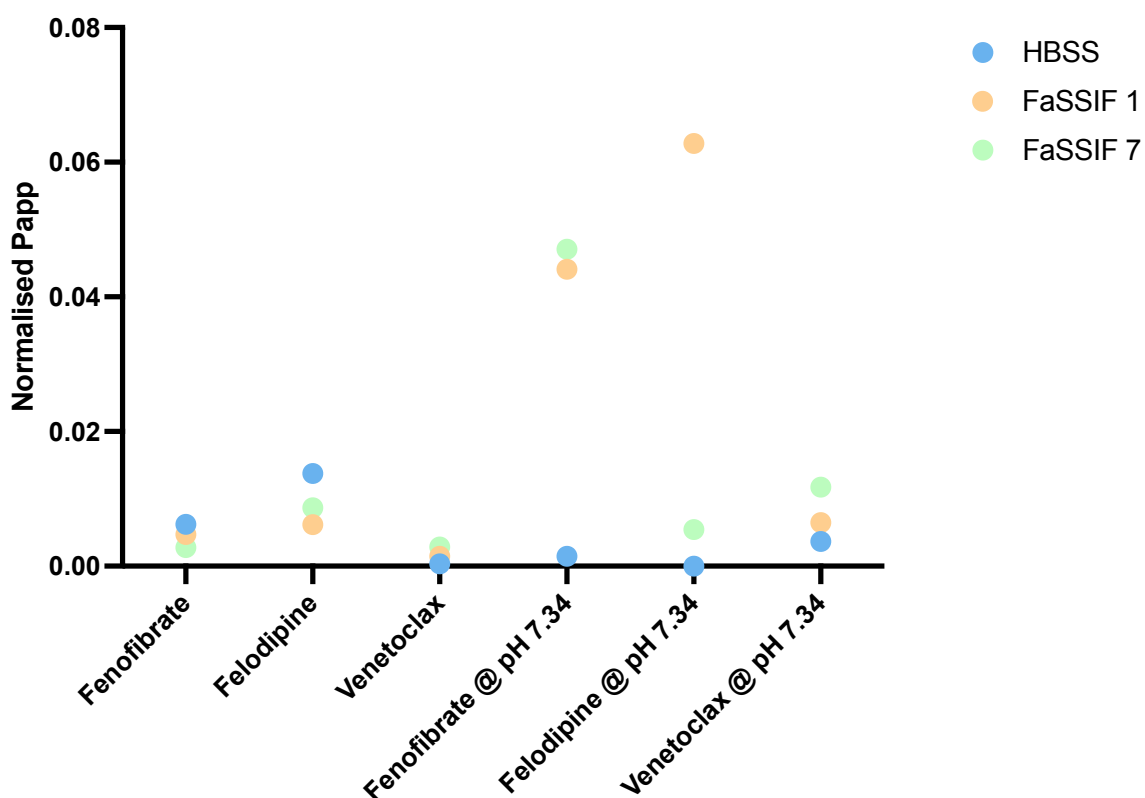


Figure 27 - Comparison of compound permeability profiles across media and pH conditions.

Normalised apparent permeability (P_{app}) values for Fenofibrate, Felodipine and Venetoclax compared across multiple biorelevant media formulations at their respective physiological pH conditions, followed by a comparison of all media at a single matched pH. The first set of panels illustrates how each compound's permeability varies with media composition when pH is systematically adjusted. The second set of panels presents permeability values for all media normalised to a common pH, enabling direct assessment of the contribution of media composition independent of pH-driven ionisation effects. Data are shown as mean $\pm$ SD, demonstrating distinct permeability behaviours driven by both physicochemical properties of the compounds and the colloidal characteristics of each media formulation.

Given the observed change in the permeability profiles of fenofibrate and felodipine following pH adjustment, it was considered relevant to determine whether a comparable effect would be observed in other low-permeability compounds, such as those within BCS Class IV. Figure 27 demonstrates no notable alteration in the permeability profile of venetoclax ($pK_a = 5.5$) across the transport media when the pH was shifted from 6.64 to 7.34. However, at the biorelevant pH of 7.4, a reduction in apparent permeability was observed (Figure 27), with the compound remaining permeability-limited despite pH adjustment. This behaviour is consistent with that typically exhibited by Class IV

compounds, which display low intrinsic permeability and may rely, at least in part, on carrier-mediated transport mechanisms.

The combined influence of a compound's pKa and the surrounding fluid pH warrants careful consideration, given the direct effect this has on the proportion of ionised species present and, consequently, on the compound's capacity to permeate the Caco-2 monolayer. Adjustment of the media pH to 7.34 enabled experimental conditions to more closely approximate those encountered by compounds as they diffuse toward the cellular surface *in vivo*. To further interrogate this relationship, apparent permeability was compared across the two Class II compounds and the Class IV compound under varying pH conditions, reflecting their shared classification as permeability-limited.

For the two Class II compounds, standardising the pH of all examined media (HBSS, Strath FaSSIF 1, and Strath FaSSIF 7) to 7.34 resulted in increased apparent permeability for fenofibrate in both Strath FaSSIF 1 and Strath FaSSIF 7, rising to approximately 0.04 in each. Felodipine exhibited a more pronounced increase in Strath FaSSIF 1, from 0.01 to approximately 0.06, whereas a modest decrease was observed in Strath FaSSIF 7, from approximately 0.01 to 0.009. By contrast, venetoclax exhibited increased apparent permeability in both biorelevant media, rising to approximately 0.01 in Strath FaSSIF 1 and 0.019 in Strath FaSSIF 7, despite remaining substantially permeability-limited overall. The compounds examined in the present study, felodipine, fenofibrate, and venetoclax, are not established substrates for major intestinal transporters and exhibit permeability behaviour predominantly governed by passive diffusion.

The enhanced apparent permeability of fenofibrate and felodipine under biorelevant media conditions is likely attributable to the elevated lipophilicity conferred by these media, which promotes more favourable interactions between the compounds and the amphiphilic constituents present. As indicated in Table 9, fenofibrate and felodipine possess LogP values of 5.5 and 5.6, respectively, consistent with pronounced lipophilicity. In media containing amphiphilic components, such as Strath FaSSIF 1 and 7, lipophilic compounds of this nature exhibit greater partitioning into micellar structures. This partitioning may enhance drug transport by increasing micellar loading, thereby facilitating compound translocation toward the phospholipid membrane. This behaviour is consistent with the principle that lipophilic solutes preferentially partition into

lipophilic microenvironments; such partitioning may reduce the effective diffusional resistance of the aqueous boundary phase and increase overall flux across the cell monolayer, as illustrated in Figure 27. Micelle-mediated transport of this nature may also recapitulate aspects of *in vivo* solubilisation and absorption, wherein bile salt micelles are known to facilitate intestinal uptake of poorly soluble, highly lipophilic compounds.

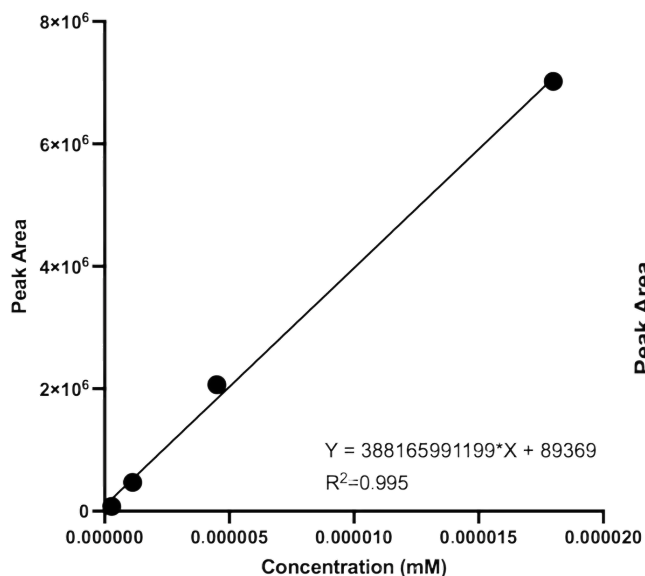
Additional physicochemical and biophysical factors inherent to the *in vitro* experimental system likewise warrant consideration. The unstirred water layer (UWL), a static aqueous layer adjacent to the intestinal epithelium, constitutes an additional diffusional barrier capable of rate-limiting drug transport. As diffusion across the UWL may proceed more slowly than transmembrane permeation, apparent permeability (P_{app}) is governed not only by membrane-intrinsic properties but also by the diffusional characteristics of this aqueous layer. These considerations bear direct relevance to the present findings: although pH adjustment modulated the apparent permeability of Class II compounds, the absence of a proportional relationship between neutral fraction and permeability, particularly evident for venetoclax, indicates that additional determinants, such as transporter-mediated processes and diffusional barriers including the UWL, may contribute to the observed permeability behaviour. Collectively, these findings suggest that solubility–permeability interplay and microenvironmental factors, rather than pH-partitioning alone, govern drug transport across these *in vitro* models.

4.4.1. Variability in permeability data

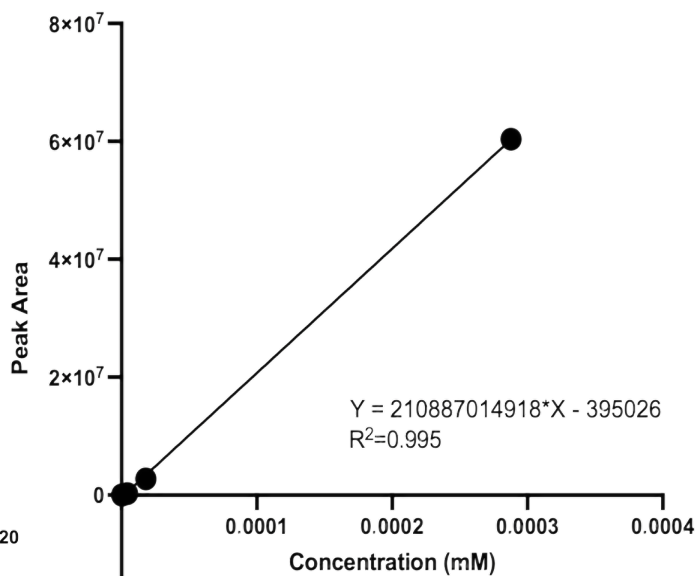
Across all apparent permeability datasets, a marked degree of variability was observed within the complex media conditions. The coefficient of variation (CV) for these datasets consistently exceeded 30%, surpassing the threshold recommended in PBPK industry guidance³³. By contrast, across all four BCS classes, the CV obtained using HBSS as the buffering system remained below this 30% threshold. This reduction in variability is attributable to the absence of complex interactions between free drug and the experimental medium under HBSS conditions. Within the simulated intestinal media, however, the presence of bile salts, lipids, free fatty acids, and cholesterol introduces additional complexity, influencing not only the solubilisation of the drug but also the interaction of the free drug fraction with the intestinal membrane.

To confirm that this variability did not arise from analytical limitations, calibration curves for each compound were generated in HBSS. As shown in Figure 28, the low analytical variability observed confirms that the increased coefficient of variation seen in biorelevant media reflects genuine physicochemical complexity, rather than artefacts of quantification.

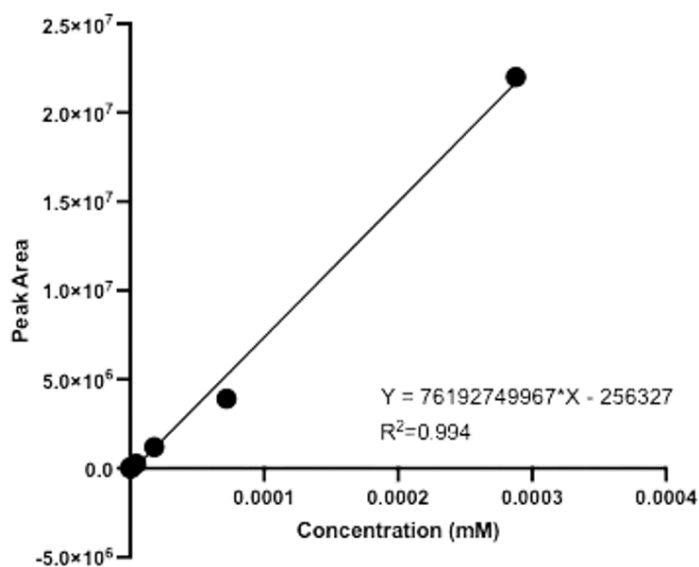
Verapamil



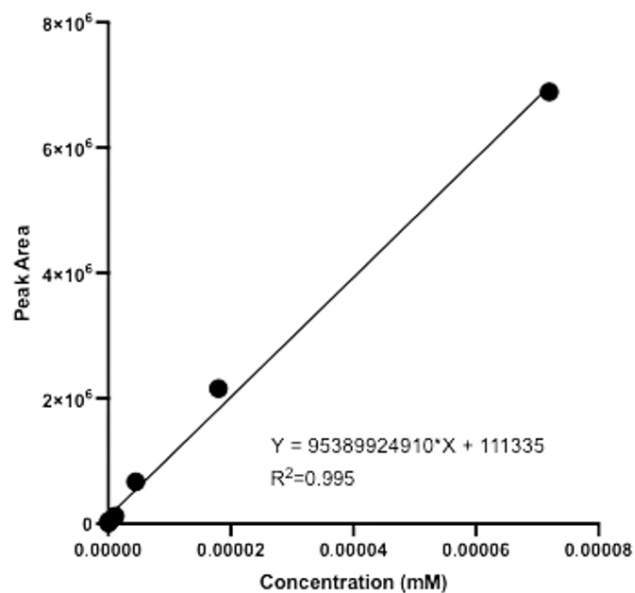
Fenofibrate

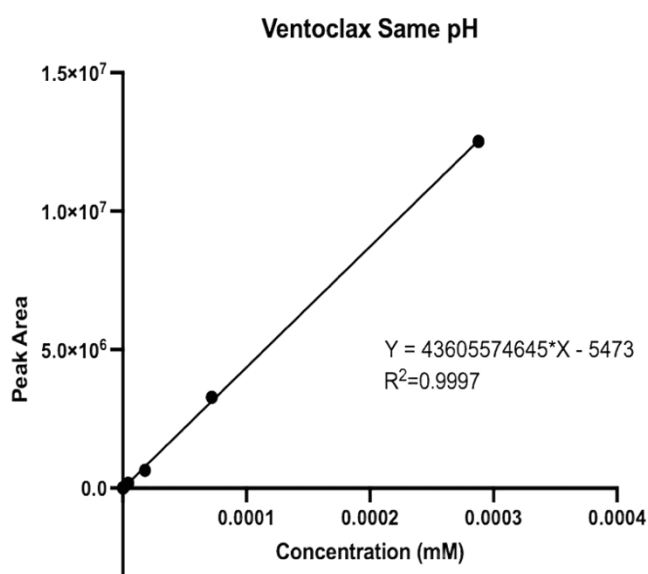
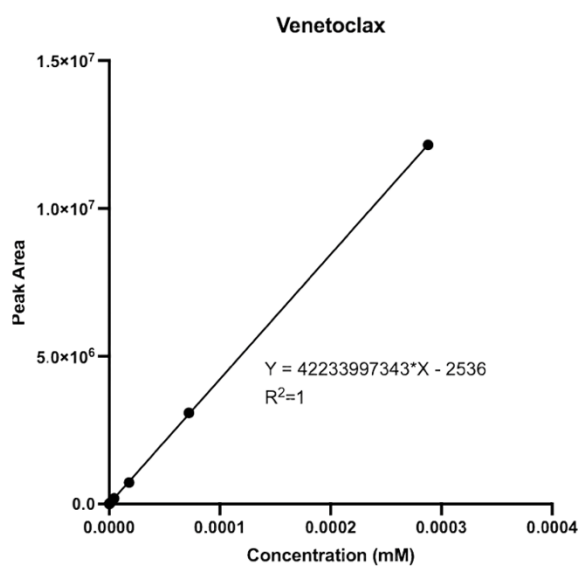
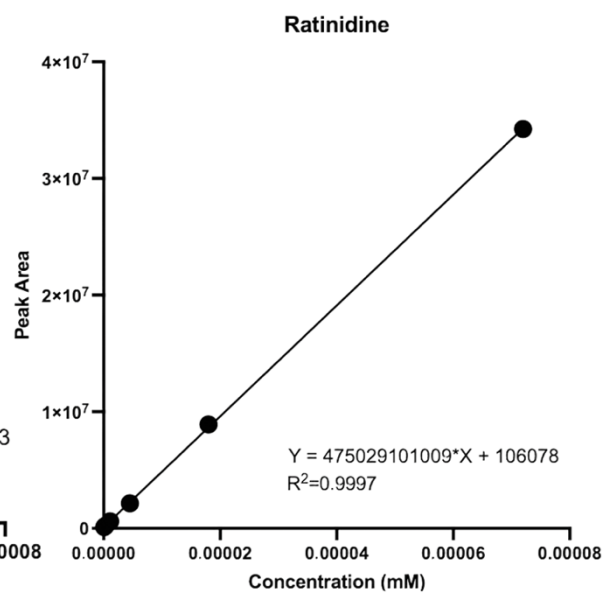
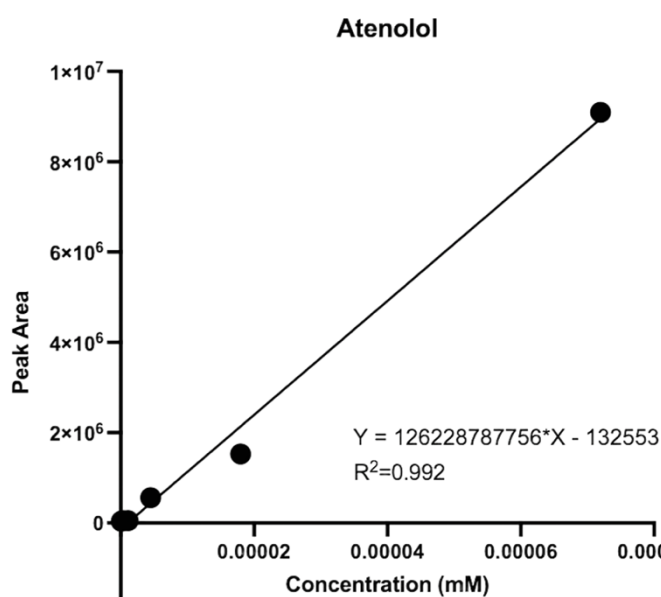
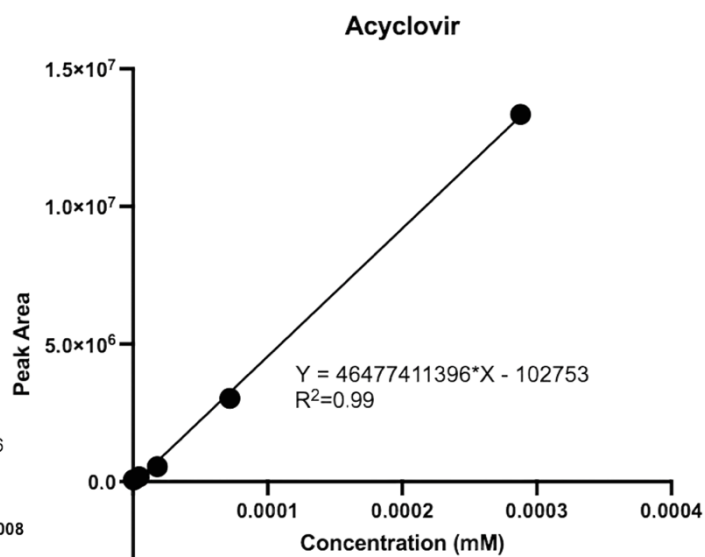
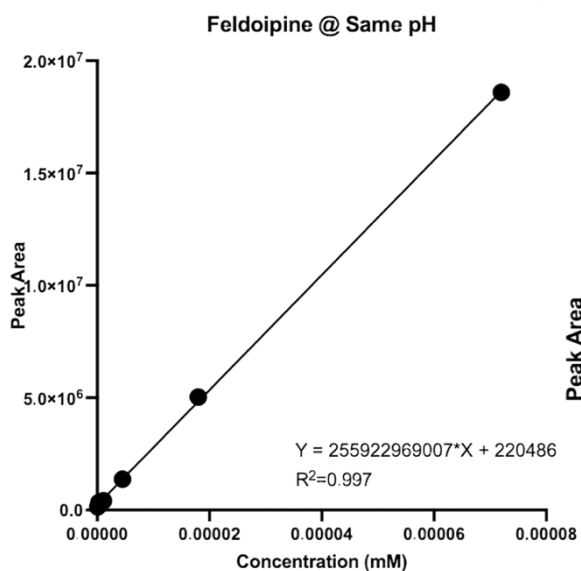


Felodipine



Fenofibrate @ Same pH





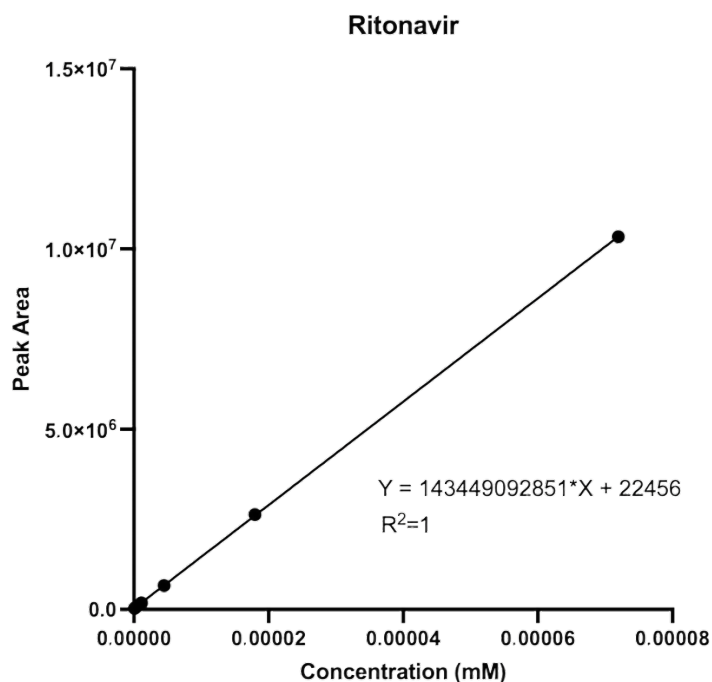


Figure 28 - Calibration curves for LC-MS/MS quantification of study analytes.

Peak area is plotted against nominal concentration (mM) for verapamil, venetoclax, fenofibrate, felodipine, ritonavir, acyclovir, atenolol, and ranitidine, including replicate curves for fenofibrate and felodipine generated under matched pH conditions ("@ Same pH"). Each point represents the mean peak area of a calibration standard; solid lines show the linear regression fit. For verapamil/venetoclax and venetoclax/fenofibrate, two concentration ranges were required to achieve linearity across the full assay range; insets show the expanded lower-concentration region with its corresponding regression line. Linear regression equations and coefficients of determination (R^2) are displayed on each plot; all curves showed acceptable linearity ($R^2 \geq 0.99$) over the concentration ranges tested, confirming suitability for quantification of these analytes in the Caco-2 permeability assay.

Current FDA guidance on Caco-2 permeability studies categorises model compounds according to their *in vivo* intestinal absorption (fa), defining low (fa < 50%), moderate (fa = 50–84%), and high permeability (fa ≥ 85%) compounds³⁴. However, within these guidelines, limited detail is provided regarding the experimental set-up. While confirmation of Caco-2 monolayer integrity is stipulated, the composition of the donor fluid is not addressed³⁵.

In the present study, HBSS as the donor medium yielded limited variability; however, this medium possesses limited biorelevance. By contrast, apparent permeability values obtained in biorelevant media exhibited considerably greater variability, ranging from 5% to 160%. While such variability is conventionally regarded as a limiting factor, within the context of complex biorelevant media it may instead reflect physiological realism, offering mechanistic insight into the interplay between drug and colloidal structures.

A review of the existing literature revealed that 40% of studies reported apparent permeability variability exceeding the industry-accepted threshold of 30%, while 12 studies reported no variability whatsoever in their Papp measurements³⁶⁻⁴². This inconsistency, ranging from unexpectedly high variability to a complete absence of reported variability, raises concerns regarding the reliability and reporting practices within published Caco-2 permeability data. Across these literature studies, the transport media employed was either standard culture media or HBSS, indicating a degree of consistency in media selection despite the divergent variability outcomes reported.

It is important to note that variability in drug performance was evident even within the present experimental set-up. While the use of more biorelevant transport media may introduce additional variability, this can be considered advantageous, as it more closely reflects physiological conditions and provides a more accurate representation of *in vivo* drug behaviour. Accordingly, such variability may be regarded as beneficial to the predictive value of *in vitro* models, rather than as a limitation to be minimised. Interactions between the compound and the transport medium likely contribute to this variability, an effect further compounded by the inherent heterogeneity of the Caco-2 cell line itself⁴³.

Current regulatory guidelines governing the Caco-2 model specify only the compounds to be used for model validation, with limited direction provided regarding the underlying experimental protocol; validation is instead demonstrated through correlation between experimental transport and human absorption data³⁴. A frequently overlooked challenge within Caco-2 permeability research concerns the treatment of magnitude and variability in relation to apparent permeability values. Caco-2 permeability data often do not conform to neat, symmetrical normal distributions, owing to the inherent biological

complexity underlying these measurements. Treating such data as normally distributed may therefore misrepresent the true extent of underlying variability.

4.4.2. *Micelle-Drug Interactions*

Drug loading of micelles is a dynamic process, whereby the concentration of free drug in solution directly influences the extent of micellar incorporation. This reflects the reversible nature of micelle–drug interactions, whereby the drug may associate with or dissociate from micelles depending on prevailing environmental conditions⁴⁴.

Table 16 – Lipophilic properties of the assessed compounds and the media that generated the highest permeability values.

<i>Drug</i>	<i>Log P</i>	<i>Chemical Property</i>	<i>BCS Class</i>	<i>Most permeable media</i>
<i>Acyclovir</i>	<i>~-1.6</i>	<i>Hydrophilic</i>	<i>3</i>	<i>HBSS</i>
<i>Atenolol</i>	<i>~0.16</i>	<i>Hydrophilic</i>	<i>3</i>	<i>HBSS</i>
<i>Felodipine</i>	<i>~4.5</i>	<i>Lipophilic</i>	<i>2</i>	<i>Biorelevant Media</i>
<i>Fenofibrate</i>	<i>~5.2</i>	<i>Lipophilic</i>	<i>2</i>	<i>Biorelevant Media</i>
<i>Ranitidine</i>	<i>~0.3</i>	<i>Hydrophilic</i>	<i>3</i>	<i>HBSS</i>
<i>Ritonavir</i>	<i>~5.6</i>	<i>Highly Lipophilic</i>	<i>4</i>	<i>Biorelevant Media</i>
<i>Venetoclax</i>	<i>~7.1</i>	<i>Very Lipophilic</i>	<i>4</i>	<i>Biorelevant Media</i>
<i>Verapamil</i>	<i>~3.8</i>	<i>Lipophilic</i>	<i>1</i>	<i>Biorelevant Media</i>

Any observed increase in apparent permeability shown in Figure 22, Figure 23, Figure 24 and Figure 25 may be attributed to increased micellar association of the free drug, whereby micelles are able to migrate toward the cell monolayer, thereby facilitating permeation of the compound across it. In this context, micelles may act as mobile carriers within the intestinal fluid, capable of diffusing toward the epithelial surface⁴⁵. Hydrophobic interactions between micelles and the lipid bilayer promote accumulation at the cell monolayer, increasing the local concentration of drug-bound components at the membrane surface. This accumulation may, in turn, enhance drug transport, an effect that would be expected to attenuate under conditions of reduced micelle density or when the critical micelle concentration is not reached.

The increase in total amphiphile concentration (TAC) within Media 7 is likely to influence not only micelle size but also the number of micelles capable of forming within the aqueous environment. Critically, the two media also differ in bile salt concentration, with Media 7 exhibiting a two-fold increase in sodium taurocholate relative to Media 1. This difference may, in turn, influence the extent of drug solubilisation, such that the relationship between micellar solubilisation and drug absorption cannot be assumed to be strictly linear:

$$Drug_{Solid} \rightleftharpoons Drug_{Dissolved} \rightarrow \text{Dissolved fraction free to permeate}$$

Rather it is more complex with the dissolved drug interacting with the colloidal structures within the media and there in turn being variable which fraction of compound is free to permeate the intestinal barrier:

$$Drug_{Solid} \rightleftharpoons Drug_{Dissolved} \rightarrow \text{Dissolved fraction free to permeate}$$



$$Drug_{Colloidal} \rightarrow \text{Bound Fraction free to permeate}$$

The amount of drug bound to the micelle can vary not only depending on the concentration of the free drug within the intestine but also size of the micelle, the larger the micelle the greater the loading capacity.

Figure 18 demonstrates that increasing TAC is associated with an initial increase in particle size, up to a maximum, beyond which a marked reduction in particle size occurs. This pattern is consistent with findings reported by McKinnon and colleagues (2024), who evaluated micelle particle size in simulated intestinal fluid (SIF) following the addition of eight model compounds (tadalafil, carvedilol, griseofulvin, fenofibrate, felodipine, phenytoin, indomethacin, and naproxen) across the Strathclyde suite of SIF media. Their findings demonstrated that the highest TAC produced the smallest micelle diameter⁴⁶. While this reduction in micelle size suggests that each individual micelle may accommodate a smaller quantity of drug, the overall solubilising capacity of the medium nonetheless increased, owing to the substantially greater number of micelles formed under high-TAC conditions. This is consistent with the observed reduction in particle size occurring specifically at maximal TAC, where the amphiphile pool is distributed across a greater number of smaller micellar structures rather than fewer, larger ones.

A consistent trend observed across this body of work is that 65% of the compounds examined displayed higher apparent permeability in Media 7 relative to Media 1. This may be facilitated by the two-fold increase in the lecithin-to-bile salt ratio in Media 7 compared with Media 1, together with its six-fold greater lipid content, both of which may support a greater overall micellar load and enable more effective packing of drug molecules within individual micelles.

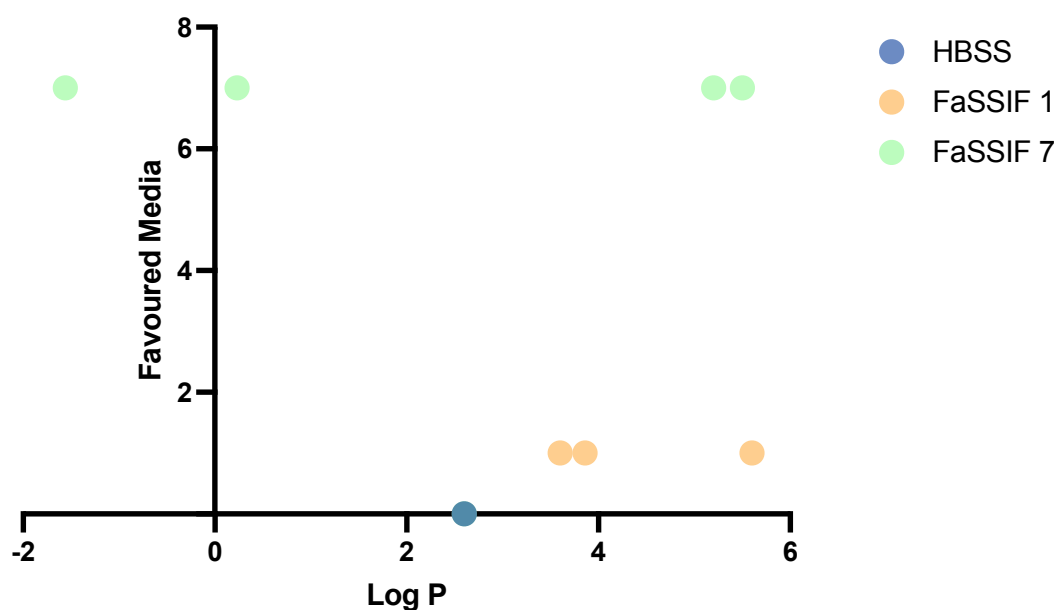


Figure 29 - Relationship between lipophilicity ($\log P$) and the highest observed apparent permeability (P_{app}) across biorelevant media.

The relationship between compound lipophilicity ($\log P$) and permeability is illustrated by plotting each compound's $\log P$ against the highest P_{app} value achieved across all tested biorelevant media formulations. This comparison highlights how increasing lipophilicity generally aligns with enhanced passive permeability, while more hydrophilic or ionised compounds exhibit lower maximal P_{app} even under the most favourable media conditions.

A trend appears to exist between the $\log P$ of the compounds examined and the media associated with their highest apparent permeability, as shown in Figure 29. Both highly lipophilic compounds ($\log P > 3$) and more hydrophilic compounds ($\log P < 3$) exhibited their highest apparent permeability in the biorelevant Strathclyde SIF formulations.

The behaviour of the high- $\log P$ compounds may be explained by the lipid content of the biorelevant media, which is at least 0.2 mM greater than that of HBSS. Additionally, the micellar binding capacity of Media 7 is 0.3 mM higher than that of FaSSIF Q3, the medium

shown in Figure 29 to display the largest hydrodynamic radius, potentially conferring an increased micellar binding capacity. It could be argued that the increase in TAC within Media 7 remains below the threshold at which increasing TAC is associated with a reduction in particle size, as discussed above. High-LogP compounds would therefore be expected to preferentially incorporate into these lipophilic micelles owing to their hydrophobic character.

Examination of the biorelevant media further revealed a consistently positive zeta potential across formulations. This positive surface charge may promote electrostatic interaction between the micelles and the negatively charged cell membrane, offering a further explanation for the enhanced apparent bioavailability of compounds observed in biorelevant media. The close proximity of micelles to the cell monolayer may generate a localised reservoir effect, sustaining elevated drug concentrations at the membrane interface. This could artificially maintain a steep concentration gradient, potentially inflating apparent permeability values relative to systems in which only free drug is considered.

It is important to note, however, that *in vivo*, micelle–membrane interactions are transient and subject to dilution, digestion, and competitive binding, whereas static *in vitro* systems may overestimate the contribution of micellar shuttling to overall drug transport. Furthermore, the assumption that micelle-mediated maintenance of concentration gradients directly equates to enhanced absorption overlooks the potentially rate-limiting step of drug release from micelles, a process typically governed by binding affinity and micelle stability⁴⁷.

It is notable that the lower-LogP compounds also displayed a preference for biorelevant media over the purely aqueous environment. Compounds such as acyclovir, atenolol, and ranitidine are either neutral or cationic in character, meaning that their incorporation is more plausibly explained by loose, surface-level interactions with the negatively charged bile-salt micelles, rather than true core solubilisation within the micellar interior. However, the observed increase in permeability for these hydrophilic compounds in biorelevant media is more likely attributable to disruption of the unstirred water layer (UWL) than to micelle-mediated transport. Hydrophilic, low-LogP compounds are typically UWL-limited, and the presence of bile salts is known to reduce UWL thickness

and, consequently, its associated diffusional resistance, thereby enhancing apparent permeability. In this context, the observed improvement in permeability reflects reduced diffusional resistance rather than enhanced membrane affinity or micelle-driven delivery. The presence of surfactants within the media disrupts the UWL, reducing the resistance it imposes on the permeation of lower-LogP compounds, which may account for the preference of these compounds for the more complex biorelevant media⁴⁸⁻⁵⁰. Further there is a potential of an increased membrane fluidity in the presence of bile salts and phospholipids. Together, these factors enhance the effective free concentration and diffusional flux of hydrophilic drugs compared with HBSS.

The dynamic interplay between drug molecules and micelles highlights a key challenge in permeability modelling: distinguishing between true molecular diffusion and carrier-mediated transport phenomena. Previous work has demonstrated that formulating drugs directly into micelles can markedly increase their uptake across Caco-2 monolayers, with a 2025 study reporting a substantial enhancement in absorption of the compound of interest across Caco-2 monolayers relative to the corresponding free-drug solution prepared in buffer⁴⁹. In that context, the micelle functions as a deliberate delivery vehicle, with the drug pre-loaded into a structured carrier system specifically designed to interact with the cell surface and facilitate transport.

By contrast, the present study examines a drug that was not formulated into the micellar system but instead partitioned spontaneously into micelles already present within a mixed biorelevant medium, a process which similarly resulted in increased absorption across an analogous system. As a result, any increase in apparent permeability observed here cannot be attributed to a designed micellar delivery mechanism, but rather reflects dynamic, equilibrium-driven binding of free drug to colloidal species within the medium. This distinction is critical, as the mechanistic basis of drug uptake differs substantially between a pre-formulated micellar delivery system and one in which micelles simply sequester the free drug fraction. Consequently, interpretation of any observed permeability enhancement must consider whether the effect reflects a genuine improvement in membrane transport, or an artefact arising from micelle-mediated maintenance of drug concentration at the cell interface.

In addition, examination of free drug compared with micelle-bound drug across a dialysis cassette revealed that, while the same overall quantity of drug ultimately diffused across the membrane, the free fraction was released in a sustained manner, taking longer to reach maximum released concentration⁵¹. Highlighting the influence that micelles have on drug availability

4.1. Conclusion

These findings underscore the importance of considering both physicochemical properties and media composition when assessing Caco-2 permeability. While pH and ionisation state remain central to the pH-partition hypothesis, the deviations observed in this study suggest that micellar dynamics and potential transporter interactions also play critical roles in governing permeability behaviour.

Analysis of apparent permeability by LC-MS/MS revealed that increasing TAC and lipophilic content were associated with increased apparent permeability for verapamil, acyclovir, ranitidine, venetoclax, and ritonavir. The introduction of complex media resulted in greater variability in apparent permeability for compounds with higher LogP values compared with more hydrophilic compounds, an effect potentially attributable to hydrophobic interactions between these compounds and micelles. As the interaction of free drug with micelles is non-linear, the quantity of drug bound to individual micelles is expected to vary, correspondingly increasing the proportion of variability introduced into the dataset.

Predictive permeability models, such as PAMPA and the HBSS-based Caco-2 assay, typically assume a linear relationship between the free drug fraction and its resulting permeability. These simplifications overlook the inherent heterogeneity of micelle–drug interactions and the dynamic interplay between drug and cell monolayer. Consequently, such models fail to capture key sources of variability, increasing the risk of either underestimating experimental variability or overestimating *in vivo* reproducibility. These assumptions ultimately give rise to divergence between predicted and observed outcomes, limiting the reliability and translational value of current predictive frameworks.

4.2. Future Work

The present study represents an important first step toward understanding the complex interplay between a compound's lipophilicity and its association with micellar structures. Future research should build upon these findings to disentangle overlapping transport mechanisms through transporter inhibition studies, which would help clarify the relative contributions of active and passive processes. Furthermore, advanced modelling approaches, such as physiologically based pharmacokinetic (PBPK) simulations incorporating dynamic micelle–drug equilibria, should be employed to predict *in vivo* behaviour under varying physiological conditions. Collectively, these efforts would deepen mechanistic insight and enhance the translational accuracy of drug disposition predictions.

Although beyond the scope of the present study, incorporation of a mucin layer across the monolayer would represent a valuable advancement to the current bench-side model, given that direct drug contact with the cell epithelium is not fully biorelevant. Furthermore, inclusion of a mucin layer may enable a broader range of biorelevant media to be tested; the present study was constrained in the suite of biorelevant fluids that could be examined due to the cellular stress these media impose, whereas mucin *in vivo* is understood to reduce such cellular stress. Incorporating a mucin layer into the monolayer model may therefore permit an expanded suite of biorelevant fluids to be evaluated.

It should also be noted that the biorelevant fluids examined in this study both fall toward the lower end of the TAC range within the wider Strathclyde SIF suite. It is possible that Papp would be more markedly influenced as TAC increases further, and further work could examine whether this relationship mirrors the trend observed for micelle size, that is, whether a corresponding reduction in Papp occurs at the highest TAC concentrations tested. Elucidating this relationship would provide further mechanistic insight into the interaction between micelles and drug molecules.

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4.4. Appendix

Assessing the percentage of cell viability:

$$\text{Cell Viability}(\%) = \frac{A(\text{Test})}{A(\text{Positive Control})} \times 100$$

**Chapter 5- Quantification of transporter
expression within Caco-2 cells at different
passage numbers.**

5.1. Introduction

The mRNA expression profile of transporters in Caco-2 cells correlates closely with that observed in the human small intestine, supporting the predictive utility of this model for studying intestinal drug transport¹⁻³. Nonetheless, achieving full cellular differentiation typically requires an incubation period of approximately 21 days, allowing for the establishment of tight junctions and the expression of relevant metabolic enzymes and membrane transporters^{4,5}. Comparative qPCR studies have demonstrated that transporter expression in Caco-2 cells sourced from different suppliers is broadly consistent with that of the small intestine, although minor discrepancies have been reported, highlighting the importance of characterising transporter expression profiles prior to undertaking drug transport assays⁶. Membrane transporters are broadly classified into two major superfamilies: the ATP-Binding Cassette (ABC) transporters and the Solute Carrier (SLC) transporters⁷. SLC transporters are predominantly involved in passive and facilitated transport processes, whereas ABC transporters mediate active transport driven by ATP hydrolysis.

A more comprehensive understanding of transporter expression at biological membranes can inform, and improve, decision-making throughout drug development. Transporter expression is known to vary according to physiological state, for example between fasted and fed conditions, and may also change with age^{8,9}. Furthermore, a number of therapeutic agents are deliberately designed to target specific transporters in order to enhance cellular uptake and efficacy. Serotonin reuptake inhibitors, for instance, exert their pharmacological effect by targeting the serotonin transporter (SERT) in the treatment of depression, while inhibition of the sodium-glucose cotransporter 2 (SGLT2) has become a cornerstone of modern diabetes management. Conversely, efflux transporters such as P-glycoprotein (P-gp), one of the most extensively studied members of the ABC transporter family, are well recognised for their role in actively exporting drug substrates out of cells, thereby reducing intracellular drug concentrations and, in some cases, contributing to multidrug resistance.

The functional significance of P-gp in modulating drug response was demonstrated in a 2020 study, in which knockdown of the ABCB1 gene, encoding P-gp, was shown to restore doxorubicin-induced immunogenic cell death; reduced P-gp expression enhanced

chemosensitivity and improved the therapeutic window of agents such as doxorubicin¹⁰. These findings reinforce the necessity of evaluating transporter expression and function during pre-clinical development, given their considerable influence on drug permeability and overall therapeutic response.

This need for careful transporter characterisation is particularly pronounced when extending Caco-2-based findings beyond the adult context. Beyond its established use in adult drug development, the Caco-2 model is increasingly explored as a tool for supporting paediatric formulation development, offering a means of assessing intestinal permeability and transporter-mediated handling of compounds intended for use in children¹¹. However, given that transporter and enzyme expression is known to vary substantially with age, following distinct, transporter-specific developmental trajectories rather than a single uniform pattern⁹, and that the Caco-2 model itself is derived from adult colonic tissue, its capacity to accurately represent the paediatric intestinal environment may be limited. Where transporter expression in Caco-2 cells diverges from that observed in paediatric intestinal tissue, permeability and metabolism data generated using this model may not fully capture the transport processes relevant to paediatric drug disposition; for example, fresh paediatric intestinal tissue has been shown to express markedly lower levels of P-gp, BCRP, and PEPT1 protein in children aged 0–2 years compared with adults⁹. Consequently, while Caco-2 remains a useful screening tool for early-stage paediatric formulation assessment, its findings should be interpreted with appropriate caution and ideally supported by direct comparison with paediatric-relevant expression data, when extrapolating results to paediatric populations.

Experimental variability inherent to Caco-2-based studies, particularly differences arising from passage number, can result in inconsistent permeability measurements, thereby compromising the assumptions underpinning pre-clinical evaluation¹². Quantifying passage-dependent variation in transporter expression is therefore essential for improving both the reliability and predictive value of *in vitro* permeability assessments. Systematic characterisation of transporter expression profiles across successive passages is consequently a critical step towards strengthening the translational relevance of Caco-2-based permeability studies.

To achieve this level of systematic characterisation, a robust and quantitatively accurate analytical approach is required, one capable of resolving absolute, rather than merely relative, differences in transporter abundance across passages. Quantification concatemer (QconCAT) technology offers such an approach, providing a targeted, mass spectrometry-based method for absolute protein quantification. The technique is founded on the principle of stable isotope dilution mass spectrometry, whereby a known quantity of an isotopically labelled internal standard, chemically identical to the endogenous analyte but distinguishable by mass, enables accurate quantification independent of variability in sample preparation or instrument response¹³.

A QconCAT construct is an artificial, concatenated gene engineered to encode a series of "Q-peptides," each corresponding to a tryptic peptide sequence unique to, and therefore representative of, a specific target protein. Peptides are typically selected according to proteotypic criteria, including uniqueness within the proteome, absence of post-translational modification sites or missed-cleavage-prone sequences, and favourable ionisation behaviour, ensuring that each Q-peptide functions as a reliable surrogate for its parent protein¹⁴. Because a single QconCAT construct can incorporate Q-peptides corresponding to numerous target proteins, this approach permits multiplexed, simultaneous quantification of multiple transporters and enzymes within a single analytical run, an advantage of particular relevance when characterising the broad transporter profile of Caco-2 cells.

The concatenated construct is expressed recombinantly, most commonly in *Escherichia coli* cultured in the presence of stable isotope-labelled amino acids, typically ¹³C/¹⁵N-labelled lysine and arginine. Consequently, every Q-peptide released following trypsin digestion of the purified QconCAT protein carries a defined, predictable mass shift relative to its endogenous, unlabelled ("light") counterpart, while remaining chromatographically and chemically indistinguishable from it. A precisely known quantity of the labelled QconCAT standard is introduced into the biological sample of interest prior to proteolytic digestion, ensuring that both the labelled Q-peptides and their endogenous light analytes are generated under identical digestion conditions. This controls for inter-sample variability in digestion efficiency, a recognised source of error in bottom-up proteomic quantification¹⁵.

Following digestion, samples are analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), typically operating in selected or multiple reaction monitoring (SRM/MRM) mode to maximise analytical sensitivity and specificity. The abundance of each endogenous target protein is determined by comparing the peak area, or integrated peak intensity, of the light (endogenous) peptide to that of its heavy-labelled (QconCAT-derived) counterpart of known concentration. As the ratio of light to heavy signal is directly proportional to the quantity of endogenous protein present, this approach yields absolute quantification, typically expressed as femtomoles of protein per microgram of total or membrane protein, rather than the relative fold-change values obtained through techniques such as qPCR or conventional immunoblotting.

This capacity for absolute quantification represents a critical advantage in the context of Caco-2 passage characterisation. While qPCR provides valuable insight into transcript-level expression, it does not necessarily reflect functional protein abundance at the plasma membrane, nor does it permit direct comparison of absolute transporter levels between passages, cell lines, or *in vitro* and *in vivo* systems. QconCAT-based targeted proteomics therefore provides a more physiologically informative and translationally relevant measure of transporter expression, and is consequently well suited to the systematic, passage-dependent characterisation of the Caco-2 transporter profile undertaken in this thesis.

5.2. Objectives

The primary objective of this study is to determine the influence of passage number on the expression of key target proteins in Caco-2 cells. Through absolute quantification of protein abundance, this work aims to characterise the ontogeny and reproducibility of transporter and enzyme expression across passages, and to identify any passage-dependent drift that may compromise the reliability of the model. Further comparison of these expression profiles with those observed in paediatric small intestinal biopsies aims to strengthen confidence in the Caco-2 model as a relevant and informative tool for predicting paediatric intestinal drug permeability.

5.3. Materials and methods

5.3.1. Cell Culture

Caco-2 cells were seeded on transwell inserts (Corning 3401) at a density of 3×10^5 . Cells were seeded 3 weeks prior to transporter expression analysis. Following the same experimental protocol as in 3.3.3, cells were washed twice with cold PBS and moved in a figure of 8 motion to ensure coating of cells. Cells used were passage 31 and passage 61.

5.3.2. Protein Extraction from Cells

The method of protein extraction used in this study is based on previous work conducted by Goelen and colleagues in 2023¹⁶. All protein extraction was conducted on ice to prevent interrupting the cold chain. The monolayers were twice washed with cold PBS which was then replaced by Radio-Immunoprecipitation Assay (RIPA) lysis/extraction buffer (R0278) supplemented with protease inhibitor cocktail (P8340) along with the phosphatase inhibitors (P2850, P5726) at a concentration of 13 μL per 1cm^2 . The monolayer plate was then placed on ice for 5 minutes. Following these cells are scraped off and transferred into a centrifuge tube. Cell resuspension then sonicated for 30 seconds at a 50% pulse. Following this samples were centrifuged at 14,000g for 15 minutes, supernatant is then removed and frozen unless needed straight away for further analysis. Of the supernatant ~25 μL is used in Pierce BCA assay to determine protein content according to manufactures guideline.

The isolated protein from the extracted cell mix was digested using a Filter-Aided Sample Preparation (FASP) protocol. The protein content was diluted with RIPA buffer to obtain a solution containing 1 μg of protein per μL . From this, 20 μg of protein was combined with 370 μL of urea (UA) buffer (8 M urea in 0.1 M Tris/HCl, pH 8.5) and 10 μL of the QconCat PaedCat solution. The mixture was vortexed for 30 seconds and then allowed to rest for 5 minutes.

Following the 5-minute incubation, 40 μL of 100 mM 1,4-dithiothreitol (DTT) prepared in UA buffer was added, and the mixture was incubated for 40 minutes at 56 °C. To prime the FASP column, 200 μL of UA buffer was added and centrifuged at 14,000 g for 5 minutes, after which the filtrate was discarded. Once the sample had cooled following the 40-minute incubation at 56 °C, the mixture was transferred to the primed column and

centrifuged at 14,000g for 20 minutes. The filtrate was discarded, and 400 µL of UA buffer along with 20 µL of 300 mM iodoacetamide in UA buffer were added to the spin column. The sample was incubated at room temperature in the dark for 30 minutes, followed by centrifugation at 14,000g for 20 minutes, with the filtrate again discarded.

The sample was then washed by adding 400 µL of UA buffer to the column and centrifuging for 20 minutes at 14,000g. To remove excess bound urea, three wash steps were carried out using 400 µL of 50 mM ammonium bicarbonate (ABC) in ultrapure water, with each wash followed by centrifugation at 14,000g for 20 minutes at room temperature.

The digestion of proteins was achieved through tryptic digestion aided by Lys-C. Enzyme mix of (Trypsin/Lys-C Mix, Mass Spec Grade, Promega) was made up to 0.2 mg/mL through addition of 100 µL trypsin resuspension buffer with 20 µL of enzyme. Ten microlitres of enzyme solution is added to 30 µL of 25mM ABC-solution which is then added to spin column. Samples are incubated overnight @ 37°C on shaking.

Elution of peptides on the following day was performed by adding 50 µL of 100 mM ABC containing 5% acetonitrile to the spin column. Samples were centrifuged at 14,000g for 20 minutes, and the filtrate was collected in a 0.5 mL LoBind tube. This elution step was repeated twice more, with each filtrate collected into the same LoBind tube.

Samples were then desalted using C18 tips. The tips were first washed twice with a 50:50 acetonitrile: water solution and subsequently equilibrated by aspirating 100 µL of 0.1% TFA twice. The acidified peptide sample was aspirated through the C18 tip ten times to allow binding to the resin. The tips were then washed twice by aspirating 100 µL of 0.1% TFA/5% acetonitrile solution. Peptides were eluted from the resin using 100 µL of 0.1% TFA/80% acetonitrile and transferred into low-bind 1.5 mL PCR-clean tubes. Samples were dried using a SpeedVac at 45 °C (Concentrator 5301, Eppendorf) and stored at – 20 °C.

5.3.3. LC-MS/MS Quantification of Peptides

Concentrations of the samples were analysed through LC-MS/MS, with the concentration being determined on light peptides in relation to heavy peptides. Dried samples from 4.2.3 were reconstituted in resuspension buffer (35 µL of 98% LC-MS-grade water supplemented with 0.1% (v/v) formic acid with 2% Acetonitrile (with 0.1% (v/v) formic acid). LC-MS/MS conditions are listed in Table 17.

Table 17 - LC-MS/MS optimised conditions for quantitative analysis.

Phase	Item	Details
Sample	Dried sample	20 µg mucosal protein
Reconstitution	Resuspension buffer	35 µL of a 98% (v/v) LC-MS-grade water (with 0.1% (v/v) formic acid (FA)) / 2% ACN (with 0.1% (v/v) FA)
Injection	Autosampler	Shimadzu Nexera, SIL-40C X3 at 4°C
	Injection volume	25 µL of the reconstituted sample
Separation	Column	C18, ACQUITY Premier Peptide, 100 x 2.1 mm, 1.8 µm, Waters, UK
	Oven	CTO-40C, Shimadzu at 30°C
	Mobile phase A	Ultrapure water with 0.1% (v/v) FA
	Mobile phase B	ACN with 0.1% (v/v) FA
	Gradient	2 min 97% A, then a ramp to 70% B over 40 Gradient min, followed by a column wash (ramp to 80-95% B over 5 min), returning to starting conditions (ramp for 5 min, maintaining starting conditions for 2 min) before the next run (Shimadzu Nexera, LC-40D xs solvent delivery module)
Instrument	Triple quadrupole	Shimadzu 8060NX
	Ionisation mode	Electron Spray Ionisation (ESI)
	MRMs per peptide	Range from 3-6
	Acquisition window	96 seconds around the expected retention time
	Dwell time	5 msec
	Pause time	5 msec
	Loop time	1.422s

Table 18 - Proteotypic peptide sequences selected for QconCat-based quantification of Caco-2 membrane transporters/proteins.

Peptides were encoded in the PaedCAT QconCAT standard, each carrying a C-terminal [$^{13}\text{C}_6$, $^{15}\text{N}_2$]lysine (R) or [$^{13}\text{C}_6$, $^{15}\text{N}_4$]arginine (K) to allow discrimination from the corresponding endogenous peptides by mass spectrometry."

Gene	Protein	Peptide
ABCB1	P08183	QEIGWFDVHVDVGELNTR
ABCB1	P08183	AGAVAEVLAIR
ABCB1	P08183	FYDPLAGK
ABCG2	Q9UNQ0	VIQELGLDK
ABCG2	Q9UNQ0	TIIFSIHQPR
ABCG2	Q9UNQ0	LFDSLTLASGR
ABCC2	Q92887	IFNSTDYPASQR
ABCC2	Q92887	QLLNNILR
ABCC2	Q92887	YLGDDLDLSAIR
ABCC3	O15438	IDGLNVADIGLHDLR
ABCC3	O15438	SQLTIIPQDPILFSGTLR
ABCC3	O15438	AEGEISDPFR
ABCC4	O15439	LTVTLFFPSAIER
ABCC4	O15439	VFVAVTLYGAVR
ABCC4	O15439	ITILVTHQLQYLK
ATP1A1	P05023	IVEIPFNSTNK
ATP1A1	P05023	NIAFFSTNCVEGTAR
ATP1A2	P50993	NICFFSTNCVEGTAR
ATP1A3	P13637	NITFFSTNCVEGTAR
CES2	O00748	FAPPEPPESWSGVR
CES2	O00748	LGVLGFFSTGDK
CES2	O00748	SFFGGNYIK
CYP2C19	P33261	IYGPVFTLYFGLER
CYP2C19	P33261	GHFPLAER
CYP2C9	P11712	GIFPLAER
CYP2C9	P11712	LPPGPTPLPVIGNILQIGIK
CYP2D6	P10635	AFLTQLDELLTEHR
CYP2D6	P10635	DIEVQGFR
CYP2J2	P51589	VIGQGQQPSTAAR
CYP2J2	P51589	FEYQDSWFQQLK
CYP3A4	P08684	EVTNFLR
CYP3A4	P08684	LSLGGLLQPEKPVVLK
CYP3A4	P08684	SAISIAEDEEWK
CYP3A5	P20815	DTINFLSK
CYP3A5	P20815	SAISLAEDEEWK
SLC15A1	P46059	HTLLVWAPNHVQVVK
SLC22A1	O15245	ENTIYLK
SLC15A1	P46059	VQQCGIHSK
SLC22A1	O15245	LSPSFADLFR
UGT1A1	P22309	GHEIVVLAPDASLYIR
UGT1A1	P22309	DGAFYTLK

UGT1A1	P22309	TYPVPFQR
UGT1A3	P35503	YLSIPTVFFLR
UGT1A3	P35503	EVSVDILSHASWWLFR
UGT1A4	P22310	GLQVPLPR
UGT1A4	P22310	YLSIPAVFFWR
UGT1A9;	O60656	TYSTSYTLEDLDR
UGT1A8		
UGT1A9	O60656	AFAHAQWK
UGT2B17	O75795	SVINDPIYK
UGT2B17	O75795	FSVGYTVEK
UGT2B7	P16662	TILDELIQR
UGT2B7	P16662	IEIYPTSLTK
VIL1	P09327	GSLNITTPGLQIWR
VIL1	P09327	LFQVQGTGANNTK
VIL1	P09327	DPETPIIVVK

5.4. Results and Discussion

5.4.1. LC-MS/MS based quantification of prototypic peptides.

Peptide selection was refined by applying the following inclusion criteria to the LC-MS/MS method. This was assessed using the Heavy Standard QconCat, which included 40 target peptides in total; of these, 24 met the inclusion criteria and were retained for quantification. The 16 excluded peptides fell into three categories, described below:

- No heavy chain detected (n = 3)
 - For three peptides, ITLVTHQLQYLK, NITFFSTNCVEGTAR, and TILDELIQR, no heavy chain signal was detected in any replicate across either of the two cell passages examined. As the heavy chain acts as the internal standard against which the endogenous (light chain) signal is quantified, its absence made it impossible to calculate a light-to-heavy chain ratio for these peptides. They were therefore excluded from further analysis Figure 30.
- No endogenous light chain detected (n = 10)
 - For ten peptides (AGAVAEVLAAIR, FEYQDSWFQQLK, VIGQGQPSTAAR, LSPSFADLFR, YLSIPTVFFLR, EVSVVDILSHASVWLFR, YLSIPAVFFWR, and TYTSYLTLEDLDR isoforms UD18 and UD19), no endogenous light chain was detected: more than 50% of the expected product ion masses were undetectable, and the corresponding chromatographic peak was absent. As a reliable light-to-heavy ratio could not be calculated, these peptides were excluded from quantification.
 - Where only one peptide of a pair (heavy or light) was detected, the corresponding protein was still retained for quantification, with detection of the heavy chain peptide alone treated as confirmation that the target protein was present at that peptide site (Figure 31 and Figure 32).
- Co-eluting peptides with unresolved transitions (n = 2)
 - The remaining two peptides, SAISIAEDEEWK (CYP3A4) and SAISLAEDEEWK (CYP3A5), co-eluted, and the optimised transition method was unable to resolve their peaks. This is because isoleucine and leucine share an identical molar mass, making the two peptides indistinguishable under the current method. As peaks could not be reliably assigned to their corresponding peptide, both were excluded from further analysis.

- Additionally, for two peptides, LFDSLTLASGR and LGVLGFFSTGDK, heavy chain signal was detected at the higher passage number (p-61) but was absent in the earlier passage cell line (p-31) (Figure 33).

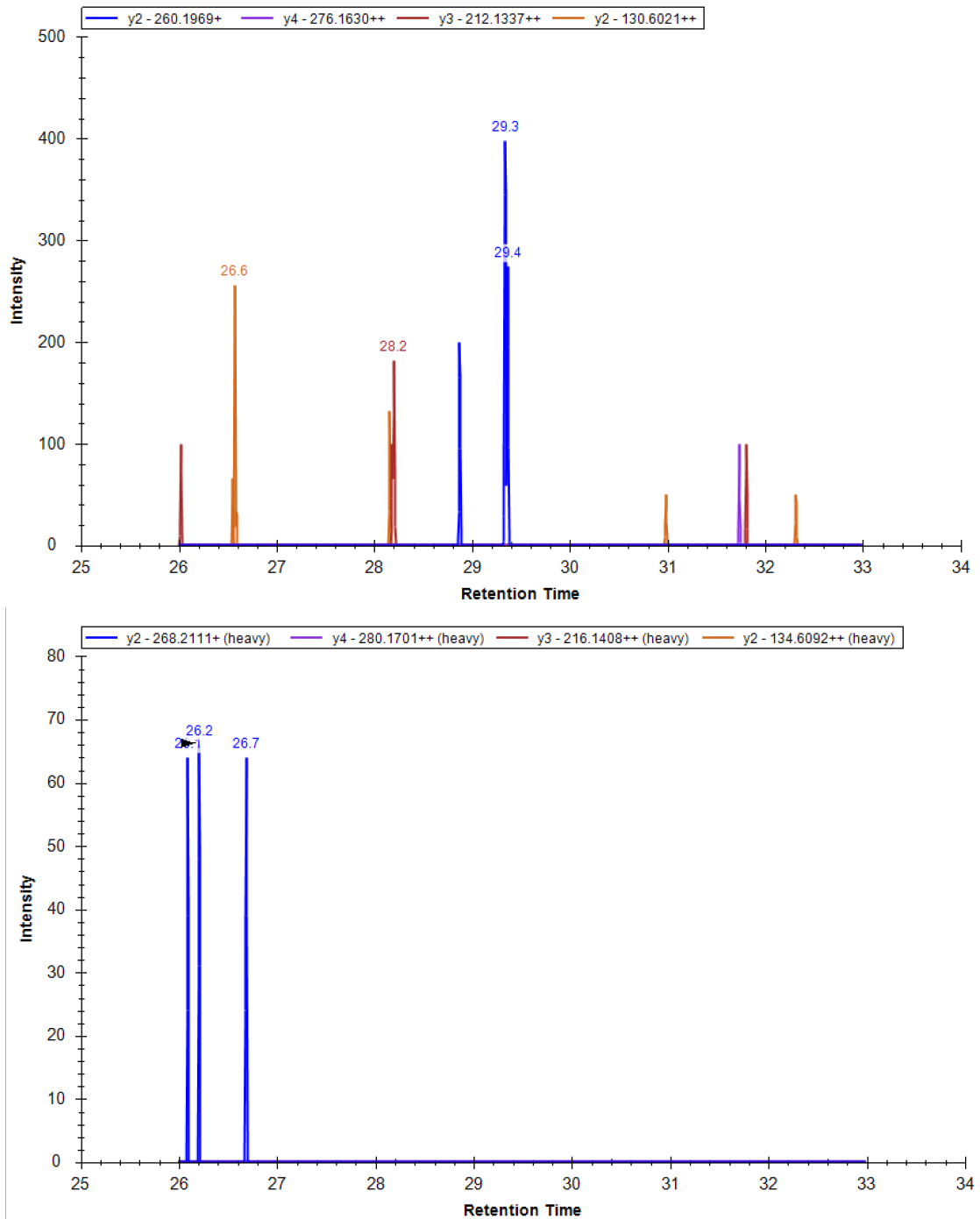


Figure 30 - Missing heavy chain of peptides, given this there is no light chain observed.

Peptides ITILVTHQLQYLK for MRP4 protein. A) shows the endogenous light chain B) shows the heavy chain (PaedCAT standard) channel.

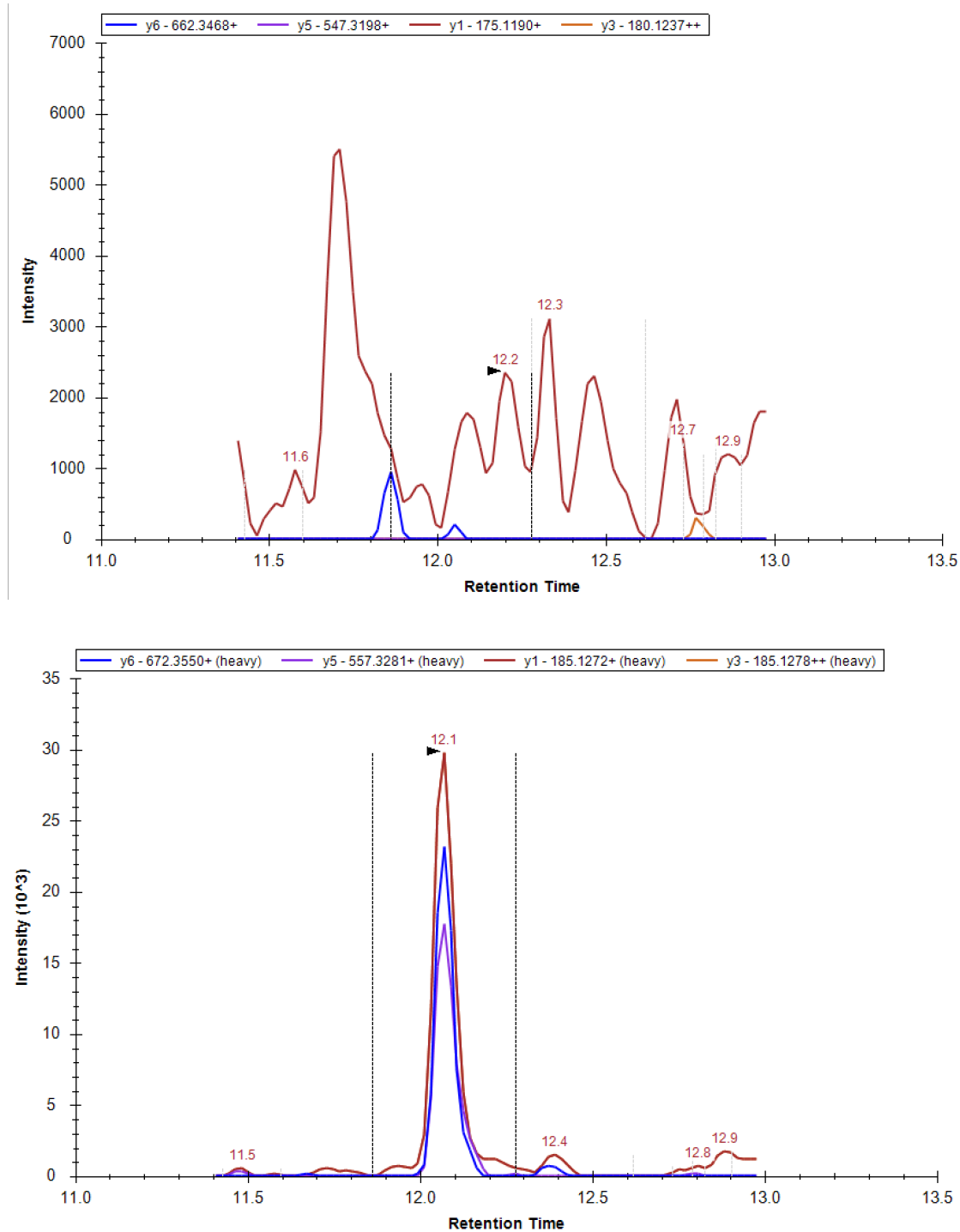
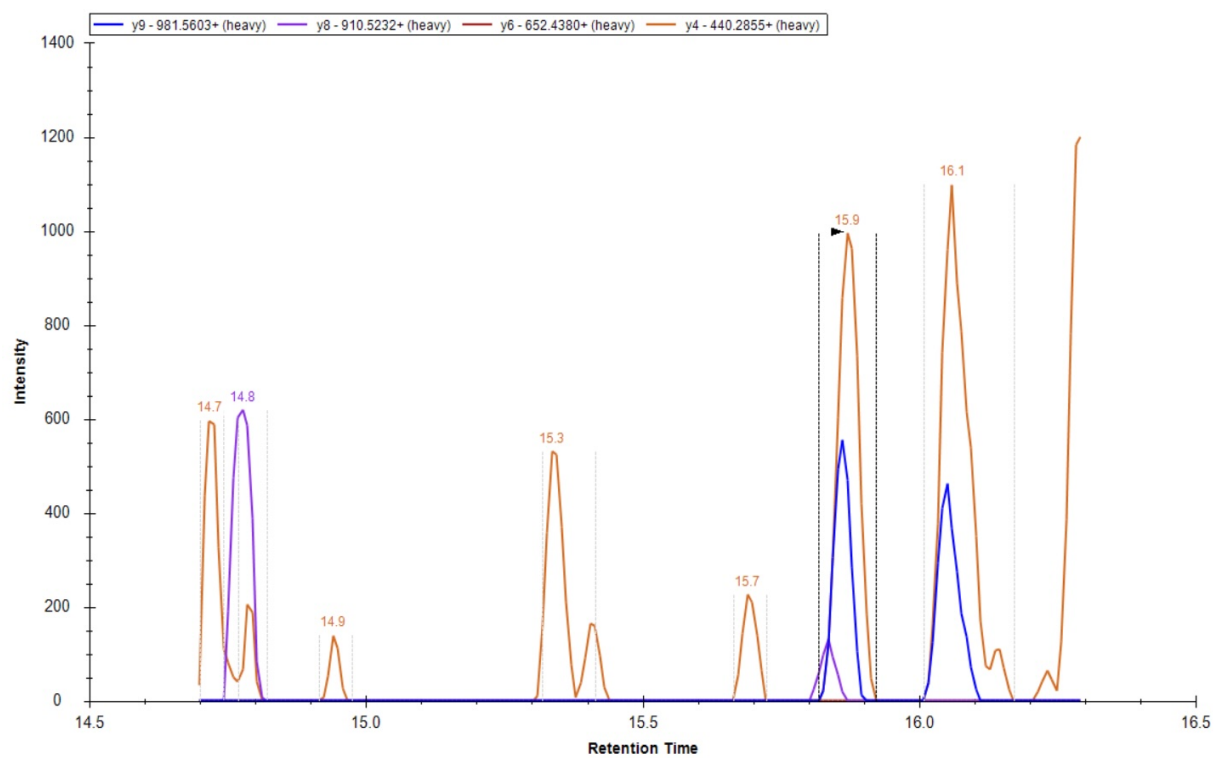
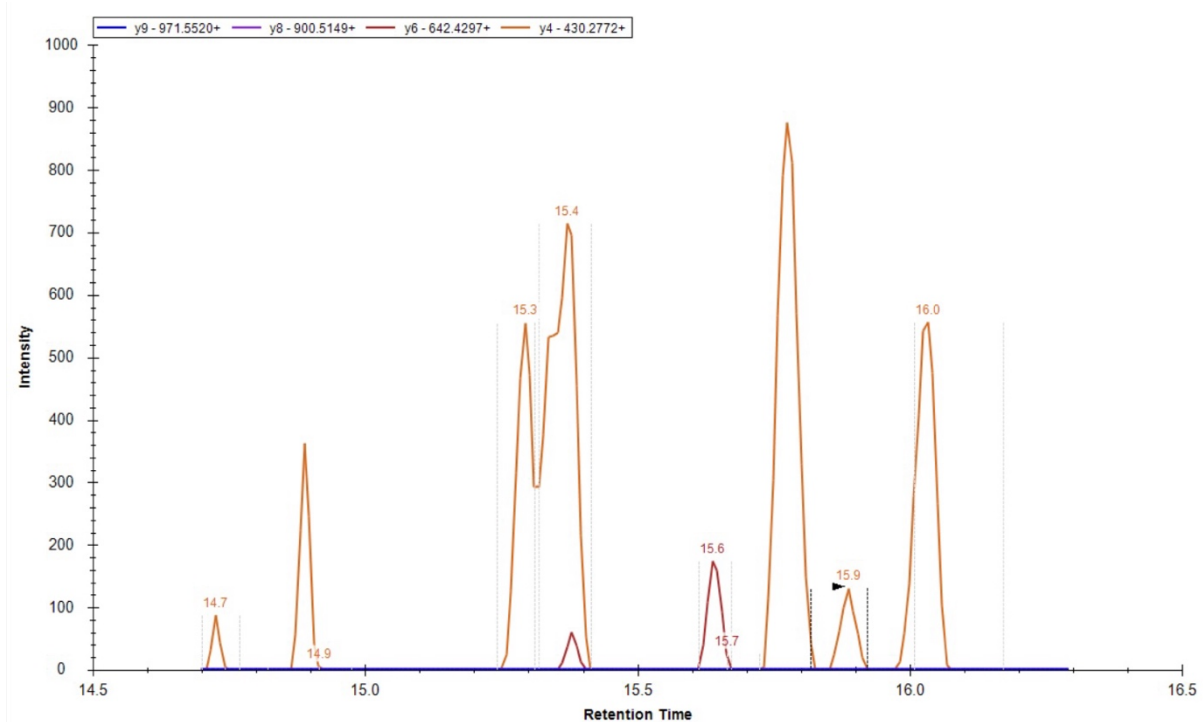


Figure 31 - Detection of heavy chains, however there is no endogenous light chain detected in the sample.

Protein MRP2, peptide YLGGDDLDTSAIR. Top panel shows the endogenous light chain, bottom panel shows the heavy chain (PaedCAT standard) channel.

A)



B)

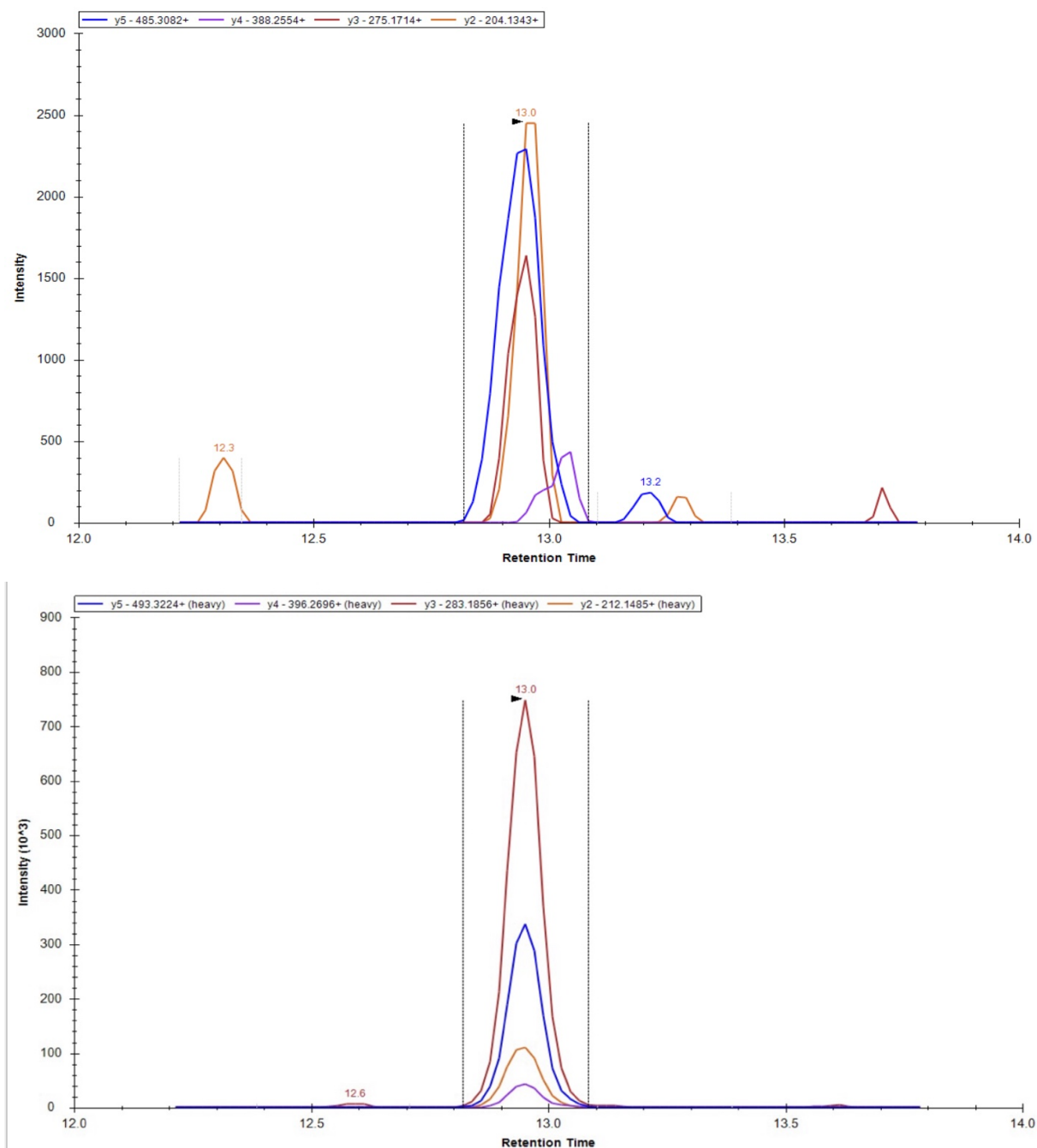


Figure 32 - Example of missing signal for one of target peptide P-GP in passage 31 but the peptide is detected in later passages.

(A) Peptide AGAVAEVLAAIR and (B) peptide FYDPAGK, two proteotypic peptides for MDR1. Within each panel, the upper trace shows the endogenous (light) peptide and the lower trace the corresponding heavy-labelled peptide from the PaedCAT standard.

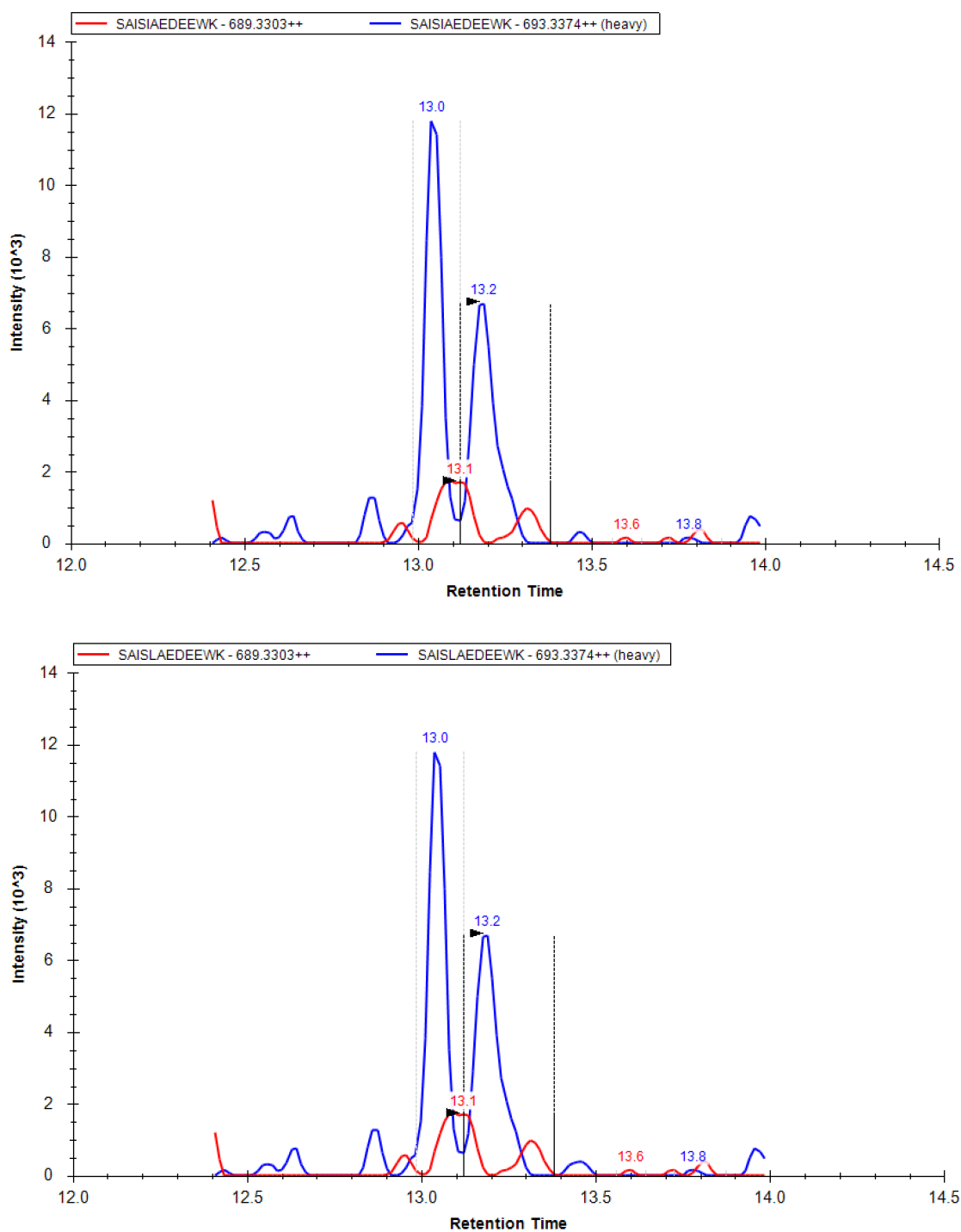
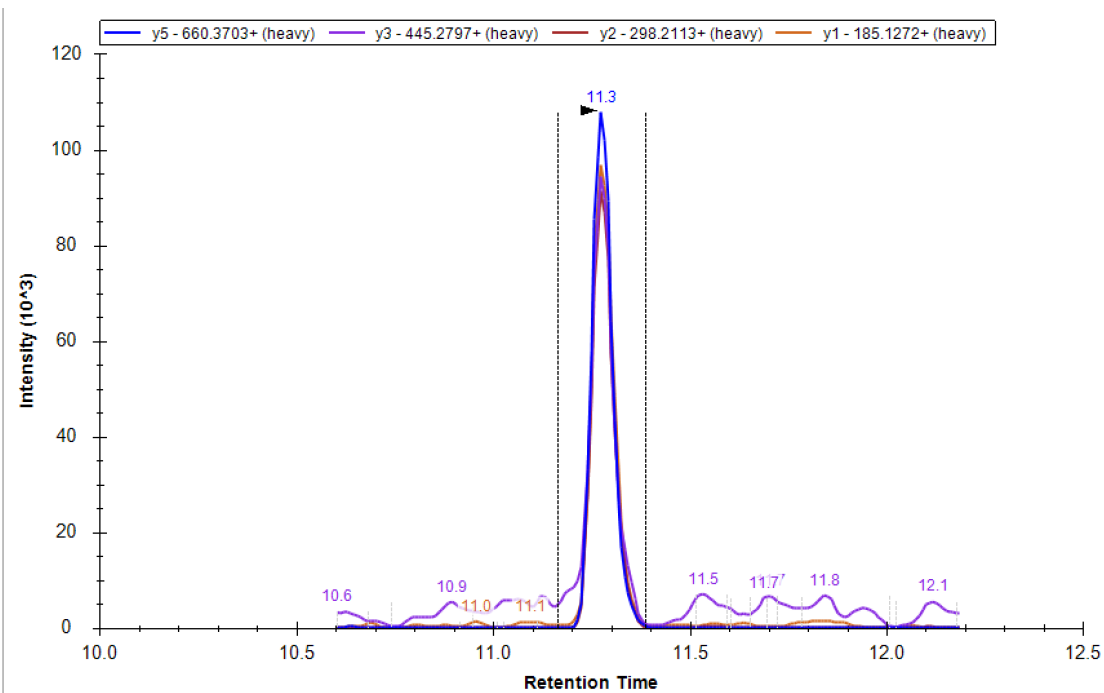
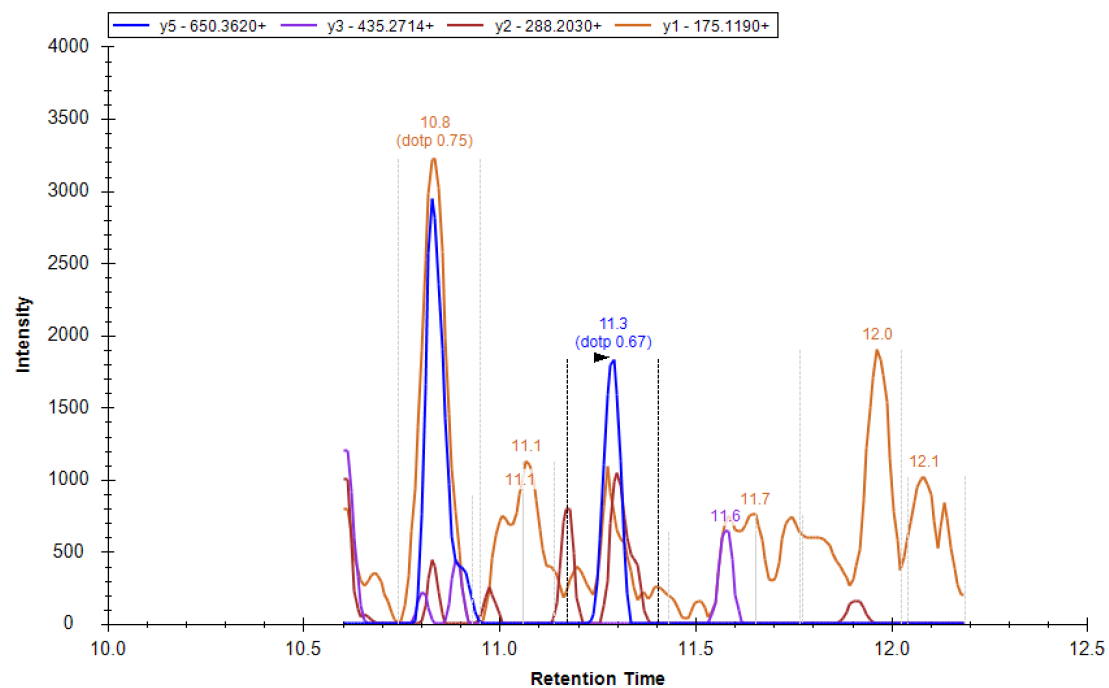


Figure 33 - Co elution of peptides at same retention time.

peptide top panel is SAISIAEDEEWK, bottom panel SAISLAEDEEWK. Both peptides elute at the same retention time and share an identical precursor mass and so cannot be resolved chromatographically. Within each panel, the red trace shows the endogenous (light) peptide and the blue trace the corresponding heavy-labelled peptide from the PaedCAT standard



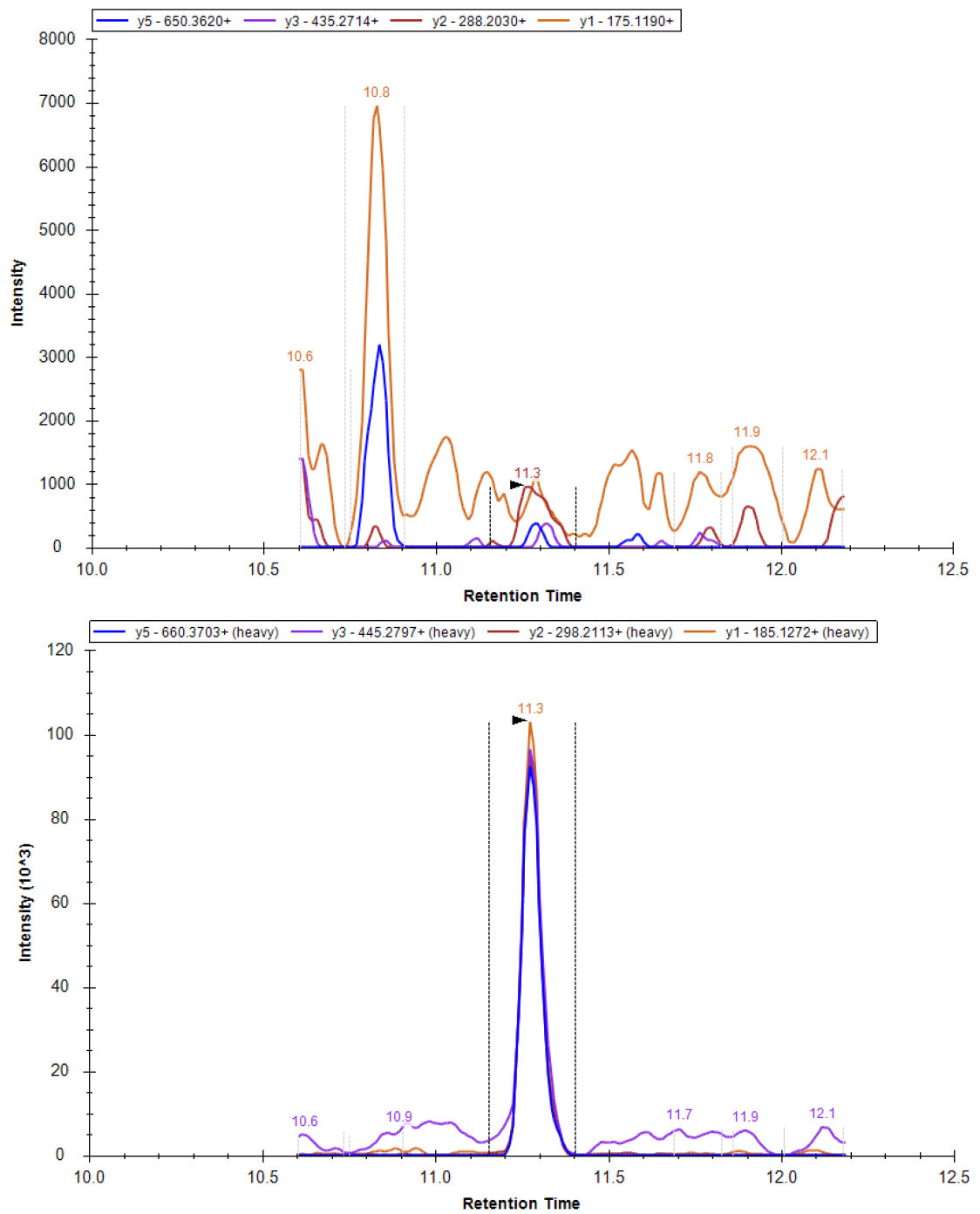


Figure 34 - Heavy chains detected in the later passage but not at the earlier one.

Peptide EVTNFLRR A) is peptide EVTNFLRR for the CP3A4, i) is p-31 light chain and ii) is corresponding heavy chain. B) Top panel is endogenous light chains; the bottom graph shows the heavy chain (PaedCAT standard) channel.

For proteins in this study where two peptides could be quantified, with both light and heavy chains present for each, correlation between the resulting light-to-heavy ratios was assessed as a quality control step. In principle, strong agreement between two independently quantified peptides from the same protein provides confidence that the measured signal reflects true protein abundance rather than peptide-specific artefacts, such as differences in ionisation efficiency or susceptibility to matrix effects and allows the two values to be averaged to improve overall quantification accuracy. A correlation coefficient ($r^2 > 0.8$) was set as an internally defined acceptance criterion for considering paired peptide ratios sufficiently reliable to average, reflecting a strong linear relationship between replicates while accounting for the technical variability inherent to the assay.

However, in this dataset, the paired peptides did not meet this threshold and did not show sufficient correlation Figure 35. This indicates that peptide-specific variability was too great to justify averaging, as doing so risked introducing greater uncertainty rather than improving reliability. Consequently, no averaging across peptide pairs was performed; each peptide was instead quantified individually, with reported values based solely on the signal from that specific peptide.

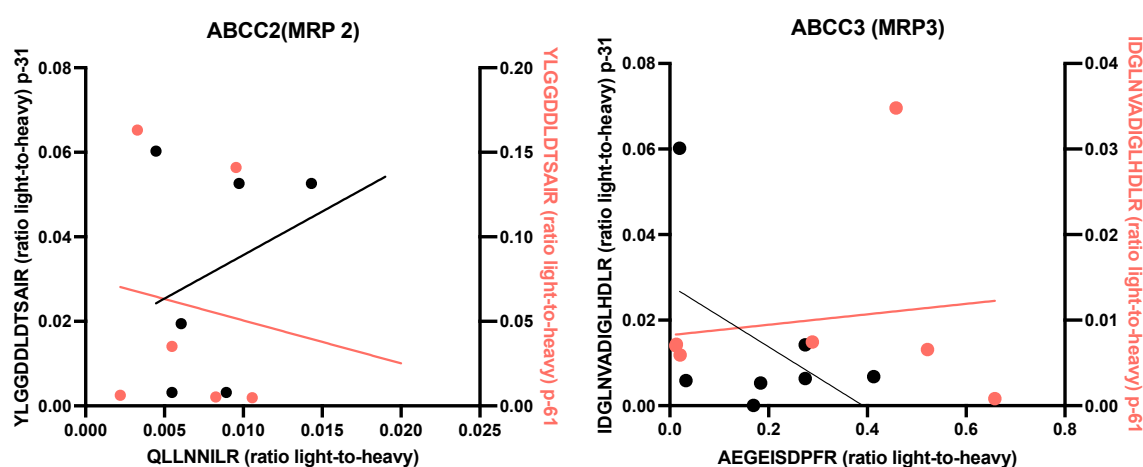


Figure 35 - Correlation of the light-to-heavy ratios for two peptides.

(A) UGT2B17 and (B) UGT1A1. The black line depicts the trend for linear regression for passage 31 Caco-2 cells and the red line depicts the linear regression for p-61 Caco-2 cell line with the colour of the points corresponding to colour of the line.

5.5. Abundance of drug metabolism and transporter proteins

5.5.1. Transporters

During this study, nine transporters were screened, of which seven could be quantified across both cell passages. Endogenous light-chain peptides were not detected for MDR1 (AGAVAEVLAIR), ABCG2 (LFDSLTLASGR), MRP4 (ITILVTHQLQYLK), ATP1A3 (NITFFSTNCVEGTAR), and SLC22A1 (LSPSFADLFR). In addition, heavy chain signal was absent for both MRP4 and ATP1A3, and for ABCG2 (LFDSLTLASGR) at passage 31, though heavy chain signal was present for this peptide at passage 61.

The peptide transporter SLC15A1 (PEPT1) exhibited the greatest overall abundance at both cell passages, consistent with its established role as the primary route for di- and tripeptide uptake across the intestinal brush border membrane, where it also mediates the absorption of a wide range of peptidomimetic drugs owing to its broad substrate tolerance¹⁷. In contrast, ATP1A1 maintained a stable level of protein abundance across passages, suggesting minimal variation in its expression during cellular ageing. Among the efflux transporters, ABCC3 demonstrated the highest abundance at both passages, followed by ABCC2, indicating a consistent rank order of expression across passage number. In contrast, ATP1A1 maintained a stable level of protein abundance across passages, suggesting minimal variation in its expression during cellular ageing. Among the efflux transporters, ABCC3 demonstrated the highest abundance at both passages, followed by ABCC2, indicating a consistent rank order of expression across passage numbers (Figure 36).

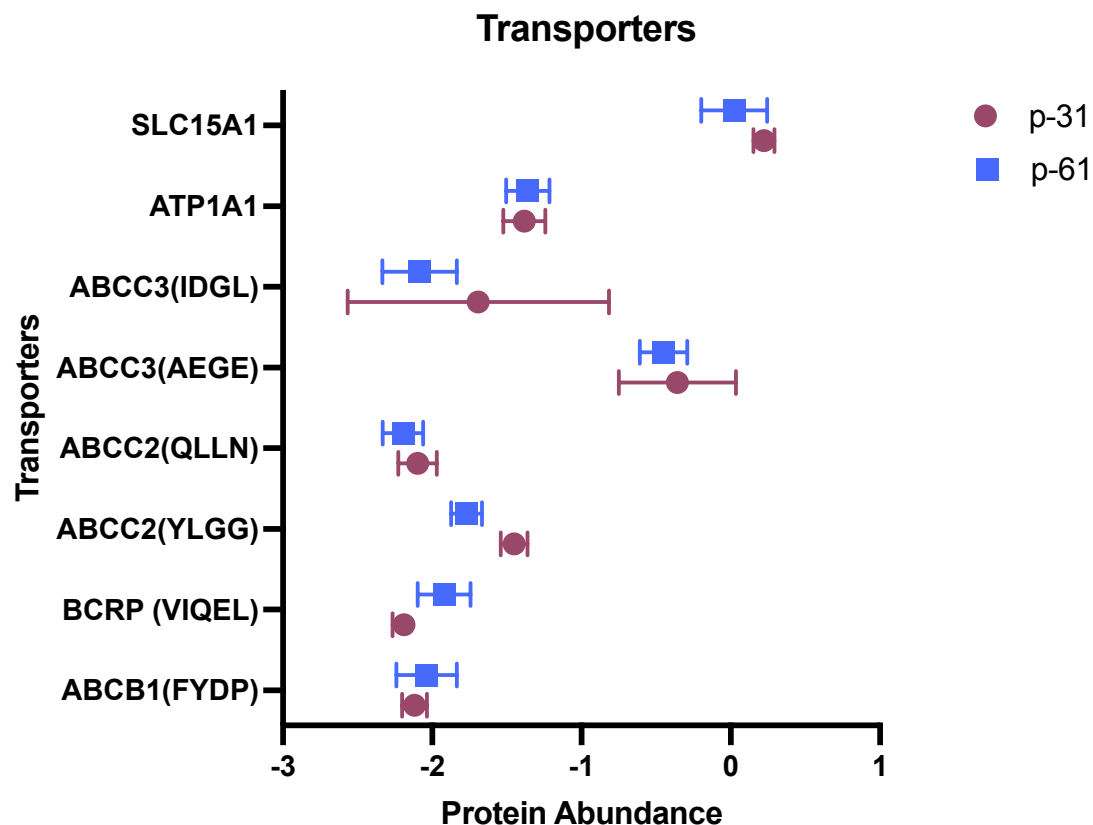


Figure 36 - Transporter abundance in Caco-2 cells across different cell passages.

Quantification of transporter abundance in the Caco-2 cell line across multiple cell passages. Values represent mean protein expression levels with error bars indicating the standard deviation ($n = 3$).

In this study, ABCC2 levels were observed to be similar across both cell passages, and the same trend was observed for ATP1A1, ABCG2 (BCRP), and ABCB1 (MDR1). As shown in Figure 36, SLC15A1 (PEPT1) expression was significantly higher at both cell passages than any other transporter quantified. This observation is consistent with existing literature reporting SLC15A1 expression in the Caco-2 cell line¹⁸⁻²⁰. Work published in 2004 evaluated PEPT1 transporter expression by RT-PCR and reported that protein abundance varied according to the clonal origin of the cell line²¹. In that study, Behrens *et al.* observed that as passage number increased from 32 to 42 in Caco-2 cells sourced from ATCC, there was a considerable reduction in SLC15A1 expression; this was confirmed by fluorescence analysis, which showed sparsely populated transporters at higher passage numbers. This finding is consistent with the present study, in which a reduction in SLC15A1 protein abundance was similarly observed at p-61 relative to the earlier passage²¹.

A similar trend was observed across both cell ages for the ABCC family, with ABCC3 displaying consistently higher protein abundance than ABCC2. This rank order is consistent with findings from adult intestinal biopsy analysis reported in 2005, which examined expression of the ABCC family (multidrug resistance-associated proteins) and found that ABCC3 exhibited higher gene expression than ABCC2 across all intestinal regions examined (duodenum, ileum, and colon)²². Despite this overall pattern, ABCC2 expression in the colon is notably supported by further literature from 2007, in which blotting analysis of regional intestinal sections identified the most pronounced regional variance in distribution for MRP2 (ABCC2), with expression decreasing progressively toward more distal regions of the intestine²³. Given that Caco-2 cells are derived from a colon adenocarcinoma, their transporter expression profile would be expected to reflect distal intestinal characteristics; the findings of the present study align with this pattern of transporter distribution reported in human intestinal tissue.

A consistent downward trend in expression was evident across all transporters examined with increasing passage number, suggesting a broader biological pattern worth noting (Figure 36). Similar trends have been reported in the literature, with transporter protein expression shown to vary with passage number²⁴⁻²⁶. PCR-based analysis of cells grown as monolayers over a 21-day incubation period reported a reduction in abundance for 66% of the MRP transporters examined as passage number increased, with the growth protocol itself also shown to influence transporter expression within cellular monolayers²⁴. Notably, all of these studies quantified transporter expression using PCR-based methods; however, transcript levels do not necessarily reflect protein abundance, as post-translational modifications and other regulatory processes can substantially alter the relationship between mRNA expression and functional protein levels. By contrast, the present study employed QconCat technology, which directly quantifies peptide abundance and therefore provides a more accurate representation of true protein levels than transcript-based approaches. Our findings demonstrate a clear decrease in protein abundance for four of the transporters examined with increasing passage number, reinforcing the importance of accounting for cell age when evaluating drug transport, given the potential impact of passage-dependent changes in transporter expression on drug performance.

5.5.2. CYP enzymes abundance

Among the CYP enzymes assessed in this study, approximately 50% were present above the limit of quantification. CYP2D6 (AFLT) was the most abundant enzyme detected at both Caco-2 passages, followed by CYP2C9, CYP2D6 (DIEV), and CYP2C19. Notably, this rank order mirrors the expression pattern reported in adult intestinal tissue²⁷, as illustrated in Figure 37. The similarity in expression hierarchy between Caco-2 monolayers and human intestinal sections further supports the suitability of Caco-2 cells as an *in vitro* model of the small intestine.

However, it is important to highlight that CYP3A4, the predominant CYP isoform in human intestinal tissue, was not fully captured in this model: one of its two quantifying peptides was not detected in either Caco-2 passage.

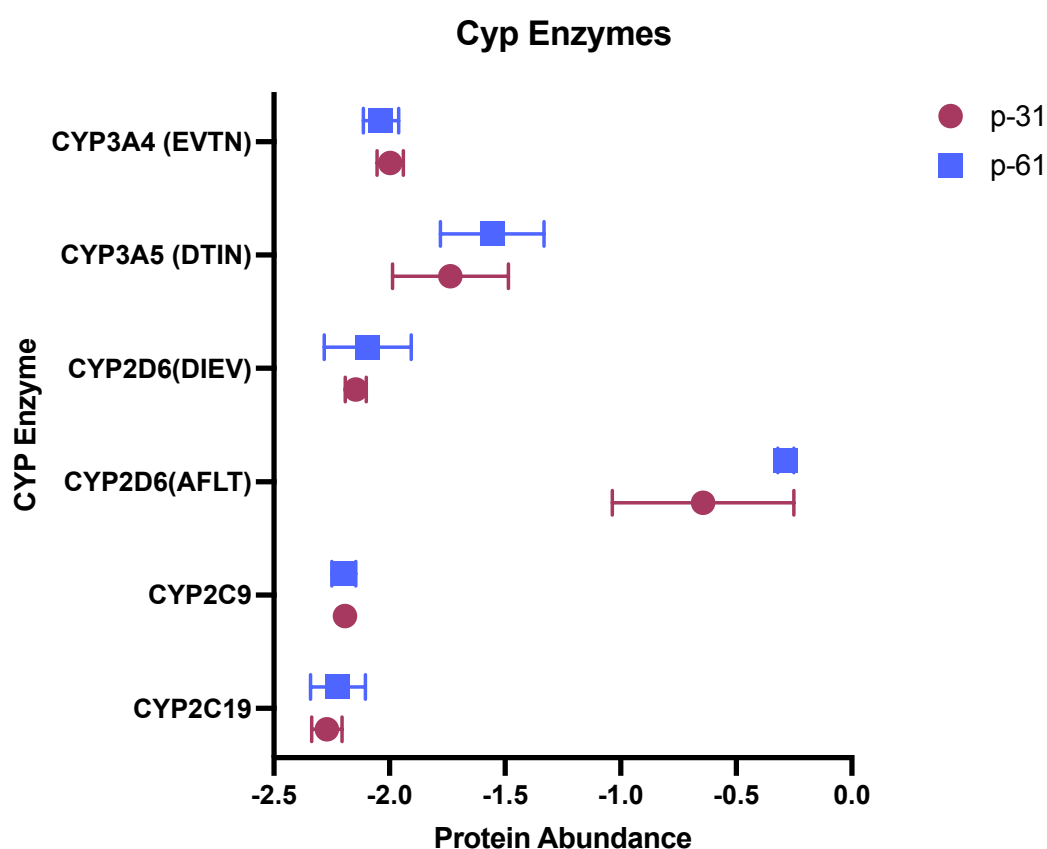


Figure 37 - CYP enzyme expression in Caco-2 cells across different cell passages.

Expression levels of cytochrome P450 (CYP) enzymes in the Caco-2 cell line measured across multiple cell passages. Data are presented as mean protein abundance with error bars indicating the standard deviation ($n = 12$). The figure highlights passage-dependent variability in

CYP expression, reflecting changes in cellular differentiation and metabolic phenotype over extended culture.

Given that the parental Caco-2 clone originates from colonic epithelium, its capacity to fully recapitulate the enzyme profile of the small intestine is inherently limited. Literature consistently demonstrates that CYP3A4 expression declines progressively along the gastrointestinal tract, with the ileum exhibiting the lowest levels among adult intestinal regions²⁷⁻²⁹. Given the high physiological abundance of CYP3A4 within the small intestine, coupled with its clinical relevance, its near-undetectable expression within the Caco-2 model, as shown in Figure 37, represents an important limitation of this system. Its low expression in the Caco-2 passages examined in this study, despite its critical role in the biotransformation of many drug candidates, may therefore compromise the predictive utility of this cell line for modelling intestinal drug metabolism. Owing to this characteristically low expression, wild-type Caco-2 cells are frequently modified to overexpress CYP3A4 in order to better represent the *in vivo* intestinal environment^{30,31}. As the cells examined in this study were wild-type, the below-limit-of-quantification levels of this enzyme observed here are consistent with expectations for this cell line.

In contrast, CYP2D6 was the most abundantly expressed enzyme at both cell passages, with no significant change in expression observed between the two passages examined. This lack of passage-dependent variation was not unique to CYP2D6; all other quantifiable CYP enzymes in the Caco-2 cell line similarly showed no significant difference in expression with increasing passage number. This is consistent with existing literature, which indicates that CYP enzyme expression in this cell line remains low regardless of passage number^{32,33}. More broadly, colonic epithelial cells are known to express lower levels of CYP enzymes than those found in the small intestine; given that Caco-2 cells originate from a colon adenocarcinoma, this reduced CYP expression carries metabolic implications that must be considered when interpreting Caco-2-derived apparent permeability data.

CYP enzymes play a central role in drug metabolism, functioning as the principal enzyme family responsible for the biotransformation of pharmaceuticals *in vivo*, with an estimated 70–90% of marketed drugs metabolised, at least in part, via CYP-mediated pathways underscoring the central role of CYP enzymes in drug metabolism³⁴.

Across the two passages examined in this study, no significant difference in CYP enzyme protein expression was observed. While a passage difference of this magnitude can, in principle, be sufficient to shift cell phenotype away from its original characteristics, the specific passage range tested here did not produce measurable changes in CYP expression. Any variation observed at higher passage numbers may reflect broader passage-associated drift in Caco-2 phenotype, which has been attributed to differences in culture conditions and cumulative passage history across laboratories, rather than genuine biological drift³⁵. The absence of a detectable difference between passages provides confidence in the stability of the model within this passage range, suggesting that the impact of CYP-mediated metabolism on the drugs examined is unlikely to differ substantially as the cells age. Within the tested passage window, therefore, the cells appear to maintain comparable CYP expression levels, supporting the reliability of this system for drug metabolism studies conducted within this range.

The limited change observed in CYP enzyme expression with increasing passage number may, in part, reflect the inherently low baseline levels of CYP enzymes within this cell line. As each sample was derived from a single well of a 12-well plate, the resulting CYP expression levels may lie close to the lower limit of detection of the instrument, reducing sensitivity to small changes in expression. It should also be noted that this study examined only a 30-passage interval, which may fall within a range insufficient to induce statistically significant changes in protein expression; the absence of a measurable difference may therefore reflect the limited passage interval tested, rather than genuine long-term stability of CYP expression.

Collectively, the relatively low CYP enzyme levels observed in this study suggest that Caco-2-derived permeability measurements may differ from the *in vivo* situation as a result of limited CYP-mediated metabolism of the compound *in vitro*. Low CYP expression may also limit the model's capacity to detect drug–drug interactions, enzyme induction, or inhibition effects that would normally be observed *in vivo*. Overall, these low CYP levels should be taken into consideration when applying the Caco-2 model, which may be better suited to permeability-focused applications than to metabolism-related predictions.

5.5.3. UGT enzymes abundance

The other class of enzymes assessed in this study was UGT (UGT-glucuronosyltransferase) enzymes, which catalyse glucuronidation reactions that increase the solubility and clearance of many xenobiotics and endogenous compounds. The following section examines UGT expression and its implications for drug metabolism within this model. Of the seven UGT enzymes examined, fewer than 30% were detected using the LC-MS/MS QconCat method.

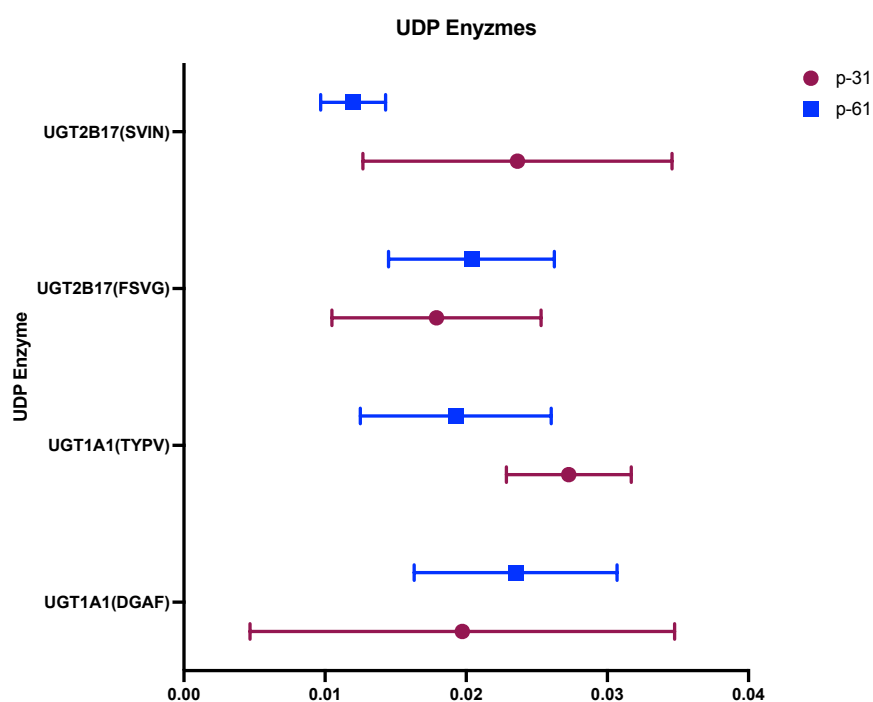


Figure 38 - UGT enzyme expression in Caco-2 cell line at different cell passages; error bars dictate the standard deviation.

Expression levels of UGT-dependent metabolising enzymes in the Caco-2 cell line measured across multiple cell passages. Data are presented as mean protein abundance with error bars indicating the standard deviation (n = 12). The figure highlights passage-related variability in UGT enzyme expression, reflecting changes in cellular differentiation and metabolic capacity as cultures progress through successive passages.

UGT protein levels within the Caco-2 cell line were low, with abundance across all tested samples falling below 0.1, lower than any other protein class examined in this study. Consistent with the pattern observed for CYP enzymes, no significant increase or decrease in UGT enzyme abundance was observed with increasing passage number.

A review of the existing literature indicates that UGT enzyme expression in Caco-2 cells increases substantially upon cellular differentiation^{24,36}. This is reflected in Figure 38, where two of the peptides examined showed a modest shift in average expression, with higher values observed at p-61 than at p-31. However, the overall level of UGT protein detected in this study was sufficiently low that the analytical method may lack the sensitivity required to reliably resolve small changes in protein abundance between passages.

Consistent with the pattern observed for CYP enzymes, UGT enzyme expression in the colon is markedly lower than in the small intestine. This reduced baseline expression likely contributes to the minimal detectable differences observed between passages, given the inherently low levels of these enzymes in colonic tissue. The colonic origin of Caco-2 cells may further constrain the extent to which UGT enzymes can be expressed within this model^{36,37}. Overall, *in vitro* data indicate that UGT enzyme expression across the colon is substantially reduced, suggesting a limited detoxification capacity in this region. Consequently, the metabolic breakdown of compounds in the colon may occur at a slower rate. When evaluating compounds using the Caco-2 model, these constraints must be considered, as *in vivo* behaviour may differ from *in vitro* observations due to the restricted metabolic activity of the model system. This is consistent with prior work showing that even in their differentiated state, Caco-2 cells exhibit UGT activity considerably lower than that observed in human intestinal or liver microsomes, indicating that the model does not fully replicate first-pass glucuronidation capacity *in vivo*^{24,38}.

Overall, these *in vitro* findings indicate that UGT enzyme expression across the colon is substantially reduced, suggesting a limited detoxification capacity in this region and a correspondingly slower rate of metabolic breakdown for glucuronidated compounds. These constraints should be taken into account when evaluating compounds using the Caco-2 model, as *in vivo* metabolic behaviour may differ from that observed *in vitro* due to the model's restricted metabolic activity.

5.5.4. Caco-2 vs. paediatric samples

A direct comparison of the Caco-2 protein expression profile with paediatric intestinal samples provides a meaningful means of assessing how well Caco-2 cells can function as a paediatric-relevant *in vitro* model. The paediatric dataset was generated previously within the research group using the same experimental workflow, offering a uniquely controlled benchmark: identical methods and identical analytical conditions allow for a true like-for-like comparison¹⁶. This alignment enables evaluation of where Caco-2 monolayers successfully recapitulate features of the paediatric intestine, and where their adult, colonic phenotype diverges, ultimately clarifying the strengths and limitations of applying Caco-2 cells as a surrogate for paediatric intestinal biology. Paediatric intestinal tissue displays distinct, age-dependent patterns of CYP expression, with several isoforms present at markedly higher and more functionally relevant levels than those observed in Caco-2 monolayers¹⁶. Figure 39 illustrates these differences, highlighting the metabolic divergence between Caco-2 cells and paediatric biopsy samples, and providing essential context for interpreting ADME predictions derived from Caco-2-based studies.

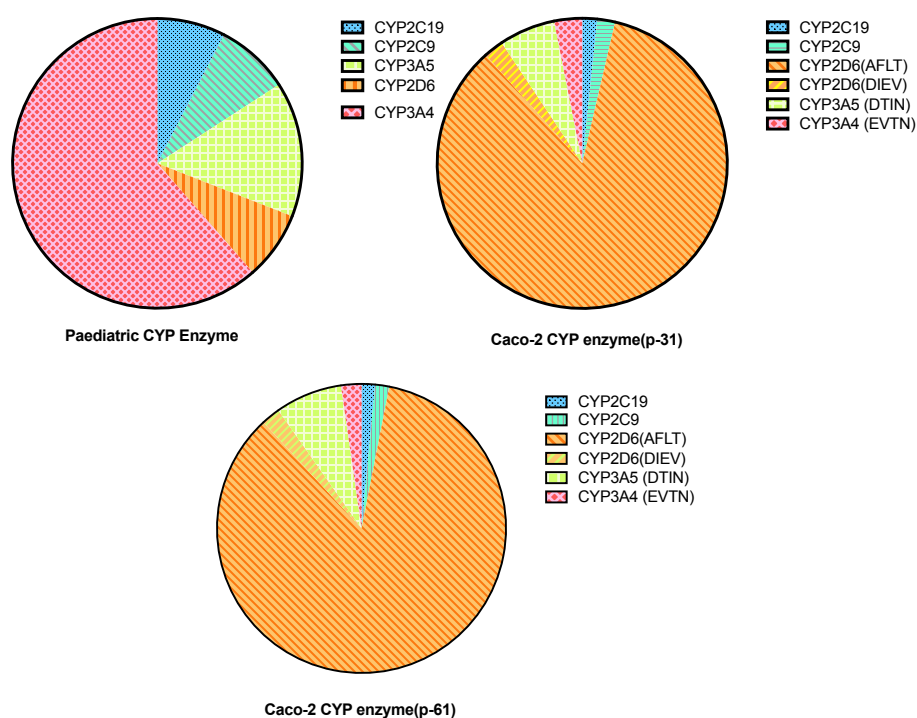


Figure 39 - Mean Relative Abundance of Quantified CYP enzymes.

Representation of transporter expression profiles in paediatric duodenal sections (ages 2–12 years) and Caco-2 cell monolayers. Paediatric samples reflect *in vivo* transporter abundance within the developing small intestine. Caco-2 samples correspond to two independent passages, P-31 and P-61, collected following 21 days of differentiation under standard culture conditions. All Caco-2 monolayers were untreated.

Paediatric samples express CYP isoforms in an age-dependent manner, whereas Caco-2 cells display a comparatively restricted CYP profile, with several CYP enzymes present in human tissue notably underrepresented in the Caco-2 model. This distinction can influence the interpretation of *in vitro* metabolism data and the extrapolation of Caco-2-derived findings to paediatric populations. A clearer understanding of these differences is therefore essential for accurately interpreting metabolic findings in this model.

CYP3A4 is the most abundantly expressed enzyme in human intestinal samples¹⁶. This enzyme is primarily responsible for metabolising approximately 50% of marketed drugs and represents one of the principal enzymes involved in detoxification via first-pass metabolism³⁹. CYP3A4 expression is characteristically high in the small intestine, whereas RNA-seq analysis has identified only low expression within the colon; this

distinction may account for the differing CYP profiles observed between the paediatric intestinal samples and the Caco-2 models³⁹.

By contrast, the predominant CYP enzyme expressed in Caco-2 cells is CYP2D6, a hepatically expressed cytochrome enzyme responsible for metabolising only approximately 20–25% of drugs. The range of substrates metabolised by CYP2D6 is narrower than that of CYP3A4, and CYP2D6 exhibits a greater degree of inter-individual variation in activity⁴⁰. Given the lack of physiological homogeneity within the paediatric population, careful consideration must be given to the predictive value of Caco-2-derived data when extrapolating how paediatric patients may respond to active pharmaceutical ingredients (APIs).

The metabolic behaviour of drugs in Caco-2 cells may therefore differ substantially from that in human intestinal tissue, owing to the contrasting CYP expression profiles of the two systems: Caco-2 cells exhibit minimal CYP3A4 expression, but detectable levels of CYP2D6, an enzyme that is not significantly expressed in the human intestine. Given the differences in substrate specificity, catalytic activity, and regulatory mechanisms between these enzymes, the metabolic pathways observed in Caco-2 cells may not accurately reflect those occurring *in vivo*. As a result, drugs that undergo extensive CYP3A4-mediated metabolism in the human gut may appear more metabolically stable in Caco-2 cells than they are *in vivo*, whereas compounds that are CYP2D6 substrates may exhibit *in vitro* metabolism that does not occur to the same extent within the intestine.

These discrepancies underscore the need for careful interpretation of Caco-2-derived metabolic data, particularly when modelling paediatric oral drug absorption. Consequently, metabolic parameters derived from Caco-2 cells may need to be adjusted, or supplemented with human-relevant CYP3A4 data, when incorporated into PBPK models, in order to avoid underestimating intestinal first-pass metabolism.

In contrast to CYP enzymes, which are primarily involved in first-pass (Phase I) metabolism of compounds entering the body, UGT enzymes mediate Phase II metabolism via glucuronidation. Comparing UGT enzyme levels between the paediatric human samples and Caco-2 cells therefore offers further insight into the overall appropriateness of the Caco-2 model for representing paediatric small intestinal metabolism.

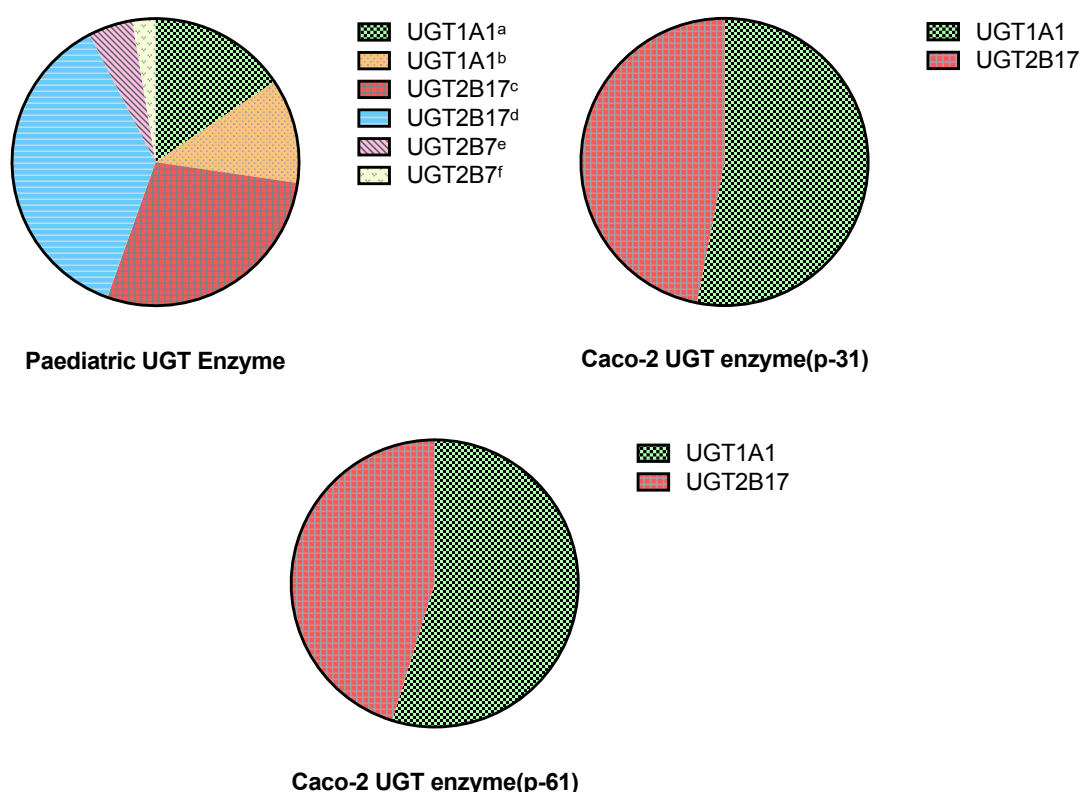


Figure 40 - UGT enzyme comparison between Paediatric duodenum profile, and those of Caco-2 cells cultured in monolayers for 21-day incubation. Monolayers were harvested under basal conditions, without drug treatment or other stimulation.

Representation of transporter expression profiles in paediatric duodenal sections (ages 2–12 years) and Caco-2 cell monolayers. Paediatric samples reflect *in vivo* transporter abundance within the developing small intestine. Caco-2 samples correspond to two independent passages, P-31 and P-61, collected following 21 days of differentiation under standard culture conditions. All Caco-2 monolayers were untreated.

The UGT enzyme profile differs substantially in architecture between human and cell-based assays. Human duodenal biopsy samples from Goelon et al. displayed three distinct UGT enzymes¹⁶. whereas only two of these enzymes were detected in the Caco-2 monolayers examined in this study. *In vivo*, these enzymes were represented by six unique peptides, all of which were measurable in every one of the 30 human samples, indicating a stable and reproducible UGT expression profile *in vivo*.

By contrast, the Caco-2 monolayer system exhibits a markedly restricted repertoire of UGT enzymes. Although Caco-2 cells undergo enterocyte-like differentiation during the standard 21-day culture period, their metabolic machinery remains incomplete, reflecting both their colonic carcinoma origin and the well-documented limitations of this cell line in recapitulating intestinal Phase II metabolism. Within the present dataset, only two peptides associated with UGT enzymes were detectable, indicating a substantial reduction in both the number and diversity of UGT isoforms relative to human tissue. This restricted profile highlights the simplified, and less physiologically representative, metabolic environment of the Caco-2 model, particularly with respect to glucuronidation capacity.

In the adult small intestine and colon, UGT2B17 is the most abundantly expressed UGT isoform⁴⁰, a pattern that aligns with the paediatric tissue dataset¹⁶, in which combined UGT2B17 peptides similarly showed the highest abundance across samples. In contrast, the most abundant UGT isoform detected in Caco-2 cells was UGT1A1. While UGT1A1 ranks second in paediatric tissue and fourth in adult tissue^{16,41}, its dominance in Caco-2 cells reflects neither physiological profile. This indicates partial concordance between adult and paediatric intestinal UGT expression patterns but also demonstrates that the Caco-2 model fails to reproduce either *in vivo* distribution.

The inability to detect several UGT-associated peptides in Caco-2 lysates further complicates assessment of the model's suitability for representing paediatric UGT-mediated metabolism. Given the limited number of detectable enzymes, data generated using this system should be interpreted with caution, as glucuronidation activity in Caco-2 cells is likely to underestimate the metabolic capacity observed *in vivo*. Consequently, while Caco-2 cells remain valuable for permeability studies, their utility for modelling intestinal glucuronidation, particularly within paediatric contexts, is inherently limited.

The restricted detection of UGT enzymes observed here may also, in part, reflect technical limitations, including low sample volume and reduced total protein yield. UGTs are membrane-associated enzymes that are often present at low abundance, making their detection particularly sensitive to constraints in sample preparation. Increasing the number of cells per sample, for example by pooling lysate from a full plate, may improve protein yield and enhance the sensitivity of the analytical method by increasing the

amount of analyte available for detection relative to the assay's limit of detection/quantification, thereby improving signal-to-noise ratio for low-abundance protein, enabling more reliable detection of low-abundance UGT isoforms in future work.

While the preceding section characterises the metabolic capacity of each model through UGT enzyme expression, metabolism alone does not determine the ultimate fate of a molecule. The subsequent step, transport and efflux of resulting conjugates, is governed by membrane transporters. The following section therefore examines the expression of key transporters, in order to assess how well each model reflects the intestinal handling of drug metabolites.

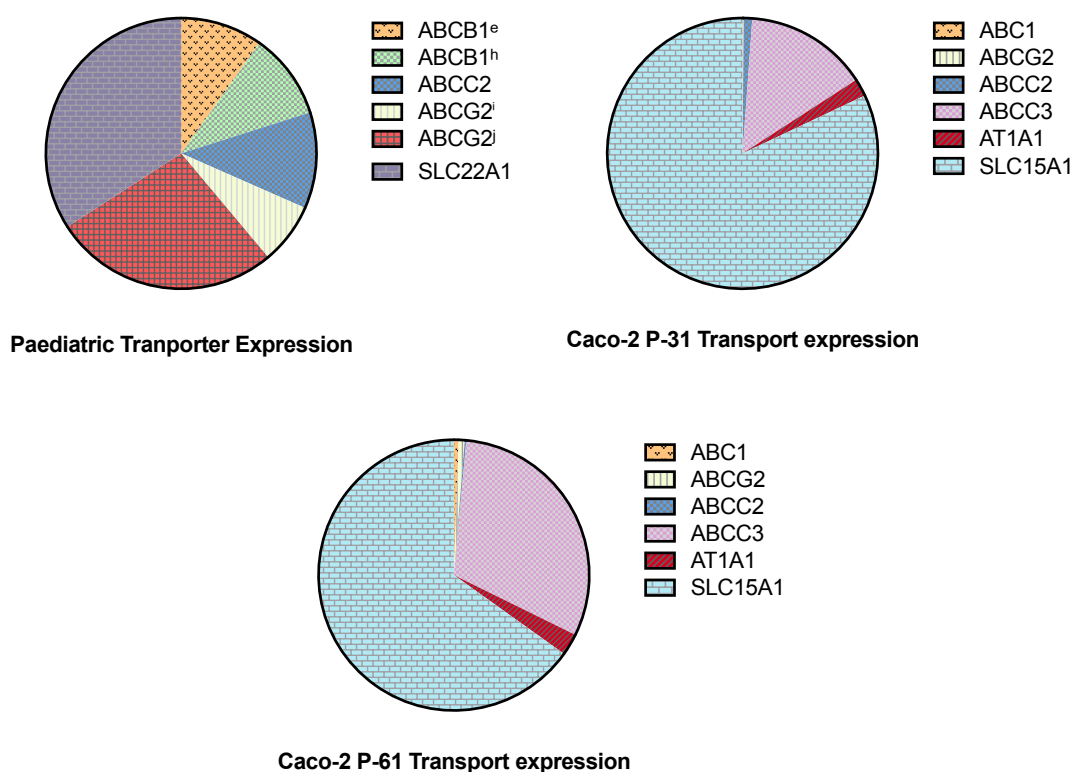


Figure 41 - Proportional Expression of Transporters in Paediatric Duodenal Tissue and Caco-2 Cell Monolayers.

Representation of transporter expression profiles in paediatric duodenal sections (ages 2–12 years) and Caco-2 cell monolayers. Paediatric samples reflect in vivo transporter abundance within the developing small intestine. Caco-2 samples correspond to two independent passages, P-31 and P-61, collected following 21 days of differentiation under standard culture conditions. All Caco-2 monolayers were untreated.

Evaluation of transporter expression in the Caco-2 model reveals a pattern similar to that observed for the enzyme families previously assessed. As illustrated in Figure 41, Caco-2 monolayers display a far less diverse transporter distribution, with a single transporter accounting for the majority of expression at both passage 31 and passage 61, while the remaining transporters are present only at low levels. In contrast, the paediatric duodenal samples exhibit a more balanced and diverse distribution of transporter expression across intestinal segments, reflecting the broader physiological repertoire present *in vivo*.

One of the major transporters responsible for effluxing substrates across the epithelial membrane is ABCB1, also known as P-glycoprotein (P-gp), which actively transports its substrate compounds out of the cell against their concentration gradient, thereby

reducing the proportion of susceptible drug molecules able to cross the epithelial membrane. ABC transporters primarily drive efflux, whereas SLC transporters can mediate either influx or efflux, with their principal role generally being drug uptake⁴². Notably, Figure 41 shows that SLC transporters constitute a higher proportion of total transporter expression in Caco-2 cells than in the paediatric samples: over 50% of transporters detected across Caco-2 passages belonged to the SLC family, compared with approximately 43% in the *in vivo* samples. As the greater proportion of ABC transporters observed *in vivo* may play a substantial role in driving drug efflux and resistance within the intestine, the relative shift toward SLC-family dominance in Caco-2 cells may limit the model's ability to predict the significant contribution of efflux transporters *in vivo*, potentially leading to overestimation of a compound's bioavailability where efflux is underrepresented *in vitro*.

This divergence between the transporter diversity observed in paediatric tissue and the more restricted Caco-2 profile raises important considerations for the use of Caco-2 as a surrogate for paediatric intestinal absorption. A model dominated by SLC transporters, with limited ABC-family representation, may not accurately reflect the absorption pathways relevant to children. Therefore, while Caco-2 remains a valuable tool for assessing permeability, its transporter composition does not fully align with the physiological environment of the paediatric duodenum, and results derived from this model should be interpreted with caution in this context.

5.6. Conclusion

This study examines differences in protein expression as Caco-2 cells increase in age. The use of two distinct cell passages, both of which formed coherent monolayers prior to lysis, allows for a more informed understanding of, and a more evidence-based approach to, the use of Caco-2 cells in drug development. Protein quantification was performed using an optimised LC-MS/MS method, employing a heavy chain standard containing three distinct sequences for each target DMET protein.

Analysis of both transporter and enzyme protein classes across the two cell passages revealed no significant differences in expression. This limited variation is consistent with stable expression between passages 31 and 61, suggesting that compound testing across this passage range is unlikely to be substantially confounded by passage-dependent variation in enzyme or transporter expression. While this finding is based on only two passages and would benefit from confirmation across a wider range, it lends some support to the interpretation that observed changes in compound potency across the Caco-2 monolayer reflect properties of the compound itself, rather than underlying variation in protein abundance.

In this study, only limited similarity was observed between the Caco-2 protein expression profile and that of the paediatric intestinal samples reported by Goelen et al. this divergence indicates that the Caco-2 model fails to fully replicate the protein landscape of the paediatric small intestine¹⁶. As each Caco-2 sample was derived from a single monolayer well, the total protein content obtained was considerably lower than that of the paediatric biopsy samples. This reduction in sample quantity likely positioned several proteins close to the analytical limit of detection, potentially limiting the instrument's ability to resolve small changes in expression.

5.7. Future Work

Further work should include increasing the range of passage numbers examined in the Caco-2 monolayer. This may help identify the point at which changes in the protein profile emerge, as increasing passage number could result in either an increase or decrease in key proteins that influence compound transport across the monolayer. Mapping these passage-dependent changes would allow clearer interpretation of permeability data and help determine whether observed differences arise from the compound itself or from underlying biological drift within the model.

The reduced protein mass obtained in this study likely pushed several low-abundance proteins toward the analytical limit of detection, restricting the instrument's ability to reliably quantify subtle changes in expression. Increasing the number of Caco-2 wells pooled per sample would enhance total protein input, improve detection of low-abundance transporters and enzymes, and generate a more robust and representative proteomic profile. Such an approach would strengthen cross-tissue comparisons and may help clarify whether the apparent dissimilarity between models reflects true biological differences, or simply analytical under-sampling of the Caco-2 proteome.

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Chapter 6- Conclusions and future work

6.1. Thesis Conclusions

This thesis represents the latest advancement in research at the University of Strathclyde aimed at developing more biorelevant *in vitro* methods for assessing the performance of oral dosage forms. It builds on the group's previous work characterising drug solubility under conditions representative of the human intestine. This began with design of experiments (DoE) studies that established the influence of individual media components on solubility, followed by reduced DoE approaches that minimised the number of experiments required while still capturing a physiologically relevant solubility range. This work was subsequently extended through multidimensional analysis of human intestinal fluid (HIF), leading to the development of media recipes that describe the variation in intestinal fluid composition observed across real-world populations. The present thesis is the first to apply these media to the assessment of permeability. In doing so, it consolidates the group's previous solubility-focused research and introduces a novel *in vitro* method for measuring permeability in the fasted state, contributing to the advancement of predictive tools in pharmaceutical science.

The primary aim of this thesis was to apply a fasted-state biorelevant media system to the Caco-2 Transwell™ model in order to evaluate drug permeability under fasted intestinal conditions, and to further understand the suitability of Caco-2 cells as a model for *in vivo* predictions.

The first objective Chapter 2 was to develop a gas chromatography-mass spectrometry (GC-MS) method for the simultaneous quantifications of the four principal SIF components: bile salt, lecithin, cholesterol and sodium oleate. Preliminary LC-MS investigations were hindered by strong retention of several analytes on the stationary phase, resulting in incomplete recovery, prolonged wash-out and carry-over that compromised column performance. Preliminary LC-MS investigations were hindered by strong retention of several analytes on the stationary phase, resulting in incomplete recovery, prolonged wash-out and carry-over. GC-MS was therefore explored as an alternative, as derivatisation and higher operating temperatures reduce carry-over and improve chromatographic performance in complex matrices. Following optimisation of temperature programmes, injection parameters and derivatisation protocols, all four components were successfully detected within a single analytical run. However, signal losses indicated that derivatisation was not yet complete for all analytes, and full

quantification, including assessment of sensitivity in receiver compartment samples, remains to be achieved.

The second objective of this study (Chapter 3) was to apply the fasted SIF to Caco-2 cells to understand the media compositions that maintained Caco-2 cells viability and monolayer integrity without inducing cellular damage, thereby enabling development of an experimental protocol for assessing drug permeability under physiologically relevant conditions. Initial screening indicated that specific media components were driving cytotoxicity, with bile salts and free fatty acids identified as the primary contributors, whereas cholesterol and lecithin appeared to exert a protective effect, potentially by stabilising cell membranes. Following screening, the selected media were shown not to induce apoptosis Strath FaSSIF 1 and Strath FaSSIF 7, and further assessment of tight junction integrity TEER confirmed that the monolayer remained intact in their presence. These findings established that Strath FaSSIF 1 and Strath FaSSIF 7 could be carried forward for drug transport studies and highlighted the need to consider cellular tolerance alongside physiological relevance when designing biorelevant media for use in cell-based models.

The objective of Chapter 4 was to evaluate the impact of the biorelevant media selected in Chapter 3 on drug permeability across Caco-2 monolayers in the absence of a mucus layer, using HBSS, the industry-standard transport medium, as a comparator. Eight BCS-classified drugs were studied, selected because they had previously been characterised in SIF solubility studies. Reliable quantification required substantial method development: the addition of 4% BSA to the basolateral compartment to replicate *in vivo* sink conditions reduced HPLC sensitivity, necessitating a transition to LC-MS/MS, and carry-over of highly lipophilic compounds was subsequently resolved by adopting HILIC LC-MS/MS. Increasing TAC was associated with increased apparent permeability for verapamil, acyclovir, ranitidine, venetoclax and ritonavir. The biorelevant media also introduced greater variability in permeability, particularly for more lipophilic compounds, likely reflecting heterogeneity in drug-micelle interactions. Adjusting all media to a uniform pH of 7.34 showed that, for fenofibrate and felodipine, permeability changed despite a negligible change in neutral fraction, indicating that media composition rather than drug ionisation was the dominant factor. Together, these findings suggest that micelle-mediated transport across the unstirred water layer is an important determinant

of permeability for highly lipophilic compounds, and that the pH-partition hypothesis alone is insufficient to predict permeability in biorelevant media. They also indicate that conventional predictive models, such as PAMPA and HBSS-based Caco-2 assays, which do not account for micelle–drug interactions, may underestimate the variability in permeability that occurs under physiological conditions.

Finally, the objective of Chapter 5 was to determine the influence of passage number on the expression of key drug-metabolising enzyme and transporter (DMET) proteins in Caco-2 cells, and to evaluate the relevance of the model to paediatric populations. Absolute protein quantification was performed using an optimised LC-MS/MS method with a heavy-chain standard containing three peptide sequences per target protein. No significant differences in expression were observed between passages 31 and 61, indicating that expression is stable across this range and supporting the interpretation that the permeability differences observed in this thesis reflect properties of the compounds and media rather than variation in protein abundance. Comparison with paediatric small intestinal data reported by Goelen *et al.* revealed only limited similarity, although the low protein content of the single-well Caco-2 samples is likely to have placed several proteins close to the limit of detection¹. These findings provide a quantitative basis for interpreting Caco-2 permeability data, while indicating that caution is warranted when extrapolating to paediatric populations.

Overall, this thesis advances biopharmaceutics by extending the multidimensional biorelevant media system developed at the University of Strathclyde from solubility into permeability assessment. Building on a decade of research characterising intestinal fluid as a multidimensional system. Taken together, this work provides both a method and a mechanistic basis for generating more physiologically relevant permeability inputs, with the potential to improve the accuracy of PBPK and other predictive models used in drug development.

6.2. Future Work

This thesis applied the fasted-state biorelevant media system developed within the group to the Caco-2 Transwell™ model, establishing a method for measuring drug permeability under biorelevant conditions and characterising the model itself through proteomic analysis. While this work advances the understanding of how media composition influences permeability, several areas warrant further investigation. Future work could involve:

1. **Incorporate the permeability data obtained in this study into the PBPK model:**

this study was primarily focusing on generating the permeability data of some of these drugs of interest. The addition of the generated permeability data set into a PBPK model and compare the resulting predictions with clinical pharmacokinetic data, to establish whether biorelevant permeability inputs improve the prediction of oral absorption.

2. **Finalise development of the GC-MS method:** Completing the optimisation of derivatisation conditions and constructing calibration curves for each analyte would enable quantification, rather than detection alone, of SIF components. Applying the method to basolateral fluids collected from permeability studies would establish whether SIF components cross the monolayer, and whether drugs remain associated with these components once in the basolateral chamber. This would provide a more detailed understanding of micelle–drug interactions and their influence on transport. This distinction carries direct methodological implications, as the calculation of apparent permeability assumes that measured basolateral concentrations represent freely dissolved drug; if a proportion remains micelle-bound, Papp values determined in biorelevant media may require reinterpretation. Such work would provide a more detailed understanding of micelle–drug interactions and their influence on transport.

3. **Expanding the drug catalogue:** The compounds studied here were selected for their existing SIF solubility data, and a broader set would allow the mechanisms proposed in this work to be tested more rigorously. Priority should be given to ionisable drugs whose neutral fraction changes substantially across the physiological pH range, which would provide a direct test of whether pH-partitioning or media composition dominates; to compounds spanning a wider

LogP range, to define the lipophilicity threshold above which micellar interactions begin to govern transport; and to established substrates of P-gp, BCRP and other intestinal transporters, to determine whether biorelevant media modify efflux-mediated transport.

4. **Incorporation of mucin:** The permeability studies presented here were conducted in the absence of mucus, which *in vivo* forms an additional diffusional barrier overlying the intestinal epithelium. Incorporating mucin, either by applying a mucin layer to the monolayer or by adopting a Caco-2/HT29-MTX co-culture, would allow this barrier to be represented. Including mucus would therefore test whether the enhanced permeability of highly lipophilic drugs in biorelevant media persists under more physiologically representative conditions and would extend the model towards a more complete representation of the intestinal barrier.
5. **Enhanced SIF catalogue:** The absence here of a mucin layer is likely to have increased the susceptibility of the monolayer to toxicity. Incorporating mucin into the model may therefore allow media of higher amphiphile concentration to be tolerated, widening the range of formulations that can be studied. Future work should define concentration thresholds for individual amphiphiles in the presence and absence of a mucus layer, in order to establish the limits within which monolayer integrity can be maintained and to expand the catalogue of biorelevant media available for permeability assessment.

6.3. Reference:

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