Mechanistic Studies and Modeling of Effects of Ingested Lipids on Oral Drug Absorption

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ABSTRACT

Ingested lipids, typically originating from food and potentially used as delivery agents, can enhance absorption of compounds by several hundred percent, but they can also decrease absorption or have no effect. The influence of lipids on compound absorption originates from colloidal structures they form, compound trafficking between these colloidal structures and aqueous medium, and affects on transport through the intestinal mucosa. However, these effects are typically documented as empirical, compound-specific observations and not predictable a priori.

The overall goal of this project was to gain quantitative mechanistic insight into and to predict the influence of lipids, mainly triglycerides, in the gastrointestinal (GI) tract on compound absorption. Previously, the impact of lipid ingestion on co-administered compound absorption (compound solubility enhancement, change in intestinal permeability) has been studied in isolation. The proposed research aimed to thoroughly characterize and model kinetics of parallel processes occurring in the GI tract upon co-dosing a compound with lipids and to relate the kinetics to chemical composition and colloidal structure of intestinal contents. The study included experimental characterization and computational modelling of kinetics of parallel processes occurring in the GI tract after lipid ingestion – namely, compound dissolution, lipid digestion, compound partitioning into colloidal phases, absorption. The specific four aims of the proposed experimental approach were the followings. (1) Design and characterize biorelevant in vitro lipid digestion models able to simulate fundamental features of human intestinal contents in post-prandial conditions. Basic back-titration has been used to establish associated chemical composition characterizing products of the lipolysis
(2) Investigate the ultra-structure and composition of colloidal species existing in the GI tract upon ingestion of lipids and their dynamic behavior. Dynamic light scattering (DLS), and small angle neutron scattering (SANS) have been employed to characterize the colloidal structures (emulsion droplets, vesicles, micelles) present throughout digestion. (3) Establish kinetics and thermodynamics of drug transport into and out of colloidal structures in the GI tract, based on a model drug that was selected to represent poorly water-soluble drug compounds. High-pressure liquid chromatography (HPLC) has been used in solubility/dissolution experiments, while electron paramagnetic resonance (EPR) has been employed to track the model drug in different phases formed during the digestion process (oil droplets, vesicles, micelles, free in aqueous solution, etc.). (4) Examine kinetics of compound transport across the intestinal membrane in the presence of food-associated lipids. The Caco-2 and HT29-MTX cell culture models were employed to investigate drug permeability properties in post-prandial conditions. The outcome was a mechanistic and kinetic model of intestinal absorption in the presence of ingested lipids.

Developed kinetic expressions were integrated into a systems-based model that simulated the influence of ingested lipids on oral compound absorption. Mechanistic understanding of how ingested food lipids altered effectiveness of orally delivered compounds was also gained. The final outcome was an experimental and theoretical framework able to quantitatively describe and predict the effects of ingested food lipids on the overall oral drug absorption.
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1.0 Introduction

The oral route is the most desirable and common method for drug administration because it is generally well accepted by the patients, and it does not require involvement of health care assistance. However, orally delivered drugs are often challenged by the complex nature of the gastrointestinal (GI) tract, which might cause insufficient drug absorption and bioavailability. Thus, drug absorption, good and reproducible bioavailability, and pharmacokinetic profiles in humans are nowadays recognized as some of the major challenges in oral delivery of new drug candidates [2]. The issue has especially emerged in the last two decades, when the introduction of combinational chemistry and high throughput screening determined a shift in the properties of new drug candidates towards higher molecular weight and increasing lipophilicity [3, 4], resulting in decreasing aqueous solubility. Approximately 40–70% of all new drug candidates have been estimated to have very poor water solubility [6, 7] with medium/high lipophilicity. Therefore, they are very likely to show low bioavailability when orally administered, and it is not expected that this figure will change in the future [2]. Generally speaking, any orally delivered compounds, including dietary supplements, nutrients, and toxins might face similar problems of incomplete and insufficient absorption due to their lipophilic nature. For example, many fundamental nutrients, including vitamins, cholesterol, and sterols are lipid soluble. Besides poor solubility and slow dissolution in aqueous-based gastrointestinal fluids, the permeability through membranes of the intestinal wall is a second critical aspect for oral absorption of substances. In fact, pharmaceutical compounds have been classified based on their solubility and permeability in the Biopharmaceutical Classification System (BCS) since mid-1990s [10].
The capability of food to affect the absorption and the bioavailability of co-administered compounds has been generating academic and commercial attention for the past 30 years [11-16]. Food intake is followed by a wide range of changes in the GI tract, which can impact the absorption and the pharmacokinetics of drugs. Depending on the physicochemical properties of the drug substance, the type of dosage form, the general state of the GI tract, and the composition of the ingested food, the overall drug absorption can be increased, decreased, delayed, or accelerated by food intake. Although it is recognized that other food components, such as proteins and sugars, can influence overall compound absorption, the impact of food on oral compound absorption has been attributed mainly to the lipid components of food intake or delivery agents, especially to triglycerides (TG). For example, fat-rich meals have been observed to greatly enhance the oral bioavailability of poorly water-soluble drugs administered in solid forms. Ingested lipids are able to induce larger changes in the GI tract physiology, which turn to produce favorable conditions for enhancing dissolution, solubility and absorption processes of hydrophobic compounds. This has been the case not only for lipids present in food but also lipids used as delivery vehicles (e.g., in oral formulations [17-19]). Thus, ingested lipids could offer valuable opportunities for enabling oral delivery of poorly water-soluble drugs and drug candidates. However, although numerous studies have been performed correlating hydrophobic drug bioavailability and lipid vehicles, only few successful lipid based formulations have been marketed so far, including Sandimmum Neoral® (cyclosporine), Norvir® (ritonavir), Fortovase® (saquinovir) [20] and lipid soluble vitamins.

The influence of lipids on oral absorption has been typically documented as
empirical and compound-specific observations [21], which are generally not predictable a priori. A lack of a general in vitro model that is able to predict a priori the in vivo performance of drug–lipid (from oily vehicles and/or food) systems might be due to: i) the limited understanding of in vivo mechanisms of drug bioavailability enhancement by lipids; ii) the difficulty of mimicking in vitro the high complexity and dynamicity of the GI environment; iii) the consequent poor match between physiological physicochemical properties of in vitro systems and physiological features of GI fluids.

Designing proper models is crucial for correct evaluation and quantitative prediction of dissolution and absorption profiles of oral compounds in vitro. In fact, physicochemical and physiological features of GI fluids, such as pH, buffer capacity [22], bile component concentrations and states of aggregation, and enzyme activity, can greatly influence the drug dissolution process. Furthermore, permeability and modes of transport through the intestinal mucosa might be affected by changes in fluid properties between fasted and fed states, leading to altered absorption. In addition to that, processes involved in lipid impact on overall absorption, studied in isolation and on disparate systems, frequently did not take into account the dynamic, interconnected nature of the digestion/partitioning/absorption processes. Therefore, it is desirable that an integrated experimental approach that would include quantitative analysis of dynamic processes occurring simultaneously rather than single processes studies in isolation or at equilibrium. Such global approaches would also elucidate how physical and chemical system properties relate to function, which would lead to a general rather than compound-specific understanding, and therefore would allow mathematical modeling and quantitative predictions.
1.1 The importance of mathematical modeling in oral drug delivery

Mathematical modeling and predictability of drug delivery is a field of increasing academic and industrial importance with an enormous future potential in product development. Due to the significant advances in information technology, the in silico models in this field can be expected to significantly improve in accuracy and easiness of application, and computer simulations are likely to become an integral part of future research and development in pharmaceutical technology. One of the major driving forces for the use of mathematical modeling in oral drug delivery is to save time and to reduce costs in developing a new drug or in optimizing an existing drug product. In addition, the quantitative analysis of the physical, chemical and potentially biological phenomena, which are involved in the control of drug absorption, offers the fundamental advantage of understanding mechanisms involved. This knowledge is not only of academic interest, but a pre-requisite for an efficient improvement of the safety of new pharmaco-treatments and for efficiently addressing potential challenges encountered during production.

Specifically, mathematical systems-based models have shown considerable promise with respect to predicting bioavailability of orally delivered compounds [23]. There are several examples of such models (Simulations Plus, Inc.’s GastroPlus™ software, Intellipharm® Software) that are currently used in the pharmaceutical industry for predicting oral bioavailability and pharmacokinetic profiles. However, these models generally do not consider the influence of agents that interact with drug molecules, such as completing agents or lipids. This is related to lack of mechanistic and quantitative understanding of the function of lipid based systems in the GI tract, leading to simplified
consideration of the food effect as resulting from enhancement in equilibrium solubility in fed state intestinal contents [24]. Thus, there is a significant need for enhanced understanding of the dynamic nature of processes occurring in the GI tract in the presence of ingested lipids, and their dependence on colloidal structures formed by ingested lipids. This understanding will enable development of models predicting the impact of lipids, the framework of which is included in the proposed project.

1.2 Project goals and significance

The overall goal of this project was to gain quantitative mechanistic insight into and predictive capability of the influence of lipids in the GI tract on compound (drug, nutrient, and lipid) absorption. As described in the sections above, these effects are not currently amenable to any form of quantitative prediction, yet quantitative understanding and predictability of the fate of ingested lipids has tremendous implications pertaining to oral drug delivery and to diet-related diseases (e.g., such as obesity).

Previously, while aspects of lipid ingestion impact on co-administered compound absorption (e.g., compound solubility enhancement, change in intestinal permeability) have been studied in isolation, the relevant dynamic, interconnected processes, and their dependence on dynamic system colloidal structure and composition, have not been studied in a comprehensive, integrated fashion conducive to enabling quantitative prediction. The present research aimed to thoroughly characterize and model kinetics of parallel processes occurring in the GI tract upon co-dosing a compound with lipids (compound dissolution, lipid digestion, compound partitioning into colloidal phases, absorption) and relate the kinetics to chemical composition and colloidal structure of
intestinal contents. The result was a systems-based model of the influence of ingested lipids on compound bioavailability.

It is well recognized that lipid digestion and absorption are highly complex processes with multiple intricacies that are not currently possible to capture in mechanistic in vitro studies in a single project. Therefore, the approach followed was to start with a simplified system from which an experimental and theoretical framework was developed, feasibility of quantitative prediction was established, and considerable insight into complex effects of lipids on compound absorption was gained. The specific aims of this project were:

**Aim 1** - Design and characterize biorelevant in vitro lipid digestion models able to simulate fundamental features of human intestinal contents in post-prandial conditions. Basic back-titration was used to quantify the extent of the lipolysis process. The outcome was suitable in vitro models mimicking the in vivo lipid digestion process.

**Aim 2** – Investigate the ultra-structure and composition of colloidal species existing in the GI tract upon ingestion of lipids and their dynamic behavior. Dynamic light scattering (DLS), and small angle neutron scattering (SANS) were used to characterize the colloidal structures (emulsion droplets, vesicles, and micelles) present throughout digestion. The outcome was a physical and chemical conceptual model of the dynamic intestinal milieu post lipid ingestion.

**Aim 3** - Establish kinetics and thermodynamics of drug transport into and out of colloidal structures in the GI tract, based on a set of test compounds that will be selected to represent broad physicochemical properties. High-pressure liquid chromatography (HPLC) was used in solubility/dissolution experiments, while electron paramagnetic
resonance (EPR) was used to track the drugs (oil droplets, vesicles, micelles, free in aqueous solution, etc.). The outcomes were kinetic expressions describing drug dissolution and partitioning into intestinal lipid digestion phases.

**Aim 4** - Examine kinetics of compound transport across the intestinal membrane in the presence of food-associated lipids. The Caco-2 and HT29-MTX cell culture models were employed to investigate drug permeability properties in post-prandial conditions. The outcome was a mechanistic and kinetic model of intestinal absorption in the presence of ingested lipids.

Developed kinetic (mass transport) expressions were integrated into a systems-based model that simulated the influence of ingested lipids on oral compound absorption. Mechanistic understanding of how ingested food lipids altered effectiveness of orally delivered compounds was also gained.

The final outcome was an experimental and theoretical framework able to quantitatively describe and predict the effects of ingested food lipids on the overall oral drug absorption.
2.0 Critical Literature Review

The overall GI absorption of compounds can be considered a combination of several processes, some of which occur simultaneously (Figure 1, [23]). Upon oral administration, solid pharmaceutical substances are absorbed in the gastrointestinal tract after first disintegrating and then dissolving into GI fluids. Drugs that do not dissolve in the GI fluids are not absorbed and a primary chemical property affecting dissolution is the drug’s solubility. Once dissolved in GI fluids, drug can be decomposed, excreted into the feces, or be available for absorption. Absorption across the membranes of the GI tract is influenced by the permeability of the drug (related to passive diffusion mechanisms) and the presence of transporters that absorb the drug into cells or excrete the drug from cells (related to active transport routes). Once a drug is absorbed into the blood of the GI tract it must first pass through the liver where it could be metabolized or excreted back into the GI tract via biliary excretion. This process is generally referred to as the first-pass effect [23].

2.1 Impact of ingested lipids on oral absorption of compounds

Lipids, either derived from food or used as drug delivery agents, can have significant effects on dissolution, absorption, and bioavailability of orally delivered compounds. However, the influence of lipids on oral absorption is typically documented as an empirical and compound-specific phenomenon. For example, while absorption and bioavailability enhancement are associated with lipid intake for certain compounds, decreases in bioavailability of up to several hundred percent are observed for other compounds [25, 26] Therefore, despite numerous studies about fat-rich food/drug
interactions occurring during the lipid digestion, there is still an incomplete understanding of the influence of ingested lipids on oral bioavailability, and a lack of a general *in vitro* model that is able to predict a priori the *in vivo* performance of drug–lipid systems. It is believed that the influence of lipids on oral compound absorption, bioavailability, and distribution originates from colloidal structures formed during the lipid digestion, compound trafficking between these colloidal structures and aqueous medium, and affects on transport through the intestinal mucosa. How the altered intestinal lumen composition in the presence food digestion influences all the processes involved in oral absorption is addressed in details in the following sections.

2.1.1 Lipid digestion process: an overview
An average daily western adult diet contains about 150 g of lipids, 95% of which are long-chain triglycerides (or triacylglycerols, TG), and 4–8 g of phospholipids [27], mainly composed of lecithin [28]. TG cannot be absorbed into enterocytes in their native form, but need to be hydrolyzed, producing in total two fatty acids (FA) and one 2-monoacylglycerol (MG) for each TG molecule. The overall lipid digestion can be considered as the result of three sequential steps [29]:

i) Dispersion of fat globules into fine emulsion particles;

ii) The enzymatic hydrolysis of TG;

iii) Dispersion of lipid digestion products into a more absorbable form.

In humans, hydrolysis of lipids starts in the stomach, by the enzymatic action of lingual and gastric lipases, which are secreted respectively by the salivary glands of the tongue and the chief cells of the gastric mucosa [30]. These lipases are responsible for 30% of the total lipid hydrolysis [29], but some authors report lower lipolysis activity of 15% [31] and higher values up to 60% [32]. The activity of lipase is expressed in term of tributyrin units (TBUs): 1 TBU is the amount of enzyme that can liberate 1 μmol of FA from tributyrin per minute [33]. The gastric lipase has an activity of about 1300 TBUs and its concentration in the fed stomach is 17 μg/ml [34]. Shear forces, produced by contractions of the stomach muscles and gastric emptying, and partially ionized FA, crudely emulsify different lipid digestion products (TG, DG, MG, FA), which pass into the duodenum. In response to intestinal presence of digestion products, bile salts (BS), biliary lipids, and pancreatic juice are secreted into the lumen of the upper small intestine [29]. These compounds alter drastically the lipid emulsion, leading to the formation of complex colloidal structures, such as mixed disc-shaped micelles with a hydrodynamic
radius of about 20 nm [29] and liquid crystalline unilamellar vesicles around 40–60 nm in radius [29, 35]. Most of the lipid digestion is carried out by another lipase, called pancreatic lipase, provided in the upper part of the intestinal lumen by pancreatic juice secretions. Lipid hydrolysis is heavily dependent on the action of pancreatic lipase, which works at the interface between the aqueous medium and insoluble lipid droplets [36]. Pancreatic lipase degrades remaining TG into free FA and 2-monoacylglycerol (2-MG) [37], which might transform into 1-monoacylglycerol (1-MG) by isomerization in aqueous media [38]. Additional hydrolysis of both forms of MG by pancreatic lipase is also possible but to a small extent, leading to the formation of glycerol and FA [39]. Pancreatic lipase requires the formation of a complex with co-lipase, promoted by FA [40], in order to be active. Alvarez and Stella [41] reported Ca$^{2+}$ ions to have a significant influence on the lipolysis activity in presence of bile (in vitro observation), although this phenomenon was not observed by some other researchers [33] and there is no mention of a calcium binding site in the 3-D structure of pancreatic lipase/co-lipase complex [42]. Another lipase present in the pancreatic juice, phospholipase A$_2$ [29], is responsible for the hydrolysis of PL, mainly of endogenous origin, that arrive in loco via bile. The pancreatic lipase has an activity of 500–600 TBUs/ml in the fasted state, and of 800–1800 TBUs/ml in the fed state [41, 43, 44]. The specific mechanism of lipid absorption is still not completely clear. In particular, the mechanism by which the products of lipid digestion, in their different colloidal forms, can pass through a thin unstirred water layer (UWL) that covers enterocytes [45] and represents a barrier for lipids and hydrophobic molecules (drugs) in general is unknown. Once taken up by the enterocytes, FA and MG can be secreted in the lymphatic system after assembly into lipoproteins, or released into
the portal blood [15]. The extent of each absorption route, portal vein blood versus lymphatic transport, depends upon the type, chain length, and degree of saturation of digested lipids. Long chain FA are preferably absorbed (about 40–60%) via the intestinal lymph, as are mono- and poly-unsaturated FA when compared to saturated ones, while medium and short chain FA are mainly transported via the portal vein blood [46].

2.1.2 Absorption enhancements during lipid digestion: current hypotheses

The presence of lipid digestion products within the GI tract, from food intake or from oily drug delivery formulations, triggers several potential mechanisms that are overall responsible for the improved bioavailability observed in co-administered compounds. Although many data indicate improved availability, the biochemical bases of multiple interacting contributing factors are not well understood. As it is well summarized in previous work [15, 17, 20, 21, 33], the most likely mechanisms are:

i) Food and lipid ability to enhance drug solubility in the GI tract, due to the drastic changes during digestion, such as reduction of speed of GI content travel through the GI tract, and highly increased amounts of gastric and intestinal secretions, in particular bile and PL [47]. Food intake, especially when it is rich in fats, significantly delays gastric emptying and transit [48, 49], giving co-administered drug a longer amount of time for dissolution and delivery to intestinal absorptive sites. Intestinal mixing of lipids, their digestion products, and bile components lead to the formation of mixed micelles and vesicles [47, 50], improving the solubilization power of the GI tract contents.

ii) Alteration of both physical and biochemical barrier functions of the intestinal wall, with subsequent improvement of permeability and absorption. It has been shown
that certain lipids, lipid digestion products, several surfactants, and combinations of them as formulation components, can act as intestinal permeability enhancers [19, 51, 52]. In addition, they are reported to attenuate and even inhibit the activity of the intestinal efflux pump (P-glycoprotein) [53-56], responsible for contributing to reduction of oral bioavailability of several drugs, which are substrates for this membrane transporter.

iii) Lipid stimulation of lymphatic transport, reducing first-pass metabolism for some highly lipophilic drugs [57, 58]. One advantage in intestinal lymphatic drug transport is that drugs (or the fraction of drug dose) absorbed via this route basically avoid or undergo reduced first-pass metabolism (associated with passage through the liver). This can lead to a significant enhancement of bioavailability, especially for drugs eventually highly metabolized when passing through the liver. Relationships between lipids, lipid-based formulation and lymphatic transport have been well reviewed [46, 59, 60].

2.1.3 Role of lipid-based colloidal particles in oral absorption

The presence of lipids and their digestion products alter greatly the intestinal lumen composition, which consequently influences compounds permeability, dissolution kinetics and solubility in the GI fluids (Figure 2). Recently, Sugano [61] well summarized the lipid effects by interactions with micelles on oral absorption based on the categories of permeability, dissolution rate, and solubility absorption. In particular, the interactions between compounds and bile micelles in fed state were proposed as the theoretical base for prediction of the lipid effects on co-administered compounds, supporting then the central role of micelles in the overall oral absorption.
For hydrophobic molecules with low water solubility, dissolution is considered the rate-limiting step of their overall absorption into the body. For example, the effectiveness of a tablet in releasing its drug for systemic absorption depends on the rate of disintegration and disaggregation of the dosage forms. However, the dissolution rate of solid drugs is generally of more importance. In particular, dissolution is the rate-controlling step in the absorption of drugs with low solubility because it is often the slowest of the several processes involved in release of the drug from its dosage form and passage into systemic circulation. Therefore, the primary role of ingested lipids and their lipolytic products is to impact the drug dissolution step by forming – with bile components – different colloidal particles, which are able to maintain a larger quantity of hydrophobic drugs in solution via micellar solubilization [62, 63].

However, micellar solubilization can also impact compounds permeability, sometimes in a negative sense. When the epithelial membrane permeability is the limiting step of oral absorption, a decrease in compound absorption is indeed expected. Since the bile micelle concentration is higher in the fed state (see Section 2.2.1 and Tables 1-2), the free drug concentration at the epithelial membrane surface is smaller in the fed state. Several studies documented a reduction of permeability by bile micelle binding [64-68], suggesting that this could be the main reason for a negative lipid effect. However, if the compound is an efflux transporter substrate, the negative food effect could be more enhanced since the reduction of the free drug fraction could eliminate a saturation of efflux transport. Compounds showing this behavior tend to be hydrophilic, although some hydrophilic bases can bind to bile micelles [69], since micelles are amphiphilic and negatively charged.
Figure 2: Enzymatic hydrolysis of ingested lipids and its interactions with all the processes involved in oral absorption of compounds.
2.2 In vivo studies on properties of gastrointestinal fluids

Since several physicochemical and physiological properties of the GI tract, such as pH, buffer capacity, osmolality [70], and BS and PL concentrations and states of aggregation, are connected to drug dissolution and absorption, it is important to have good knowledge of the usual values for these properties and of how they change over time after lipid dosing. This is required in order to set up meaningful in vitro experiments for dissolution tests and digestion. There have been several studies designed to determine GI content in fasted and fed states, and a summary is presented in the following sections. However, it is very difficult to compare and to interpret these data because experimental protocols differ widely across experiments. Therefore, the meal content, the measurement method and time, and the age of subjects involved [71] also have to be taken into consideration. In general, data for the fasted state demonstrate better agreement between studies than those for fed state. Even in the fasted state, however, small differences in protocols might lead to discrepancies among studies. For example, as noted by Kalantzi et al. [72], the volume of water administered to subjects before an experiment might highly impact the GI fluid composition. Therefore, this methodological factor (volume of water) has to be taken into account when comparing different studies.

2.2.1. Intestinal Contents in Fasted and Fed States

Tables 1 and 2 summarize data for intestine in fasted and fed conditions, respectively [73]. In this case, two different parts of the upper small intestine have been studied, duodenum (D) and jejunum (J), and they are indicated near the references in both tables. In the fasted state, reported pH values show some variation between 6.1 and 7.3, as well as in the fed state (5.0–6.6). As in the stomach, this variability could be due to
different protocols used. However, all data sets are in agreement with respect to pattern over time: after food arrival, the pH decreases more slowly than in the stomach, probably due to the natural bicarbonate buffer [74]. The high buffer capacity value in the fasting state (median 67 mmol/L DpH) is also correlated to the bicarbonate system; it decreases in the fed state as the digestion proceeds. In the fasted condition, osmolality values in the intestine are highly variable (178 and 273 mOsm/kg) compared to the analogous state in the stomach (140 and 191 mOsm/kg), while in the fed state contents are hypertonic most of the time (400–278 mOsm/kg). Surface tension is lower in the fasting state (median 32.3 mN/m) compared to the stomach, due to active surface agents such as bile and enzymes, but it remains around the same value (28–29 mN/m) over time in the fed state.
Table 1: Intestine contents in fasted state (*in vivo* measurements).

<table>
<thead>
<tr>
<th>Fasted intestine</th>
<th>PH</th>
<th>Osmolality mOsm/Kg</th>
<th>Total bile acids mM</th>
<th>Surface tension mN/m</th>
<th>Buffer capacity mmol/L DpH</th>
<th>Na⁺ mM</th>
<th>K⁺ mM</th>
<th>Cl⁻ mM</th>
<th>Ca²⁺ mM</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± s.e.</td>
<td>5.6</td>
<td>178±30</td>
<td>2.6±0.2</td>
<td>32.3</td>
<td>68±29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[72]</td>
</tr>
<tr>
<td>Median</td>
<td>6.2</td>
<td>178</td>
<td>2.6</td>
<td></td>
<td>67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Interval (5.95-6.72)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(19-122)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>7.1± 0.60</td>
<td>271±15</td>
<td>2.9± 2.9</td>
<td></td>
<td>142+13</td>
<td>5.4±2.1</td>
<td>126±19</td>
<td>0.5±0.3</td>
<td>[75]</td>
<td>J</td>
</tr>
<tr>
<td>Median</td>
<td>7.2</td>
<td>273</td>
<td>2.1</td>
<td></td>
<td>145</td>
<td>5.2</td>
<td>126</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval (5.3-8.1)</td>
<td></td>
<td>(218-292)</td>
<td>(0.1-13.3)</td>
<td></td>
<td>(111-165)</td>
<td>(1.7-11.6)</td>
<td>(92-181)</td>
<td>(0.1-1.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>6.7± 0.9</td>
<td>278± 16</td>
<td>1.52± 1.77</td>
<td>33.7± 2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[76]</td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>J</td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>6.1± 1.2</td>
<td>272± 24</td>
<td>2.00± 1.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[77]</td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>4.4± 1.8</td>
<td>1h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>J</td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>6.4± 1.3</td>
<td>before MMC^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[78]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.3± 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>J</td>
</tr>
<tr>
<td>Mean ± s.e.</td>
<td>5.0^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[80]</td>
</tr>
<tr>
<td>Median</td>
<td>6.1</td>
<td>5.8-6.5 (IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval</td>
<td></td>
<td>(0.1-14.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Data are reported as mean with standard error (a: s.e.), median, and intervals when available. Abbreviations: D, duodenum; J, jejunum.

a: MMC, migrating motor complex. b: Data extracted from graphics where error bars were not shown.
Table 2: Intestine contents in fed state (*in vivo* measurements).

<table>
<thead>
<tr>
<th>Fed intestine</th>
<th>PH</th>
<th>Osmolality mOsm/Kg</th>
<th>Total bile acids mM</th>
<th>Surface tension mN/m</th>
<th>Buffer capacity mmol/L DpH</th>
<th>Phospholipids mM</th>
<th>Na⁺ mM</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>6.6 to 5.2 in 3h 30 min.</td>
<td>400-287 variable</td>
<td>11.2 to 5.2 in 3h</td>
<td>28-29 constant in time</td>
<td>No trends, 18-30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median: 6.3</td>
<td>6.0-6.7 (IQR) (random fluctuations)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Range 5.0-7.0 in 4 h</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>10.1± 4.2</td>
<td>1h</td>
<td>6.3± 1.0</td>
<td>1h</td>
<td></td>
<td></td>
<td></td>
<td>[72] D</td>
</tr>
<tr>
<td></td>
<td>13.4± 4.3</td>
<td>2h</td>
<td>5.6± 0.5</td>
<td>2h</td>
<td></td>
<td></td>
<td></td>
<td>[81] D</td>
</tr>
<tr>
<td></td>
<td>11.8± 2.4</td>
<td>3h</td>
<td>4.3± 0.8</td>
<td>3h</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>6.7± 2.1</td>
<td>4h</td>
<td>2.5± 0.4</td>
<td>4h</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>16.2± 1.5</td>
<td>0-0.5h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[82] J</td>
</tr>
<tr>
<td></td>
<td>9.7± 1.0</td>
<td>0.5-1h</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>6.5± 0.9</td>
<td>1-1.5h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.8± 5.6</td>
<td>1h</td>
<td></td>
<td></td>
<td>105±15 LCTG</td>
<td></td>
<td></td>
<td>[78] J</td>
</tr>
<tr>
<td>LCTG</td>
<td>6.8± 1.7</td>
<td>1h</td>
<td></td>
<td></td>
<td>101±17 MCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.8± 5.6</td>
<td>1-2h</td>
<td></td>
<td></td>
<td>4.8± 1.8 1-2h</td>
<td></td>
<td></td>
<td>[35] D</td>
</tr>
<tr>
<td>MCTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.5± 9.64</td>
<td>0.5h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[83] D</td>
</tr>
<tr>
<td></td>
<td>5.2± 2.3</td>
<td>1h</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>15.0°</td>
<td>0.5 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[80] J</td>
</tr>
<tr>
<td></td>
<td>9.0°</td>
<td>1h</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

All data are presented as mean ± s.e., unless otherwise indicated.
Abbreviations: D, duodenum; J, jejunum; LCTG, long chain triglycerides; MCTG, medium chain triglycerides.

a: Data extracted from graphic where error bars were not shown.
Mean BS concentrations in fasted state range from 2.6 to 6.4 mM [43, 72, 79] in the duodenum, and from 1.52 to 5.0 mM in the jejunum [75-78, 80]. However, extreme lower and higher values, down to 0.1 mM and up to 13–14 mM, have been indeed reported for both intestinal tract regions [75, 81]. As expected, postprandial levels are higher, within a range of 10.1–16.2 mM [35, 43, 72, 78, 80, 82, 83], and they decrease over time. In studies where individual BS levels are provided, the individual variability is very high [35, 83]. PL values in the fed state are around 5–6 mM [35, 43], and they decrease over time.

2.2.2 Characterization of lipid-based colloidal particles in intestinal fluids

Bile components (BS and PL) and digestion products have also been extensively investigated in light of their ability to self-assemble into different kinds of colloidal particles, which thus have been detected in the aqueous phase of human intestinal fluids. Briefly, the first study [84] to isolate and describe phases of intestinal fluids after the ingestion of fats showed an aqueous micellar phase rich in FA and MG, with a smaller amounts of DG and TG, and an oil phase containing more DG and TG. Following studies demonstrated that this description was an oversimplification of phases generated during the digestion of fats. Porter and Saunders [85] showed a clear micellar phase was not observed, but rather a slightly turbid solution, and the presence of a third solid phase (observed upon centrifugation), called pellet, formed by precipitation of FA neutralized by ionic interactions with calcium ions. Further studies [86] on the aqueous micellar phase of intestinal postprandial fluids isolated small particles with a radius of 2.3–3.5 nm, identified as mixed micelles, formed of BS, PL, cholesterol, and FA. The most recent study analyzing human duodenal contents after a triacylglycerols-rich meal [35], confirmed the presence of three phases composed of a floating oil layer, a turbid aqueous phase, and a precipitated
pellet after ultracentrifugation of samples. Further, it showed the simultaneous presence of unilamellar vesicles with a mean hydrodynamic radius (Rh) between 20 and 60 nm, along with mixed micelles (Rh less than 4 nm) in the aqueous phase. These particles were composed of BS, PL, cholesterol, and products of lipolysis, such as FA, DG, and MG. However, a further and clean separation of micelles from vesicles as two ‘sub-phases’ of ex vivo duodenal fluid was not possible. On the contrary, the same researchers achieved the mentioned separation when they studied model systems of pure lipids, simulating in vitro the aqueous intestinal contents [87].

2.3 In vitro models simulating gastrointestinal fluids

The use of in vitro dissolution testing has two main functions: quality control and indication of the in vivo dissolution profile of drugs. The common standard pharmacopoeia media used for quality control - distilled water and aqueous buffer solutions - have limited ability to predict the dissolution profile of drugs. These simple media have few similarities with the GI environment, yet it is well established that medium composition can greatly influence drug dissolution profiles. Therefore, it is a crucial point to assess media reflecting physiological conditions, and a number of media have been proposed to date to simulate better the real conditions in the GI tract. The consideration of co-administrated food and the formulation effects on drugs dissolution complicate the situation. An accurate assessment of biorelevant dissolution models is also complex because of the high variability and dynamicity of the GI tract in postprandial conditions. In vitro lipid digestions have been performed primarily to understand better the capacity of lipid compounds, from food and/or from formulations, to maintain a co-administered drug in solution, and therefore the overall improved absorption. In order to get useful and correct information from a lipid
digestion model, the medium in which the reactions take place has to be biorelevant and match the physiological conditions of the GI tract.

2.3.1 Biorelevant media for drug dissolution testing

A simple medium representing the upper small intestine conditions is the Simulated Intestinal Fluid (SIF) without pancreatin proposed by USP [88]. Modifications by researchers over the years have included changes in pH value and composition [76, 77, 89, 90] in light of new findings from in vivo experiments. Therefore, the further dissolution media proposed have paid particular attention to osmolality, surface tension, buffer capacity, and ion contents, to better match in vivo values. All of these physicochemical properties are considered to influence the dissolution and consequently the overall absorption of drugs in the GI tract [70]. Furthermore, several studies have compared the performance of these media with respect to dissolution [27, 76, 77, 89-92] and wetting ability [93], obtaining results highly dependent on the nature of drugs tested (ionizable or not) and on media composition. This result variability shows that a proper modulation of media properties is crucial in order to evaluate correctly the dissolution of drugs tested.

Generally, in these media, the human bile acids have been modeled using mainly a single salt component, whereas few studies have used a mixture of BS to better reproduce human bile [94, 95]. The most common considered are sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC), and sodium glycocholate (NaGC) for BS and lecithin for PL. This approach might seem simplistic considering the complex composition of the human bile, and the possible effect of the nature of BS chosen on dissolution results. Correlations between solubilization of drugs and BS type have been well reviewed by Wiedmann and Kamel [96]. Media simulating the postprandial state have shown a wide variability in pH values (5.0–7.5), which only partially
reflects the physiological range (5.0–6.6) obtained from \textit{in vivo} measurements. It can be noted that media for dissolution tests commonly in use are based on non-physiological or partially physiological buffer systems, such as phosphate buffer, acetate buffer, and maleate buffer, which are not naturally produced in the GI tract. The main physiological buffer system present in the gastrointestinal lumen is bicarbonate buffer secreted by gastric mucosa, pancreas and epithelial cells of the duodenum [97]. As some studies have previously demonstrated [13, 15-17], dissolution rates of ionizable drugs can be strongly affected by not only the pH value of dissolution media, but also by type and concentration of their buffer systems, even when these buffers are used at physiological pH value. In media simulating the intestine postprandial state, mixtures of TG, DG, MG, and FA have been generally added to represent the products of partial lipid digestion that arrive from stomach. However, for intestine in fed state, simulating the continuous evolution of the content composition and properties over time is a challenge for researchers. Also in this case, different media, each corresponding to a certain time interval during digestion of the food, have been prepared [93, 98].

2.3.2 \textit{In vitro} lipolysis models

The overall performance of ingested lipids on drugs is correlated to the morphology of the intermediate phases produced during lipid digestion [99, 100]. However, traditional dissolution testing models do not generally take into account the complex drug interactions and trafficking issues related to the parallel digestion of lipids and dynamic formation of colloidal structures while lipolysis proceeds. Therefore, \textit{in vitro} lipolysis models simulating digestion in the small intestine have been proposed as useful tools for understanding the influence of lipid digestion products on drug dissolution, partitioning into colloidal phases, and ultimately
absorption over time during the lipid digestion process. *In vitro* lipolysis models consist of a biorelevant medium, similar to those used in dissolution testing for intestine, in which lipase/collipase enzymes and a lipid substrate – simple triglycerides or more complex formulation – are added. Compositions of some recent *in vitro* lipolysis models are outlined in Table 3. They differ mainly in pH value, concentration and type of BS and PL chosen to model the physiological conditions during the digestion process. The pH values show a high variability ranging from 5.8 to 8.5, which is partially in agreement with the range (5.0–6.6) reported in Table 2 for the intestinal fed state from *in vivo* studies. Higher pH values (7.5) than the physiological fed state range are often chosen in order to make the *in vitro* lipolysis more efficient [101-106], since pancreatic lipase has been shown to have the highest relative activity when tested *in vitro* around pH 8 [107, 108]. Except for a very few exceptions [41, 109], the most used buffer system is maleate buffer, although it is not completely physiological, as previous noted for buffer systems employed in dissolution media. NaTC and NATDC are commonly chosen to model human bile acids, while lecithin is added to represent the PL content of human bile. However, some authors preferred to use directly bile extract as the source of BS rather than only one salt among its components to better reproduce the human bile complexity [109-114].
Table 3: *In vitro* lipid digestion models.

<table>
<thead>
<tr>
<th>PH</th>
<th>Buffer</th>
<th>Lipid components (mM)</th>
<th>Bile salts (type and quantity in mM)</th>
<th>Phospholipids (type and quantity in mM)</th>
<th>Ratio BS/PL</th>
<th>Lipase</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>0-30 mM/ 200 mM NaCl V = 5 ml</td>
<td>Emulsion: 10% w oil 1.2% lecithin 2.25% glycerol</td>
<td>NaTDC 30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>50 mM tris-maleate/5 mM CaCl₂*2H₂O/150 mM NaCl V = 100 ml</td>
<td>2.4 g TG</td>
<td>NaTC 8</td>
<td>Lecithin 1.5</td>
<td>5.3:1</td>
<td>2-1000 TBUs/ml from pancreatin (8 TBUs per mg dry powder)</td>
<td>[33]</td>
</tr>
<tr>
<td>7.5</td>
<td>50 mM tris-maleate/5 mM CaCl₂*2H₂O/150 mM NaCl V = 10 ml</td>
<td>250 mg TG or emulsion</td>
<td>NaTDC 20</td>
<td>Lecithin 5</td>
<td>4:1</td>
<td>10000 TBUs/ml from pancreatin (8*USP specification, Sigma)</td>
<td>[101-106]</td>
</tr>
<tr>
<td>5.8</td>
<td>Maleic acid 55.02 mM/NaOH 81.65 mM/NaCl 125.5 mM V = 1 l</td>
<td>Glyceryl monooleate 5, Sodium oleate 0.8 Olive oil 1.5% v/v</td>
<td>NaTC 10</td>
<td>Lecithin 2</td>
<td>5:1</td>
<td>10000 U/ml (20 ml in 1 l), from pancreatin (8*USP specification, Sigma)</td>
<td>[98]</td>
</tr>
<tr>
<td>6.8</td>
<td>2 mM trizma maleate/150 mM NaCl, CaCl₂ a)4 mM b)12 mM c)20 mM V = 15 ml</td>
<td>TG 15.5</td>
<td>Bile extract</td>
<td>Phosphatidylcholine 1 a) 5:1 b) 4:1 c) 6.6</td>
<td>800 USP units/ml from pancreatin (Sigma)</td>
<td></td>
<td>a) [111], b) [112], c) [113]</td>
</tr>
<tr>
<td>5.8</td>
<td>2 mM trizma maleate/150 mM NaCl V = 300 ml</td>
<td>3 g SNEDDS&lt;sup&gt;b&lt;/sup&gt; formulation</td>
<td>a) Bile extract 5 a) Phosphatidylcholine 1 a) 5:1 b) Lecithin 5 (94%) b) 4:1 c) 6.6</td>
<td>800 USP units/ml (100 ml in 300 ml)</td>
<td>From pancreatin (Sigma)</td>
<td>b) [110]</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BS, bile salts; PL, phospholipids; TG, triglycerides; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate.

a: TBUs, Tributyrin Units to express the activity of pancreatic lipase, where 1 TBU is the amount of enzyme that can liberate 1 µmole of FA per minute from a saturating amount of tributyrin at pH 8.5 and 25 °C in the presence of 5 mM CaCl₂ and 150 mM NaCl [33].

b: SNEDDS, defined in ref. 84,131.
Despite these differences in composition, *in vitro* lipolysis models follow a common experimental procedure, here briefly summarized. After the addition of the lipid substrate and lipase/co-lipase enzymes, the extent of lipid digestion is monitored indirectly by recording the volume of NaOH added during the experiment to titrate the FA formed from the TG hydrolysis. The number of OH− ions present in an added volume can be equated with the FA liberation during lipolysis. To take into account the additional FA produced by digestion of TG and DG contained in the lecithin, digestions of blank simulated intestinal media are performed. Blank experiments are performed in the same manner, except that lipid substrate is not added to the reaction solution. The collection of samples at specific time intervals during blank and lipid digestion experiments and their analysis can provide information on the dynamic nature of colloidal particles produced by BS/PL/lipid interactions. Another version of the *in vitro* lipolysis model [110-114], called *in vitro* dynamic lipid lipolysis, allows controlling the rate of lipolysis by continuous addition of Ca$^{2+}$ ions. Studies using this type of model have shown that the concentrations of BS and Ca$^{2+}$ ions, as well as the lipase activity, can influence the rate of hydrolysis of lipids.

### 2.3.3 Characterization of complex ordered structures in simulated GI fluids

A wide range of biophysical methods have added employed to increase understanding of the mechanisms by which lipid digestion affects drug bioavailability by investigating different features (chemical composition, morphology of intermediate colloidal phases, liquid crystal phases, drug partitioning) that characterize complex lipolysis systems. *In vitro* studies concerning complex ordered structures associated with lipid digestion can be divided in two groups, namely: i) characterization performed at
equilibrium; ii) kinetics investigations associated with an ongoing lipolysis reaction.

2.3.3.1 Equilibrated lipolysis systems

Many in vitro studies have focused on investigating dimension, shape, composition, and mechanisms of formation of mixed micelles and vesicles, as well as the structure of other crystalline phases formed by interactions between bile components and fat digestion products. In general, these systems were solutions composed of one or a few BS, PL, sometimes cholesterol, and lipids in water or buffers, and analyzed after equilibrium was reached. Analysis at equilibrium could be a limiting factor in enabling simulation of the processes occurring during fat digestion. Studies [115, 116] on lecithin/BS water systems of different relative ratios detected the presence of paracrystalline phases (lamellar, hexagonal and cubic), in addition to a micellar phase, by means of polarized microscopy and small and wide-angle X-ray diffraction. Liquid crystalline phases (lamellar and cubic) were also detected in systems containing combinations of different FA and MG in simulated intestinal fluid [100]. Based on laser light scattering analysis of mixed lipid/BS model systems and duodenal fluid contents [117-119], Carey et al. [29] proposed that mixed micelles incorporating lipids might have a large disc-like form with Rh about 20 nm, and that they co-exist with liquid unilamellar crystalline vesicles having Rh of 40–60 nm. However, investigations of in vitro systems simulating the aqueous phase of upper intestine by small-angle neutron scattering (SANS) indicated a more complex picture of particle structure and mechanisms of formation, both of which were sensitive to type of BS, total lipid concentration, and BS/PL molar ratio [120]. In the isotropic aqueous phase, globular mixed micelles were formed at the highest total lipid concentrations; then, the micelles elongated to form long
rods when solutions were diluted. Further dilution triggered a transition to vesicles, which could co-exist with mixed micelles in appropriate dilution ranges, and could become smaller as the dilution continued.

2.3.3.2 Dynamic in vitro lipolysis models

Several techniques have been coupled with the in vitro lipolysis models to analyze the nature and the state of aggregation of lipid digestion products, and then their influence on drug dissolution. High performance thin layer chromatography (HPTLC) [101, 102] provided quantification of medium and long-chain TG and their products of lipolysis, gaining insight into their different digestion kinetics. Small angle X-ray scattering (SAXS) [111] showed the presence of lamellar and hexagonal phases, whose relative amounts dynamically changed as the lipolysis proceeded. Qualitative morphological observations of unilamellar and multilamellar vesicles, along with mixed micelles and oil drops, were achieved by means of cryogenic transmission electron microscopy (Cryo-TEM) [110, 114] for both fasted and fed conditions. However, in experiments simulating the fed state [110], the complex structured vesicles (multilamellar) detected have not been observed in analysis of ex vivo postprandial intestinal fluids, which instead included unilamellar vesicles [35].

The use of paramagnetic resonance spectroscopy (EPR) [109] was able to monitor in real time the amount of a model drug that distributed between water, oil and mixed micelles during in vitro lipolysis. This study assumed the presence only of mixed micelles in the aqueous phase, and didn't mention phases other than oil, water, and pellets, although the study simulated a fed state (10 mM of bile extract) and therefore other colloidal particles (vesicles) were likely present in such conditions. This might be
relevant considering the potential role of each colloidal phase observed to date on dissolution and absorption processes has not been studied extensively and therefore has not been completely clarified.

2.4 Models for drug absorption studies

Drug molecules have to pass several physical and biochemical barriers in the GI tract before they reach the blood stream. Compounds permeate the intestinal membrane not only by passive diffusion but also by multiple and parallel processes (Figure 3, [121]). Passive diffusion can occur between the epithelial cells through water-filled pores in the tight junctional complex (paracellular diffusion) or across the lipid membrane (transcellular diffusion). Furthermore, the transcellular route can also take place via carrier-mediated processes by the use of transporter proteins, favouring influx into or efflux out of the epithelial cell (active uptake and secretion).

Several absorption models are currently used in academia and industry to assess permeability for drugs and drug candidates. Although the effort in this field, there is no model system for permeability studies that is able to perfectly reproduce all these barrier functions. The most commonly used are membrane-based, cell culture-based, and tissue-based (ex vivo and in situ) models. How these models are employed in studying different absorption routes is summarized in Figure 3 [121].

2.4.1 Cell culture-based models

The most commonly used and best-established cell cultured-based model is based on Caco-2 cells, deriving from a human colon carcinoma [122]. Other cell lines have been used for specific purposes [121], for example Madin-Darby canine kidney (MDCK) cells [123, 124] and LLC-PK1 (from pig kidney epithelial cells) [125].
Mechanisms of absorption and representative technique

Figure 3: Schematic the different mechanisms involved in transport across the intestinal membrane and examples of methods that can be used for evaluation of the specific mechanisms. Insert shows the pathways for a compound when crossing the epithelium.

Caco-2 cells are suitable for mechanistic studies, since they enable study of different transport mechanisms, including passive transcellular and paracellular diffusion, active uptake and efflux, and metabolism. Several studies employed Caco-2 model to investigate the mechanisms of drug-drug, drug-food, and drug-excipient interactions [126]. During the differentiation process, Caco-2 cells form monolayers with tight junctions at the lateral interfaces, which have of most the morphological, structural and functional characteristics of the intestinal mucosa. The cells also express various enzymes, and several efflux carriers. Although similarities with in vivo epithelial monolayers, some essential differences should be taken into account while evaluating drug transport with this model. Caco-2 model might underestimate para-cellular transport.
because of smaller tight junctions in cells derived from colon carcinoma [127]. Several studies reported altered levels of enzymes and transporters when compared with both human intestinal cells and Caco-2 clones from different laboratories [128]. In order to minimize interlaboratory variability, standardization of cell culture procedures and protocols has been proposed, as well as a set of internal reference compounds acting as controls [129].

2.4.2 Bio-relevance of Caco-2 model

As a result of intestinal fluids complexity, drug permeability does not depend only on drug properties and the barrier function, but also on the medium present at both sides of the barrier. Therefore, the bio-relevance of media used in the experiments might affect the prediction of permeability values. Particular attention has been paid to pH values, media simulating fed-state conditions, and drug concentration in the apical side.

Standard pH (7.4) of apical side media is higher than reported intestinal pH values; therefore it can affect the solubilizing capacity of micelles, the ionization and then partitioning of drugs with pKa ~7, and the activity of pH-dependent carriers.

The simulation of fed-state conditions is still under studies for Caco-2 model. Some media including bile salts and phospholipids were tested and found to be compatible with Caco-2 cells [130]. Lipolytic products could be also included in order to investigate the effect of lipids on drug permeation across the intestinal barrier.

The drug concentration in the lumen is the driving force not only for passive transport, but also for some active transport pathways. However, because of lack of knowledge of the real drug concentration at absorptive sites, permeability studies often used drug concentration estimations based on compound’s solubility and cytotoxicity,
which might bias data interpretations. A recent study showed that integrating biorelevant conditions with Caco-2 monolayers was critical in determining the relative extent of absorption mechanisms of the poorly water-soluble drug amprenavir [131]. An interesting approach, proposed by Ginski and Polli, combined the use of dissolution tests with Caco-2 system [132]. Integrated dissolution/Caco-2 systems might be particularly useful in absorption studies of hydrophobic drug molecules, for which indeed the slow dissolution process often limits the amount of drug available for absorption in aqueous-based intestinal fluids.

2.4.3 Limitations and improvements of Caco-2 model: HT29-MTX cells

Although there are many similarities of Caco-2 model with *in vivo* intestinal epithelium, several physiological differences in drug transport should be evaluated. The Caco-2 model, derived from epithelial colon carcinoma, contains tight junctions that may reduce permeability in drug absorption as well as underestimate para-cellular transports [127]. Also, compared to *in vivo* situations, the model lacks nervous system regulation, systemic blood flow and the motility found in the intestine. Furthermore, the Caco-2 model is solely compromised of absorptive enterocyte cells, while the complex intestinal epithelial tissue is composed of several kinds of cells, including mucus-secreting goblet cells [133]. As the second most frequent cell type in the intestinal epithelium, goblet cells have been shown to act as an additional barrier to drug absorption [134]. Thus, drug permeability and absorption can be affected by the mucus layer and must be taken into account when performing absorption studies.

The mucus-producing goblet cell sub-line HT29-MTX, isolated from human intestinal HT29 cells, can be co-cultured with the absorptive Caco-2 cells *in vitro* [135,
136], giving monolayers with tight junctions [137]. The mucus-secreting HT29-MTX are able to mimic physiological conditions by covering the Caco-2 cell surface with an additional layer of mucus and they can also modulate the geometry of the tight junction [138].

2.5 Mathematical models

Mathematical modeling and predictability of oral drug delivery is a field of steadily increasing academic and industrial importance with an enormous future potential. One of the major driving forces for the use of mathematical modeling in drug delivery is to save time and to reduce costs in developing a new and/or in optimizing an existing drug product. In addition, the quantitative analysis of the physical, chemical and potentially biological phenomena, which are involved in the control of drug release, offers the fundamental advantage of underlying mechanisms. This knowledge is not only of academic interest, but a pre-requisite for an efficient improvement of the safety of new pharmaco-treatments [139].

The beginning of the quantitative treatment of drug release from pharmaceutical dosage forms can be dated in the early sixties, when Professor Takeru Higuchi published his famous equation allowing for a surprisingly simple description of drug release from an ointment base exhibiting a considerable initial excess of non-dissolved drug within an inert matrix with film geometry [140, 141]. Numerous models have been proposed since then, including empirical/semi-empirical as well as mechanistic realistic ones. In the first case, the mathematical treatment is (at least partially) purely descriptive and not based on real physical, chemical and/or biological phenomena. Consequently, no or very limited insight into the underlying drug release mechanisms can be gained, and the predictive
power of empirical/semi-empirical models is often low. In contrast, mechanistic mathematical theories are based on real phenomena, such as diffusion, dissolution, swelling, precipitation and/or degradation [142-144]. This type of models allows for the determination of system-specific parameters that can offer deeper insight into the underlying drug release mechanisms.

Mathematical based-system models have shown considerable promise with respect to predicting bioavailability of orally delivered compounds. Commercial examples of such models include Simulations Plus, Inc.’s GastroPlus™ software and Intellipharm® Software, which are widely used in the pharmaceutical industry for predicting oral bioavailability and pharmacokinetic profiles. These soft wares utilize mass transport expressions to predict absorption of a compound in the GI tract over time after dosing. However, they generally do not consider the influence of agents that interact with drug molecules, such as complexing agents or lipids. This might be related to lack of mechanistic and quantitative understanding of the function of lipid based systems in the GI tract, leading to simplified consideration of the food effect as resulting from enhancement in equilibrium solubility in fed state intestinal contents [24]. Models should also consider how physical and chemical system properties relate to function, in order to gain a general rather than compound-specific understanding.

In the following sections, an overview of mathematical models related to all the processes involved in oral absorption is presented and discussed in light of their feasibility in post-prandial intestinal conditions.
2.5.1 Lipase kinetics

Lipases, or triacylglycerol acyl ester hydrolyses, are enzymes possessing an intrinsic capacity to catalyze cleavage of carboxyl ester bonds in TG, DG and MG, which are the major constituents of animal, plant, and microbial fats and oils [145]. As a result of this type of reaction, carboxylic acids (FA) and alcohols with a lower number of ester bonds (and eventually glycerol) are released. Because most fats and oils of natural occurrence are TG of long chain (or fatty) acid residues, lipases have traditionally been termed long-chain fatty acid ester hydrolyses, or alternatively esterases capable of hydrolyzing esters of oleic acid [146].

In the fifties, the pioneering work of Sarda and Desnuelle permitted the establishment of a functional criterion for distinction between lipases and esterases, namely their ability to be or not to be activated by oil/water interfaces, respectively [145]. Due to the opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipase reactions occur at the interface between the aqueous and the oil phase [147]. Interfacial activation of lipases is characterized by a sharp increase in lipolytic activity once the substrate solubility is exceeded [148, 149], and for example when the substrate starts to form an independent phase that is often dispersed as an emulsion [146] and micelles. Desnuelle et al. [150] performed quantitatively studies of interfacial activation of lipases reporting that the rates of lipase-catalyzed reactions were positively correlated with the area of available interfacial surface. Therefore, the lipase activity is independent of the total substrate molar concentration, but controlled by the concentration of substrates at interface. In attempts to model enzyme kinetics of lipases, the Michaëlis–Menten mechanism has been recurrently used, and it’s common to read
reports about $K_m$ and $K_{cal}$. However, one of Michaelis-Menten model underlying assumptions is that the enzymatic reaction must take place in an isotropic medium, i.e. both the enzyme and the substrate must be a part of the same phase. Hence, this mechanism cannot be used in its original form to model the action of lipolytic enzymes acting at the interface between a water phase and a (insoluble) lipid phase [146] and [151]. Therefore, a modified model has been proposed elsewhere [151, 152], which consists of two steps: i) the physical adsorption of lipase at the water lipid interface, which leads to activation of the lipase and hence to opening of the lid that would otherwise block the active site; and ii) the formation of the enzyme/substrate complex, which will eventually be hydrolyzed to give the product and regenerate the adsorbed enzyme (Figure 4, [145]). This second step may be described by a pseudo Michäelis–Menten mechanism occurring on an interface, rather than in a bulk level, with kinetics constants having dimension of moles per unit area. Using this approach, many kinetic experiments have been performed on the hydrolysis of medium chain TG, generating water-soluble products. Verger-De Haas model [153] is the simplest adaptation of the Michaelis-Menten kinetic model for the interfacial hydrolysis of short- and medium chain lipids, and it is described by equation (1) reported in Figure 4.
However, the natural substrates for lipolytic enzymes are long-chain lipids, generating then water-insoluble products when hydrolyzed. Therefore, kinetic models have to take into account the processes involved in the interfacial molecular reorganization and segregation of the insoluble products, which affect the interfacial quality of the interface. \textit{In vivo}, the removal of the lipolysis products from the interface can occur by their complexation and solubilization into the aqueous sub-phase: micellar solubilization of free FA and MG is carried out by bile components in the GI tract while serum albumin binds FA in the blood.

Li et al. [8] has recently proposed a different approach in the modeling of lipid digestion profiles, which is based on the net hydrolysis of TG into free FA rather than considering all the intermediate steps involved in the enzymatic reaction. According to

\begin{equation}
V_b = \frac{k_{cat}(E^*S)}{S + K_m^*} \cdot \frac{S}{V} \frac{I}{k_p S + K_m}
\end{equation}
this model, the number of free FA leaving the oil droplets per unit time \( \frac{dm_d}{dt} \) is given by:

\[
\frac{dm_d}{dt} = -kS_dV
\]

where: \( k \) = rate constant (moles free FA per unit area)

\( S_d \) = oil droplet surface area per emulsion volume

\( V \) = total bulk volume (ml).

Regardless the approach followed to express lipase kinetics, it is recognized that lipolysis is a classic example of heterogeneous bio-catalysis. Hence, the reaction rate and direction are controlled by the overall composition at the interface microenvironment. Lipolytic reactions are therefore strongly dependent on the interfacial activity, kinetics of diffusion and partitioning of substrates and products to and from the active site. However, there is a lack of understanding of all the interfacial properties of lipase in water/oil systems. It’s difficult to discriminate between the adsorption behavior of lipases and that of their products of reaction [147], or to fully describe and model all the inhibition effects on the enzyme activity.

**2.5.2 Drug dissolution and partition kinetics in presence of colloids**

When a tablet or other solid drug form reaches the gastrointestinal tract, the drug begins to pass in solution from the solid matrix, which also disintegrates into granules and then fine particles. Disintegration and dissolution might occur simultaneously with the release of a drug from its delivery form (Figure 1). The effectiveness of a tablet in releasing its drug for systemic absorption depends on the rate of disintegration and disaggregation of the dosage forms. However, the dissolution rate of solid drugs is generally of more importance. In particular, dissolution is the rate-controlling step in the
absorption of drugs with low solubility because it is often the slowest of the several processes involved in release of the drug from its dosage form and passage into systemic circulation.

Several theories have been used to build mathematical models to describe drug dissolution from solid dosage forms. Since dissolution is a kinetic process, the rate of dissolution reflects the amount of drug dissolved over a given time period. In some cases, an analytical mathematical solution can be exactly derived that describe the dissolution time dependency. In other cases, an analytical solution cannot be derived and an empirical equation is used. In 1897, Novey and Whitney proposed the quantitative analysis that correlated the rate at which a solid drug dissolved in a solvent. The equation has been elaborated subsequently by other workers, but remains based on a diffusion layer model of dissolution of drug from a particle into a large excess bulk medium (Figure 5). The current version of the Noyes-Whitney equation describing the dissolution rate of a solid compound dC_\text{b}/dt (mass/volume time) in bulk can be written as:

\[
\frac{dC_b}{dt} = \frac{DS}{Vh} \left(C_s - C_b\right)
\]  

(3)

where:  

\( C_b \) = concentration of solute dissolved (mass)  

\( t \) = time (min)  

\( D \) = diffusion coefficient of the solute in solution  

\( S \) = surface area of the exposed solid  

\( V \) = volume of solution  

\( h \) = thickness of the diffusion layer  

\( C_s \) = solubility of the solid.
The derivation of equation (3) assumed that \( h \) and \( S \) were constant, but this might not be the case during a real dissolution process. The force of agitation at the surface of the dissolving particle perturbs the static diffusion layer thickness. The surface area \( S \) does not remain constant as a powder or tablet dissolves, and it is difficult to measure and model changes in \( S \) as the dissolution proceeds. However, when dissolution occurs in such a manner that the geometric shape of dosage forms remains constant, the Hixson-Crowell cube-root model can be used [154]. In case of drug powder with uniformly sized particles, the Hixson-Crowell cube-root law can be derived to express the rate of dissolution based on the cube root of the weight of the particles.

Figure 5: Dissolution of solid drug particles according to diffusion layer model. The thickness \( h \) represents a stationary layer of solvent around the particle, in which the solute molecules exist in concentration from \( C_s \) to \( C_b \). At the solid surface-diffusion layer interface, \( x = 0 \), the drug in the solid is in equilibrium with drug in the diffusion layer. The change in concentration with distance across the diffusion is constant, as shown by the straight downward-sloping line. Beyond the static diffusion layer, at \( x \) greater than \( h \), the drug is found at a uniform concentration \( C_b \) in the bulk phase because of the mixing action.
In several processes involving diffusion of a solute, colloidal particles may play an important role, such as dissolution of solid drugs in colloidal solutions. Higuchi developed a model that quantitatively describes solute mass transport throughout a barrier in the presence of colloids [155]. The barrier may represent the diffusion layer controlling the dissolution rate of solid drugs both in vitro and in vivo. Assuming the presence of a single species of free colloid, no net changes in colloid concentration, and that solute/colloid interactions occur rapidly enough to be considerate at equilibrium, the rate of diffusion of the colloidal rate \( c \) (mass/time area) across a plane \( x \) (length) inside the diffusion layer \( h \) (Figure 5) is:

\[
rate_c = D_c(x) \left( \frac{dM}{dx} \right)
\]

(4)

where: \( D_c(x) \) = colloid diffusion coefficient (area/time)

\( M \) = colloid concentration (mass/volume)

The rate of diffusion of total solute rate \( s \) (mass/time area) across the plane \( x \) is then:

\[
rate_s = D_s(x) \left( \frac{dC}{dx} \right) + D_c(x) \left( \frac{d[C_b M]}{dx} \right)
\]

(5)

where: \( D_s(x) \) = free solute diffusion coefficient

\( C \) = free solute concentration

\( C_b \) = amount of the bound solute per mole of colloid.

The first term in equation (5) is the diffusion rate of the free solute across plane \( x \), and the second term is the rate of solute transport by the colloidal particles, acting as a carrier. Cases in which there is no net transfer of the colloid itself, \( rate_c \) is equal to zero. For example, in the dissolution of a solid drug the colloid must not be a part of the tablet,
but be present in the solution phase acting only as a carrier. Furthermore, $D_s$ and $D_c$ can be considerate constant when solutions are diluted and the colloid size does not change respectively. Under these conditions, equation (5) can be generalized for the general case of a mixture of different species of colloidal particles as:

$$\text{rate}_s = D_s \left( \frac{dC}{dx} \right) + \sum_i D_{si} M_i \left( \frac{dC_{bi}}{dx} \right)$$

(6)

where: $i =$ index representing a particular species of colloid.

This equation states that the importance of a particular species in solute transport is directly proportional to its concentration, diffusion coefficient, and the gradient of its solute binding.

In non-steady state conditions, and after multiply and dividing both sides of equation (6) by the surface area $S$ of dissolving particles and the volume of solution $V$, we have:

$$\frac{\partial C}{\partial t} = \frac{\partial \text{mass}}{\partial t} \frac{1}{V} = \frac{SD_s}{V} \left( \frac{\partial C}{\partial x} \right) + \sum_i \frac{SD_{si} M_i}{V} \left( \frac{\partial C_{bi}}{\partial x} \right)$$

(7)

The release of drugs by dissolution in the presence of lipid-based colloids (bile micelles, oil emulsion droplets) can be modeled using equation (7), which takes explicitly into account the contribution of colloidal species present in the system to the solute mass transport. such as bile components micelles and oil droplets. After proper integration between the boundary spatial conditions, equation (7) shows an evident analogy with the Noyes-Whitney law (equation 3) valid for dissolution of a solid drug in a solvent in absence of colloids.

Higuchi’s dissolution layer model developed in the presence of colloidal particles assumes no net changes in colloid concentration, and that solute/colloid interactions
occur rapidly enough to be considerate at equilibrium. Under the instant equilibration assumption between the free drug and the drug associated to micelles, the following partition coefficients [62] can also be expressed in function of the total amount of surfactant species that aggregate into micelles [156]:

\[ \chi = \frac{C_{\text{aqueous}}^{\text{sat}} - C_{\text{water}}^{\text{sat}}}{C_{0\text{surf}} - \text{cmc}} \]  

(8)

\[ K_{m/w} = \frac{C_{\text{aqueous}}^{\text{sat}} - C_{\text{water}}^{\text{sat}}}{C_{\text{water}}^{\text{sat}}} = \frac{\chi(C_{0\text{surf}} - \text{cmc})}{C_{\text{water}}^{\text{sat}}} \]  

(9)

Where: \( C_{\text{aqueous}}^{\text{sat}} \) = drug solubility in bulk (water and in micelles)

\( C_{\text{water}}^{\text{sat}} \) = drug solubility in water (mg/ml or mg/cm\(^3\))

\( \text{cmc} \) = critical micellar concentration (mol/ml)

\( C_{0\text{surf}} \) = initial concentration of surfactants (mol/ml)

However, some considerations are needed in case of the lipid digestion that takes place in parallel to the drug dissolution process. As direct effects of the enzymatic hydrolysis of lipids, the quantities representing fats and surfactants are expected to change over time. Oil amount is decreasing according to the lipid digestion kinetics, while the total surfactants concentration might change in function of FA/colloidal particles interactions leading to the formation of new colloids (vesicles). Furthermore, the dynamic evolution of colloidal structures might affect not only the number of colloidal species available for dissolution, but also the quantity of dissolved drug per mole of colloidal particles, such as the solubilization power of the medium. In light of all the changes of bulk composition occurring during the lipid digestion process, the diffusion coefficients \( D_s \) and \( D_c \) related to the drug transport in the aqueous and colloidal phases respectively, are also expected not to remain constant.
2.5.3 Drug absorption kinetics

Regardless the model system used experimentally, drug permeability properties are determined by measuring the transport of drugs across a barrier separating a donor and an acceptor compartment, which simulates the intestinal lumen and the sub-mucosal side respectively. After introduction of a drug compound in the donor compartment, the drug flux \( J \) (mg/cm\(^2\) min) in the acceptor compartment is given by:

\[
J = \frac{dQ}{dt} \times \frac{1}{A}
\]  

(10)

where: \( Q \) = drug amount transported across the barrier (nmol)

\( A \) = barrier surface area (cm\(^2\))

\( t \) = time (sec)

According to Fick’s first law, the flux \( J \) depends on the molecule diffusion coefficient, the concentration gradient between the two compartments, and the barrier as:

\[
J = \frac{D}{h} \left( C_{\text{donor}} - C_{\text{acceptor}} \right)
\]  

(11)

where: \( D \) = drug diffusion coefficient (cm\(^2\)/sec)

\( h \) = barrier thickness (cm)

\( C \) = drug concentration in compartments (\( \mu \)M)

Assuming that the concentration in the acceptor compartment is negligible if compared with the concentration at the donor side (sink conditions), the transport rate \( dQ/dt \) can be written then:

\[
\frac{dQ}{dt} = PAC_{\text{donor}}
\]  

(12)

where: \( P \) = \( D/h \), permeability coefficient (cm/sec)
When sink conditions apply, the donor concentration can be considered constant during the experiment, and the initial donor concentration \( C_0 \) is used as \( C_{\text{donor}} \).

Many drugs are substrates of different uptake/efflux carriers; therefore it is important to assess the involvement of these carriers in trans-epithelial transport. Carrier-mediated transport is characterized by concentration-dependent permeability, as increasing concentration of their substrates can saturate carriers and enzymes. Efflux can be modelled using Michaelis-Menten kinetics \([157]\), while metabolism can be expressed as first order processes in metabolite. Therefore, the total transport rate can be written as:

\[
\frac{dQ}{dt} = P_m A C_{\text{donor}} - \left( \frac{v_{\text{max}} C_{\text{cell}}}{K_m + C_{\text{cell}}} \right) - k_m C_{\text{cell}}
\]

(13)

where: \( v_{\text{max}}, K_m = \text{efflux parameters} \)

\( k_m = \text{first order metabolism constant (cm}^2/\text{sec)} \)

\( C_{\text{cell}} = \text{free drug concentration in cells (\mu M)} \)

Mechanistic transport experiments should be carried out using various donor concentrations, especially when investigating carrier-mediated pathways, since they show concentration-dependent permeability. The use of carrier inhibitors are also recommended in order to separate the measured permeability into passive diffusion and carrier-mediated components \([158]\).
3.0 Experimental

The overall goal of this project was to gain quantitative mechanistic insight into and to predict the influence of ingested lipids in the GI tract on drug dissolution and absorption. The study included experimental characterization and computational modelling of kinetics of parallel processes occurring in the GI tract after lipid ingestion – namely, compound dissolution, lipid digestion, compound partitioning into colloidal phases, absorption - and it related the kinetics to chemical composition and colloidal structure of intestinal contents. The result was a comprehensive systems-based model of the influence of ingested lipids on compound bioavailability. The specific aims of the proposed experimental approach were:

(1) Design and characterize biorelevant in vitro lipid digestion models able to simulate fundamental features of human intestinal contents in post-prandial conditions. Basic back-titration has been used to establish associated chemical composition characterizing products of the lipolysis process.

(2) Investigate the ultra-structure and composition of colloidal species existing in the GI tract upon ingestion of lipids and their dynamic behavior. Dynamic light scattering (DLS), and small angle neutron scattering (SANS) have been employed to characterize the colloidal structures (emulsion droplets, vesicles, micelles) present throughout digestion.

(3) Establish kinetics and thermodynamics of drug transport into and out of colloidal structures in the GI tract, based on a model drug that was selected to represent poorly water-soluble drug compounds. High-pressure liquid chromatography (HPLC) has been used in solubility/dissolution experiments, while electron paramagnetic resonance
(EPR) has been employed to track the model drug in different phases formed during the digestion process (oil droplets, vesicles, micelles, free in aqueous solution, etc.).

(4) Examine mechanisms and kinetics of compound transport across the intestinal membrane in the presence of lipids and the lipid digestion process. The Caco-2 cell and HT29-MTX cell culture models were used to probe kinetics of drug transport.

Developed kinetic (mass transport) expressions have been then integrated into a systems-based model in order to predict the influence of ingested lipids on oral compound absorption. A proper MATLAB® code has been developed for solving differential equations by means of the Runge-Kutta solution technique [159].

Lipid digestion and absorption are highly complex processes with multiple intricacies. It is currently impossible to try to depict these processes mechanistically, using in vitro studies in only one project. Therefore, the project approach has developed an experimental and theoretical framework from a simplified system, in order to establish the feasibility of quantitative predictions, and ultimately gain considerable insight into the complex effects of lipids on compound absorption.

3.1 Design and characterization of in vitro lipolysis models (Aim 1)

The impacts of ingested lipids on the overall drug absorption are correlated to the drastic changes occurring during the lipolysis, namely physical-chemical properties of GI fluids and morphology of the intermediate phases of lipid digestion [99, 100]. Therefore, updated in vitro lipolysis models have been used as tools for simulating GI post-prandial conditions. The developed system comprised biorelevant media, pancreatic enzymes, and lipid substrates. Medium composition was carefully designed in order to mimic fundamental features of GI fluids in the fed state [73]. Several techniques have been
coupled with the *in vitro* digestion to study lipase kinetics and to investigate the dynamic formation of the colloidal phases produced during lipolysis.

### 3.1.1 Assessment of biorelevant media for *in vitro* lipolysis studies

Biorelevant media are solutions designed to mimic properties of gastrointestinal fluids in fasted and fed states, commonly used in dissolution testing and in the *in vitro* lipid digestion models. Physical-chemical properties of biorelevant media are known to greatly affect drug dissolution and kinetics of lipid digestion results, therefore the choice of a biorelevant *in vitro* model is the first and crucial point in order to reproduce *in vitro* as much as possible the in vivo GI fluids and processes after food intake.

In order to design biorelevant media suitable to be employed in lipid digestion simulations, a careful examination of the literature was performed, mainly focused on *in vivo* experiments that examined GI tract contents in fasted and fed states [73]. Therefore, the medium compositions were based on available knowledge of *in vivo* GI fluids. The designed biorelevant media were prepared in maleate buffer (referred also as digestion buffer), which was composed of Trizma® maleate 100 mM, sodium chloride (NaCl) 65 mM, calcium chloride didydrate (CaCl$_2$*H$_2$O) 10 mM, sodium azide (NaN$_3$) mM, and sodium hyhydroxide (NaOH) up to final pH 6.5. All the salts were dissolved in distilled water according to their concentrations. As a model of the human bile components, the media also included sodium taurodeoxycholate (NaTDC or BS) 12 mM and L-alpha-phosphatidylcholine from egg yolk (PC or PL), Type XVI-E 4 mM, which were dissolved in the digestion buffer and equilibrated for at least 12 hours under magnetic stirring at 37°C. A lipid substrate – either soybean oil or partially digested lipid mixture - was added as crude emulsion 20-30 minutes before every *in vitro* lipolysis experiment.
into the solution. Soybean oil (mainly composed of C18 TG) was chosen as a model for the lipid components of the daily food intake, because ingested lipids are composed of 95% long-chain TG [28]. The selected soybean oil concentration (50 mM) reflected approximately the average lipid intake per meal [28, 160, 161]. The partially digested lipid mixture was composed of soybean oil 35 mM, glycercyloleate 15 M, and sodium oleate 15 mM, representing the mixture of partially digested lipids (TG, MG, and FA) in output from the stomach. The lipid mixture composition were calculated considering that the hydrolysis of each TG molecule produces in total two fatty acid (FA) plus one 2-monoacylglycerol (MG), and the lingual and gastric lipases are responsible for 30% of the total lipolysis in stomach [29]. After 30 minutes of equilibration of the lipid substrate into the biorelevant medium, the in vitro lipolysis experiment were started and performed as described in section 3.1.2.

Physical chemical properties of biorelevant media were determined and critically compared to in vivo GI fluids’ in order to assess the feasibility of the proposed systems. Specifically, buffer capacity, and critical micellar concentration (cmc) were investigated as described in the following sections.

3.1.1.1 Buffer capacity

A solution has a buffer capacity of 1 when one equivalent of strong acid or alkali is required to change the pH value of 1 L by one pH unit. Buffer capacity of biorelevant media was determined by titration of 1 L of sample using NaOH or HCl 1 M, under magnetic agitation ad keeping temperature constant at 37°C.
3.1.1.2 Critical micellar concentration (cmc)

The critical micellar concentration (cmc) is the concentration at which amphiphilic molecules form micelles in solution. The cmc of the sodium taurodeoxycholate (NaTDC) and lecithin system in maleate buffer (composition shown in Table 1), at pH 6.5 and temperature of 37°C, was determined using two independent methods, a tensiometric technique and a spectroscopic (absorption) technique. Three sets of NaTDC/Lecithin solutions (molar ratio α (NaTDC) = 1, 0.75, 0.50) were prepared and analyzed accordingly. The total surfactant concentration ranged from 0.1 mM to 8 mM for each set. In the tensiometric method, surface tension values of each solution were measured using a Kibron MicroThrough X tensiometer and reported in function of the logarithm of total concentration. The concentration at which the surface tension showed a break is considered the cmc at this particular molar ratio. In the absorption method, the dye Rhodamine 6G was added in each NaTDC/Lecithin solution up to a final concentration of 2.5 μM. The maximum wavelength of absorbance for Rhodamine 6G was detected using a Bio-Tek UV-Vis spectrophotometer, and reported in function of the logarithm of total concentration. The concentration at which the maximum wavelength of absorbance for Rhodamine 6G showed a shift is considered the cmc at this particular molar ratio.
3.1.2 *In vitro* simulation of the lipid digestion process

*In vitro* lipolysis models consisted of a biorelevant medium, as described in the previous section, kept in a beaker under magnetic stirring (350 rpm) and at 37°C on a heated stirring plate throughout the duration of lipolysis experiments. 2 ml of pancreatin extract - source of lipase/co-lipase enzymes - and a lipid substrate – simple triglycerides or more complex formulation – were added in order to start lipolysis experiments (Figure 6).

Pancreatin extract was prepared freshly for every *in vitro* lipolysis experiment following the procedure proposed by Sek *et al.* [101]. Briefly, 2 g of porcine pancreatin powder were dissolved in 10 ml of digestion buffer (composition in section 3.1.1) and magnetically stirred for 15 minutes at room temperature. The solution was centrifuged at 1600g for 15 minutes at 5°C; the supernatant was collected and stored at 4°C until use.

![Figure 6: In vitro lipolysis experimental set up, comprising simulated GI fluids in the fed state.](image)
Measured enzyme activity of pancreatic lipase prepared in this manner from this supplier was similar to human pancreatic lipase activity in vivo [162]. Detailed chemical composition of the system is presented in Table 4.

**Table 4: Composition of the proposed media for in vitro lipolysis models that comprised two types of lipid substrates.**

<table>
<thead>
<tr>
<th>Digestion Buffer</th>
<th>Maleate buffer at pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model for human bile: BS and PL (biorelevant medium)</td>
<td>BS: NaTDC 12 mM</td>
</tr>
<tr>
<td></td>
<td>PL: Lecithin 4 mM</td>
</tr>
<tr>
<td></td>
<td>Ratio BS/PL 3:1</td>
</tr>
<tr>
<td></td>
<td>i) Triglycerides</td>
</tr>
<tr>
<td></td>
<td>Soybean oil 50 mM</td>
</tr>
<tr>
<td>Lipid substrates modelling food intake</td>
<td>ii) Lipid mixture</td>
</tr>
<tr>
<td></td>
<td>Soybean oil 35 mM</td>
</tr>
<tr>
<td></td>
<td>Sodium oleate 30 mM</td>
</tr>
<tr>
<td></td>
<td>Glyceryl monooleate 15 mM</td>
</tr>
</tbody>
</table>

**Start of lipolysis:** adding pancreatin extract (10,000 TBU/ml)

**Dilution:** 1:10 (1,000 TBU/ml)

After the addition of the lipid substrates and pancreatin extract, the change in pH was constantly monitored with a pH meter (Seven Multi pH meter, Mettler Toledo, Columbus, OH), and FA produced due to the lipolysis reaction were titrated manually with 0.2 mM NaOH by using a digital titration unit (VWR international, Plainfield, NJ). The extent of lipid digestion was determined indirectly by basic back-titration method, such as by recording the volume of the titrant NaOH (0.2 M) added during the experiment needed to titrate the FA formed from the TG hydrolysis. The number of moles of OH⁻ ions present in an added volume can be equated with the moles of FA produced during lipolysis, since they will react at 1 to 1 molar ratio:

\[
\text{Moles (FA)} = \text{Moles (NaOH)} = M \times V_t
\]  

(14)

where: \( M = \) titrant molarity (mol/L)
\[ V_t = \text{titrant total volume added at time } t \ (L) \]

Assuming that the enzymatic hydrolysis of one molecule of TG produces 2 molecules of FA and 1 molecule of MG, the consumption of 2 moles of NaOH corresponds to the formation of 2 moles of FA and to the hydrolysis of 1 mole of TG (Figure 7) [39]. To take into account the additional FA produced by digestion of TG and DG contained in the lecithin, digestions of blank simulated intestinal media were performed. Blank experiments were performed in the same manner, except that lipid substrates were not added to the reaction solutions. Digestion rate was related to the droplet surface area available to the enzyme, according to Equation 16, Section 4.1. Digestion rate constants were estimated accordingly via non-linear regression fitting in MATLAB®.

Samples were collected at specific time intervals during blank and lipid digestion experiments, mixed with the appropriate amount of an enzyme inhibitor solution, prior to analysis. The enzyme inhibitor was a solution of 4-bromobenzeneboronic acid (0.2 g/ml in methanol [114]), which is a known inhibitor of porcine pancreatic lipase [163].

![Figure 7: The net enzymatic hydrolysis of one mole of TG gives one mole of MG and two moles of FA.](image)
3.1.2.1 Sensitivity of in vitro lipolysis models

Despite being a common experimental procedure, previous in vitro lipolysis models depict many differences in the composition of biorelevant media used, such as pH, buffer type and concentration, ionic strength, type and quantity of lipid substrates (Table 3). These experimental parameters are known to affect the enzymatic activity of the lipase, and consequently the rate of lipolysis. Further, the high variability among the proposed in vitro lipid digestion models makes it difficult to compare results obtained from different studies. In order to evaluate the sensitivity of the proposed in vitro lipolysis models, experiments were conducted in which pH, calcium concentration, and type of substrate were varied, and the resulting change in digestion kinetics were assessed. In the quantitative assessment of system sensitivity to experimental parameters, in vitro lipolysis experiments were performed according to a balanced $2^4$ multifactor design (Table 5).

<table>
<thead>
<tr>
<th>Level / Factor</th>
<th>A: pH</th>
<th>B: Ca$^{2+}$</th>
<th>C: medium</th>
<th>D: substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low ( - )</td>
<td>6.5</td>
<td>5 mM</td>
<td>Buffer (no micelles)</td>
<td>Soybean oil</td>
</tr>
<tr>
<td>High ( + )</td>
<td>7.5</td>
<td>10 mM</td>
<td>Biorelevant medium (micelles)</td>
<td>Lipid mixture</td>
</tr>
</tbody>
</table>

The impact of various experimental factors on lipid digestion was investigated over 50 minutes of enzymatic reaction. The lipolysis profiles were analysed statistically during the digestion experiments as a function of different treatments, in order to evaluate the impact of the experimental factors selected on enzymatic kinetics parameters.
3.2 Characterization of colloidal particles associated to the lipolysis process (Aim 2)

In vitro lipid digestions of ingested lipids were coupled with dynamic light scattering (DLS), and small angle neutron scattering (SANS), in order to characterize respectively: particles’ size distribution, particles’ morphology, and particles’ internal structure. These investigations aided in understanding how endogenous micelles evolve into vesicles, and how these different colloidal structures impacted drug solubility and drug dissolution kinetics during the lipolysis process. Samples were taken before and during in vitro lipolysis experiments at defined time points, and they were analysed with the appropriate technique. Details concerning sampling procedures and instrument settings are described in the following subsections.

3.2.1 Size and Zeta-potential measurements by means of DLS

Size distribution, and Zeta-potential of colloidal particles formed before and during in vitro lipolysis of soybean oil and of lipid mixture experiments were measured using DLS (Brookhaven 90 Plus). Samples of 2 ml were collected before and during the in vitro lipid digestion at specific time intervals, and mixed with a proper amount of enzyme inhibitor solution inside plastic cuvettes [101]. Mean hydrodynamic diameters and mean Zeta-potential values were determined from five readings of 20 seconds per sample.

3.2.2 Investigation of colloidal structures by means of SANS

Small angle neutron scattering (SANS) were used to characterize in detail the structure of colloidal structures (micelles, vesicles and oil droplets) that were present throughout the digestion process. Shape and internal structural information were obtained
by SANS measurements with the contrast-matching technique, using deuterated PL and deuterated water.

3.2.2.1 Preparation of samples for SANS measurements

The investigated model system contained a biorelevant medium simulating intestinal fed state conditions (water, salts, BS and PL), as described in Table 4. Triolein were used as lipid substrate instead of soybean oil, to reduce the complexity of the system. Samples were prepared and maintained at a single temperature (37°C, body temperature). The sample cells were made of quarts, with windows of thickness 1 mm and path length of 0.8 mm. Sample cells were mounted on a brass container whose temperature was maintained at 37°C during the measurements. Three sets of samples were investigated as following:

1) Samples representing pre-digestion conditions were prepared by dissolving all the components of the biorelevant medium in either H₂O or D₂O. Intermediate H₂O/D₂O ratios were prepared by mixing appropriate amounts of these solutions. Deuterated lecithin was used in this set of experiments instead of the not deuterated compound, in order to apply the external contrast variation. This method can enable to see different particles components better (internal structure).

2) Samples were prepared by dissolving all the components of the biorelevant medium in 100% D₂O. Deuterated triolein were added in the concentration range of 50 mM - 0.05 mM in order to represent different stages of the lipid digestion process (variable amount of lipid digested). No lipase was added in this set of samples.

3) Solutions prepared as 2) were mixed to pancreatic lipase, in order to initiate the lipid digestion reaction. Samples were collected at defined time points (5 min, 30 min,
and 60 min) in order to investigate different stages of digestion. The enzymatic reaction were stopped with proper solution of enzyme inhibitor prior to analysis [101].

### 3.2.2.2 SANS data collection and SANS data analysis

Two series of experiments were conducted at the High flux Isotope Reactor (HFIR) in Oak Ridge National Laboratory (ORNL). All the measurements were performed using a broader Q range of 0.002 Å⁻¹ and 1 Å⁻¹, which was separated into a long configuration (0.002 Å⁻¹ to 0.07 Å⁻¹), medium configuration (0.005 Å⁻¹ and 0.2 Å⁻¹), and short configuration (0.2 Å⁻¹ to 1 Å⁻¹). A SANS instrument based on thermal neutrons beam for general purposes was employed with a neutron wavelength λ = 4.72 nm and a sample-to-detector distance of 1.112 m (short configuration), 9.812 m (medium), and 19.312 m (long). Measurement time was set up to 1 hour for each sample. The collected SANS scattering patterns were reduced and analyzed by means of Igor Pro software. Reduced data were plotted as absolute intensity (A.U.) versus the scattering angle Q (Å⁻¹) and fitted by means of SAS view.

### 3.3 Drug dissolution and partition between phases during lipolysis (Aim 3)

Studies pertaining to drug dissolution and drug partitioning into colloidal species present the GI fluids prior to and during the in vitro digestion of lipids were performed by means of HPLC and EPR spectroscopy. EPR is a spectroscopic technique able to detect and quantify radicals in different phases - namely aqueous, micellar, and oil phases. Since only paramagnetic molecules are visible to EPR, an appropriate spin probe, Tempol benzoate (TB) was selected represent a compound with low aqueous solubility and moderate lipophilicity (logP: 2.5) (Figure 8). Solubility and dissolution kinetics of the model compound TB in simulated GI conditions were measured by HPLC. The amount
of the model drug TB partitioned in each phase before and during *in vitro* lipid digestions was measured by EPR, and partition coefficients were determined accordingly. Results from both techniques were combined in order to test the model predictions (Section 3.3).

![Molecular formula of the stable nitroxide radical Tempol Benzoate (TB).](image)

**Figure 8: Molecular formula of the stable nitroxide radical Tempol Benzoate (TB).**

### 3.3.1 Drug solubility and dissolution measurements

The equilibrium solubility of the model drug TB was measured by adding excess amount of compound dissolved into maleate buffer at pH 6.5, in the fed state biorelevant medium, and in soybean oil according to the shake-flask method. After preparation and equilibration, 5 ml of each medium were dispersed into 8 ml borosilicate glass tubes containing excess drug. Tubes were stored at 37°C, mixed at 300 rpm for the first 12 hours, and sampled every 24 hours over a period of 72 hours (or until equilibrium solubility was attained). Samples were filtered through 0.200 µm Nylon syringe filters prior to measuring drug concentration by means of HPLC. Experiments were conducted in triplicate for in each dissolving medium and standard errors were calculated.

In dissolution experiments, the solid model drug TB was added to a stirred beaker (0.003618 mmol/ml) containing the dissolving medium (maleate buffer at pH 6.5, or fed state biorelevant medium with and without soybean oil) at 37°C and 250 rpm.
Experiments in fed state biorelevant medium with soybean oil were conducted in the presence of pancreatin extract, containing lipases, in order to assess the impact of the lipid digestion process on drug dissolution. Samples were withdrawn from the dissolution beaker at defined time intervals over a period of 3 hours. Dissolution only samples were filtered through 0.45 μm nylon syringe filters to remove undissolved drug; simultaneous dissolution and digestion samples were centrifuged at 1700 g and 37°C for 10 minutes instead of filtered to remove undissolved drug and not oil emulsions [164]. All samples were analysed via HPLC in order to determine the drug concentration in solution. Experiments were conducted in triplicate for each dissolving medium and standard errors were calculated.

The concentrations of the model drug TB in samples collected during solubility, and dissolution experiments were determined using HPLC with a photodiode detector (Shimadzu, Japan), and a wavelength of 232 nm. The analytical column used was Agilent Zorbax RX-C18 4.6 × 75 mm, 3.5 μm. The column temperature was maintained at 40°C, and the flow rate was 1 mL/min. The mobile phase contained distilled water with 0.15% TFA: methanol (70:10 to 10:70 over 16 min).

3.3.2 Drug tracking and partition experiments

In this study, EPR was used to monitor model drug transport into and out of colloidal structures (such as emulsion droplets and micelles) present in simulated intestinal environment in the presence and absence of the lipid digestion process. Since EPR spectra are highly sensitive to changes in local polarity and viscosity, the spin probes in different phases can be resolved and quantified so that the partitioning and translocation of probes between different phases can be monitored and quantified in real-
time. In order to simulate different scenarios of the presence of food lipids in the GI lumen, the model drug TB was dosed either already dissolved in the fed state biorelevant medium or as a solid form, in the presence of the digestion of the model food lipid (soybean oil). Samples collected throughout the course of soybean oil digestion were analysed by EPR spectrometer (9.1-9.9 GHz, X-Band; Bruker EMX; Figure 9) in order to track and quantify the model drug transport between phases (oil droplets, vesicles, micelles, buffer) in a time-sensitive manner.

Figure 9: EPR spectrometer Bruker EMX, working between 9.1-9.9 GHZ of magnetic field (X-band).

3.3.2.1 Drug partitioning in lipid-containing systems in the absence of digestion

The biorelevant medium simulating the intestinal fasted and fed states contained maleate buffer that consisted of 100 mM tris-maleate, 65 mM NaCl, 5 mM or 10 mM (fasted or fed) CaCl$\cdot$2H$_2$O, 3 mM NaN$_3$ at pH 6.5. In order to mimic fasted and fed state conditions 5 mM NaTDC/1.25 mM PC and 12 mM NaTDC/4 mM PC were added to
maleate buffer, respectively [98, 165]. Other biorelevant media were also prepared in a concentration range from 6mM NaTDC/2mM PC to 60mM NaTDC/20 PC in order to study TB partitioning as a function of surfactant concentration and type. For comparison, an alternative fed state biorelevant medium was prepared as above except for the addition of 16 mM of bile extract [109] instead of NaTDC and lecithin. Model bile components were mixed with maleate buffer on a stirring plate at 37°C.

In order to simulate the food-associated lipid intake, soybean oil (50 mM) was added to the fed state simulated intestinal fluid containing pre-dissolved TB (0.145 mg/ml) in order to form a crude emulsion under continuous mixing (300 rpm) at 37°C for 3 hours. Samples were taken at specific time points and analyzed via EPR immediately in order to monitor drug partitioning between different phases.

3.3.2.2 Drug partitioning in lipid-containing systems during in vitro lipid digestion

Digestion of soybean oil was carried out in two different ways: i) The model drug TB was completely dissolved in the fed state biorelevant medium (0.145 mg/ml) prior to the addition of soybean oil and the beginning of the digestion process, in order to study drug partitioning during the lipolysis; ii) Soybean oil (50 mM) was dispersed in the fed state biorelevant medium at 37°C and 300 rpm for 20 minutes prior to the simultaneous addition of the solid model drug TB (1 mg/ml) mM and the lipase enzyme, in order to investigate drug partitioning during the dissolution and the lipid digestion processes. The enzymatic hydrolysis of lipids was initiated by adding 2 ml pancreatin extract into 18 ml simulated intestinal fluids containing the lipid substrates (soybean oil emulsions). The change in pH was constantly monitored with a pH meter (Seven Multi pH meter, Mettler Toledo, Columbus, OH), and fatty acids produced due to the lipolysis reaction were
titrated with 0.2 mM NaOH using a manual digital titration unit (VWR international, Plainfield, NJ). Fatty acid production was then calculated by measuring the total volume of NaOH added to maintain the pH at 6.5 during lipolysis. Samples were also collected at different time points throughout digestion for immediate EPR analysis.

3.3.2.3 Drug partitioning in individual media - single and two component samples

EPR spectra of TB in separate environments (oil, micelles, buffer solution) were used to obtain spectral simulation parameters to determine distribution of the spin probe between the different phases in multi-component solutions (lipids dispersed in biorelevant media). Samples of the model drug TB in separate environments, namely digestion buffer (0.02 mg/ml), fed state biorelevant medium (0.145 mg/ml), and pure soybean oil (1 mg/ml) were prepared gravimetrically, kept at 37°C, and analysed under the same experimental conditions described in Section 3.3.2.4. Separate phases EPR spectra were used to obtain simulation parameters for spectral simulation to determine distribution of the spin probe between the different phases.

3.3.2.4 EPR data acquisition and analysis of spectra

Collected samples were analysed by EPR spectrometer (9.1-9.9 GHz, X-Band; Bruker EMX) in order to quantify model drug in different phases in single- to three-component solutions. Samples (25 µl) were deposited via micro-syringe in EPR quartz capillary tubes (Sigma, cat. # LG-9060-100), and they were analysed by EPR at 37°C. The parameters used during EPR measurements were as follows: microwave bridge frequency: 9.38 GHz, modulation frequency: 100kHz; microwave power: 1 mW; sweep width: 70 G, sweep time: 83.89 s; time constant: 327.68 ms; modulation amplitude: 1 G.

The recorded EPR spectra of pre and post-digestion samples represented complex
spectra containing three different components (aqueous, oil, micellar). Quantitative
determination of the ratio of the spin probe in different environments over time was
performed via multi-component fitting analysis of spectra using the Multicomponent EPR
Labview module of Altenbach [166] to perform Simplex fitting of the spectrum. In order
to reduce the amount of variable simulation parameters during the simulation process,
EPR spectra of the model drug TB in the separate environments (digestion buffer,
biorelevant medium, and lipid substrate) were first fitted in order to determine the
contemporary simulation parameters. The EPR spectra of the model drug TB recorded in
multi-component systems at different times were then fitted by using the simulation
parameters determined from spectral fitting of TB in separate environments. The dynamic
parameters that were varied for each component during the fitting procedure included
log_{10} of the rotational diffusion constant (R) and Heisenberg spin exchange frequency
(ω_{ss}). The magnetic parameters varied included the isotropic g-value (g_{iso}) and ^{14}\text{N}
hyperfine coupling constant (a_N). For all components, the underlying pattern of ^{1}\text{H}
hyperfine lines consisted of 12 equivalent protons with a splitting a_{\text{H}}/\gamma_{\text{c}} = 0.04 \text{ G}, (methyl
protons), 4 equivalent protons with a_{\text{H}}/\gamma_{\text{c}} = 0.20 \text{ G} (protons on ring carbons 3 and 4), and
a single proton (carbon 4) with a_{\text{H}}/\gamma_{\text{c}} = 0.16 \text{ G}. Three independent EPR measurements
were taken for each time point in order to estimate standard deviations in the fitted
parameters and probe distributions obtained from the lineshape analysis.

3.3.3 Dissolution and partition modelling using HPLC and EPR data

HPLC outcomes represented the total amount of drug dissolved in the bulk, which
could be allocated in different phases present in the system – namely aqueous, colloidal,
and oil phases. The dissolution kinetics were expressed according to a layer model that
takes into account the contribution of colloidal particles to the drug transport rate, in a similar fashion to a model derived by Higuchi [155] and discussed in Sections 2.5.2 and 4.2. Equilibrium solubility terms were determined experimentally by means of HPLC, as described in section 3.1.1., while the partition coefficient \( K_{em/\text{aq}} \) determined from EPR spectra analysis of samples in the absence of lipid digestion reaction.

3.4 Intestinal absorption of compounds in the presence of lipids (Aim 4)

Intestinal contents may alter intestinal permeability and transport by changing the barrier properties of the intestinal wall or the transport mechanisms across the intestinal wall. Therefore, aim 4 examined mechanisms and kinetics of the model compound TB transport across the intestinal membrane in the presence of lipids and lipid digestion products. The Caco-2 cell mono-cultures, the HT29-MTX cell mono-cultures, and co-cultures of Caco-2 and HT29-MTX cells were used as \textit{in vitro} models to probe kinetics of drug transport in the presence of simulated GI fluids. In all transport tests, transepithelial resistance (TEER) measurements and cytotoxicity assays (cck-8 tests) were conducted to assess potential effects of fed state simulated intestinal fluids on cell viability and cell monolayers’ integrity. The formation of a mucus layer on HT29-MTX cell mono-cultures, and on co-cultures of Caco-2 and HT29-MTX cells was determined by staining.

3.4.1 Cell cultures

Caco-2 cells (purchased from ATCC, USA) were cultured in Dulbecco’s Modified Eagle's Medium (DMEM, Life Technology, USA) containing 4.5 g/l glucose, and 10% fetal bovine serum (FBS, ATCC, USA), or Minimum Essential Medium with Earle’s salts (EMEM, Life Technology, USA) containing 1.0 g/l glucose, and 20% FBS. Both media
contained 100 U/ml of penicillin and 100 μg/ml of streptomycin. HT29-MTX cells clone E12 (purchased from HPA, UK) were cultured and maintained in complete DMEM containing sodium pyruvate (1 mM) [167], at 37 °C under a 5% CO₂ atmosphere with changes in media every 2 days. Caco-2 and HT29-MTX cells were used from passages 22 to 37 and 49 to 59, respectively.

3.4.2 Cytotoxicity Tests

Toxicity of the model drug TB, dosed in HBSS, and in fed state biorelevant medium with and without digestion products (TG, DG, MG, and FA) on Caco-2, HT29-MTX, and co-cultured cell monolayers was tested by means of the cell counting kit – 8 (cck-8) cytotoxicity assay (Sigma, USA). Cells were seeded on 96 well cell culture plates (seeding density of 2.5 x 10⁵ cells/ml) in 100 μl complete growth medium for 21 days prior to the cytotoxicity assay. On the 22nd day, the cell culture medium was removed, and the cells were rinsed with HBSS twice and incubated with media to be tested, such as HBSS, and media simulating intestinal conditions (with and without the model drug TB) for three hours at 37°C, 5% CO₂. Biorelevant media simulating intestinal contents in post-prandial conditions were tested with and without the model drug TB. After three hours, plate wells were rinsed twice with HBSS and incubated with a tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] solution (provided in the kit) at 10ul/100ul in complete growth medium for three-four hours at 37°C, 5% CO₂. WST-8, highly water-soluble salt, is reduced by dehydrogenases in living cells to produce a yellow colored compound (formazan), which is soluble in the culture medium. After three-four hours of incubation, UV-VIS absorbance of plate wells was measured at 450 nm. Plate wells incubated with
only HBSS were used as a positive control, while a solution of 1% sodium dodecyl sulfate (SDS) in HBSS was used as a negative control. Percent viabilities were determined in relation to the positive and the negative controls. Measurements were performed using four wells for each solution to be tested on the cell monocultures and the co-cultures.

3.4.3 Measurements of transepithelial electrical resistance TEER

The integrity of the cell monolayers was checked on the 7th, 14th and 21st days after seeding by measuring TEER using a volt-ohmmeter (EVOM, World Precision Instruments) equipped with Endohm chopsticks electrodes. Three TEER measurements were acquired in three different positions for each insert. The integrity of the cell monolayers was also controlled at the beginning and at the end of each transport experiment. The background TEER (insert) was subtracted from the total TEER (cell monolayers and insert) detected. These values were multiplied with the area of the insert in order to obtain the monolayers resistance.

3.4.4 Staining of mucus layer and differentiated cells

For the cell staining experiment specifically, both Caco-2, HT29-MTX and a co-culture of Caco-2:HT29-MTX at 90:10 and at 75:25 ratios were seeded on transparent PET 12-well inserts at an initial cell density of 1X10^5 cells/cm² and cultured for 21 days in DMEM supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Complete growth media in both the apical (500 μl) and basolateral (1500 μl) sides were replenished every two days. Secreted mucus localized to HT29-MTX cells was stained using Alcian blue (10 mg/ml), as reported by Chen et al.[168]. Briefly, complete culture media were aspirated and the cells were rinsed once with PBS. Cells
were fixed by adding a 4% solution of paraformaldehyde (PFA) in PBS for 1 hour. Mucus associated with HT29-MTX cells was stained with Alcian blue for 1 hour at room temperature and washed three times with PBS.

3.4.5 Compound transport measurements and modelling across cell-based systems

To test the influence of intestinal contents on mucosal barrier properties, Caco-2 monolayers, HT29-MTX monolayers, and co-culture Caco-2/HT29-MTX monolayers were employed. The model compound TB was chosen to ensure consistency to the drug dissolution and partitioning studies (Section 3.3, Figure 8). Transport studies were combined with TEER measurements before and after exposure to simulated intestinal fluids, in order to assess effects of lipids on the integrity of cell monolayers.

In details, Caco-2 cells were seeded at 2 x 10^6 cells/ml and cultured on a 12 Transwell® permeable support (0.4 um pore size) for 21 days (Figure 10, [169]). Cell culture medium was removed from both the apical and basolateral compartments, and cells were rinsed once with HBSS. Apical compartment solution were replaced with biorelevant media (with and without the model drug TB), while basolateral compartment solution was replaced with HBSS. Plates were placed inside an incubator at 37°C, 5% CO₂, for three hours. Solutions of TB in HBSS at pH 7.4 and pH 6.5 were also used as controls. Samples were collected from the basolateral compartments (0.750 ml) every 30 minutes, replaced with fresh HBSS, and then analyzed for drug content using HPLC, following the method described in Section 3.3.1.
Relative kinetics of transport (with and without lipids) in each case was measured, and the change in the apparent permeability coefficient $P_{app}$ (cm/sec) was assessed from cumulative mass transported-time profiles assuming a simple first order absorption process and sink conditions at the receptor side, according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

(15)

where: $Q$ = drug transported across the barrier (ug),

$t$ = time (sec),

$A$ = barrier surface area (cm$^2$),

$C_0$ = initial drug concentration in donor side (ug/ul).

Estimated apparent permeability coefficient $P_{app}$ (cm/sec) were used to calculate the human effective intestinal permeability $P_{eff}$ (cm/sec) needed in kinetic expression describing the drug absorption across the intestinal mucosa (Section 4.3).
4.0 Model Development

This project included mathematical and computational models of parallel processes occurring in the GI tract after lipid ingestion – namely, compound dissolution, lipid digestion, compound partitioning into colloidal phases, and compound absorption. The models related the processes’ kinetics to the changes in chemical composition and in colloidal structures occurring in post-prandial intestinal fluids. Developed kinetic expressions (mass transport) were integrated into a comprehensive systems-based model, which was able to that describe and predict the influence of ingested food lipids on the overall oral compound bioavailability. A proper MATLAB® code was developed in order to solve the obtained kinetic differential equations by means of the Runge-Kutta solution technique [159].

4.1 Lipid digestion kinetics

Lipid digestion rate was expressed using a modification of equation recently proposed by Li et al. relating the rate of FA production to the droplet surface area available to the enzyme, assuming that the droplet size of oil-in-water emulsions decreases as digestion proceeds, and as FA, products of digestion of oil and surfactant, leave oil droplets [8]. In many cases, only a fraction of the total digestible FA present initially in the oil droplets is released because the lipolysis reaction is inhibited by FA formation in vitro [170]. In order to take this into account, we modified the expression proposed by Li et al. to include inhibition proportional to the concentration of FA [171]:

\[
\frac{dC_{FA,aq}}{dt} = k_{dig} \frac{A_{em}}{V_{aq}} - k_{inh} C_{FA,aq}
\]  

(16)
where: $C_{FA,aq}$ = concentration of FA produced during lipolysis (mmol/sec*cm$^3$),

$k_{dig}$ = digestion rate constant (mmol/sec*cm$^2$),

$A_{em}$ = total oil droplet surface area (cm$^2$),

$V_{aq}$ = total solution volume (cm$^3$),

$k_{inh}$ = inhibition rate constant (1/sec).

It is assumed that during the lipid digestion, oil droplets would shrink in size over time due to the enzymatic reaction and this change in particle diameter, $D(t)$ can be related to the fraction of FA released from the droplets due to digestion. Hence, the time-dependent emulsion particle diameter $D(t)$ and the emulsion surface area $A_{em}(t)$ are:

$$D(t) = D_0 \sqrt[3]{\frac{m_{FA,em}}{m_{FA,0}}}$$

(17)

$$A_{em}(t) = N \pi \left( D_0 \sqrt[3]{\frac{m_{FA,em}}{m_{FA,0}}} \right)^2$$

(18)

where: $N$ = number of oil droplets present

$m_{FA,em}$ = mmoles of digestible FA remaining in the oil droplet,

$m_{FA,0}$ = initial mmoles of digestible FA in the oil droplet,

$D_0$ = initial diameter of oil droplets (cm).

Using a mass balance, we can relate the mass of digestible FA remaining in the emulsion droplet with $m_{FA,aq}$ (mmol), the mass of FA digested and released in the aqueous phase volume $V_{aq}$ (cm$^3$), which is an experimentally measured variable in our system:

$$m_{FA,em} = m_{FA,0} - m_{FA,aq} = m_{FA,0} - C_{FA,aq} V_{aq}$$

(19)
Inserting this expression into Equation 18, we get:

\[ A_m(r) = N \pi \left( D_0 \sqrt[3]{\frac{m_{FA,0} - C_{FA,aq} V_{aq}}{m_{FA,0}}} \right)^2 \]  

(20)

4.2 Solid drug dissolution

Kinetic expressions describing the rate of drug entering the aqueous environment after dosing a solid drug co-administered with food lipids were developed considering major kinetic processes that take place simultaneously in vivo (Figure 11): lipid digestion, solid drug dissolution, drug partitioning into micelles, and drug uptake into food lipid emulsion droplets. Food-associated lipids were assumed to enter directly to small intestine upon oral administration.

Figure 11: Schematic representation of the studied lipid system, which was comprised of a solid drug dosed with food-associated lipids. Processes relevant to the system appear in blue text. \( C_{D,water} \): free drug concentration, \( C_{D,aqueous} \): free and micelle-associated drug concentration, \( C_{D,em} \): drug concentration in food-associated oil emulsions, \( h \): static layer around the dissolving drug particles.

The dissolution kinetics was expressed according to a static layer model that takes into account the contribution of colloidal particles to the drug transport rate, derived by
Higuchi [155]. Assuming the presence of a single species of free colloid (micelles), no net changes in colloid number, and that solute/colloid interactions occur rapidly enough relative to other processes to be considered at equilibrium, the rate of drug dissolution in the aqueous phase (water and micelles) is:

$$\frac{dC_{D,aq}}{dt} = \frac{S_p}{V_{aq}h} \left[ D_s \left( C_{eq,D,water} - C_{D,water} \right) + D_m \left( C_{eq,D,micelles} - C_{D,micelles} \right) \right]$$

where: $C_{D,aq}$ = drug concentration in the aqueous phase (mg/ml)

$S_p$ = surface area of $N$ dissolving drug particles (cm$^2$)

$h$ = stationary diffusion layer around a dissolving particle (cm)

$V_{aq}$ = solution total volume (ml)

$D_s$ = drug diffusion coefficient in water (cm$^2$/s)

$C_{eq,D,water}$ = drug solubility in water (mg/ml),

$C_{D,water}$ = free drug concentration in the total volume (mg/ml)

$D_m$ = micelle diffusion coefficient in water (cm$^2$/s)

$C_{eq,D,micelle}$ = drug solubility in micelles (mg/ml)

$C_{D,micelles}$ = drug concentration in micelles (mg/ml).

The drug concentrations in micelles ($C_{eq,D,micelle}$ and $C_{D,micelles}$) are expressed as mass of drug associated to micelles per total volume of solution, at the concentration of micelles present in the solution.

If the dissolving drug particles are assumed to be mono-dispersed and uniform spheres with an initial radius $r_0$ (cm), and the total number of particles $N$ does not vary with time, then the time-dependent expression for $S_p$ can be written as [172]:

$$S_p = \frac{3M_g^{3/5}M_s^{7/5}}{\rho r_0}$$

(22)
where: $M_0 =$ initial mass of drug dosed (mg),

$M_s =$ mass of undissolved drug (mg),

$\rho =$ drug density (mg/cm$^2$).

Equilibrium solubility terms were determined experimentally by means of HPLC, as described in Section 3.3. The quantities $C_{D,\text{water}}$ and $C_{D,\text{micelles}}$ were calculated from the total measured drug in the aqueous phase (buffer and micelles) according to the partition coefficient $K_{m/w}$ as defined in Section 4.2.1). Diffusion coefficients for compounds and colloidal particles were calculated according to the Wilke-Chang and the Stokes-Einstein equations, respectively (Section 4.2.2).

Equation 21 was used to model solid drug dissolution data from experiments performed in biorelevant media without lipids or lipid digestion. In experiments performed in the presence of a lipid substrate, oil droplets (soybean oil) were detected by DLS to be bigger than micelles (200 nm versus 5 nm), and consequently to have a calculated diffusion coefficient of $1.64 \times 10^{-12}$ m$^2$/s (according to the Stoke-Einstein equation, section 4.2.2) and an estimated diffusion time $t$ through $h$ ($t = h^2/D$) of ~ 4 minutes, compared to ~ 6 seconds for micelles. Therefore, oil droplet contribution to the drug transport rate within the static diffusion layer $h$ was neglected. However, some dissolved compound at each time $t$ did transfer into the oil phase. Compound partitioning into oil droplets was considered using an interfacial barrier-limited model [173], rather than a diffusion-driven process as described by Equation 21. The rate of drug partitioning into the oil phase (mg/sec*cm$^3$) was expressed as:

$$\frac{dC_{D,\text{em}}}{dt} = \frac{A_{\text{em}} P_{\text{li}}}{V_{\text{em}}} \left( C_{D,\text{aq}} - C_{D,\text{aq}'} \right)$$

where: $A_{\text{em}} =$ surface area of $N$ oil droplets (cm$^2$),

$$73$$
\[ P_{rel} = \text{permeability (cm/s)}, \]
\[ V_{em} = \text{total oil volume in aqueous solution (cm}^3), \]
\[ C_{D,aq} = \text{drug concentration (mg/ cm}^3) \text{ in } V_{aq}, \]
\[ C'_{D,aq} = \text{hypothetical aqueous drug concentration (mg/ cm}^3) \text{ in equilibrium with the drug concentration inside oil droplets } C_{D,em} \text{ (mg/ cm}^3). \]

Calculation of \( C'_{D,aq} \) as a function of the drug partition coefficient between the aqueous phase and the oil phase, \( K_{aq/em} \), is explained in Section 4.2.1. \( P_{rel} \) was experimentally determined by means of EPR during drug partitioning experiments in the absence of lipolysis (Section 3.3).

Combining the two kinetic expressions (Equations 21 and 23) describing the drug dissolution rate in the aqueous phase \( C_{D,aq} \), and the concurrent lipid digestion, the main equations utilized to describe FA and dissolved drug concentration profiles, respectively, are Equation 16 and:

\[
\frac{dC_{D,aq}}{dt} = \frac{S_P}{V_{aq} h^2} D_z \left( C_{D,water}^{eq} - C_{D,water} \right) + D_n \left( C_{D,nicelles}^{eq} - C_{D,nicelles} \right) - \frac{A_{em} P_{rel}}{V_{em}} \left( C_{D,aq} - C_{D,aq}^{'} \right)
\]

(24)

4.2.1 Definition of partition coefficients

In experiments combining dissolution of the solid drug compound and \textit{in vitro} lipolysis processes, specific parameters in the above expressions (Equations 21 and 23) are expected to change, reflecting the dynamic nature of the GI fluids during the digestion process. \( A_{em} \) is expected to decrease over time according to Equation 20, taking into account lipolysis kinetics [8]. Furthermore, as the lipase enzyme digests the lipids,
micelles are expected to evolve into new colloidal structures due to the interaction with lipid digestion products FA and MG liberated in the aqueous solution. The dynamic evolution of colloidal structures affects their solubilization power $C_{eq, D,micelle}^{D}$ in Equation 21, expressed as the quantity of dissolved drug associated with micelles per bulk volume. Therefore, the colloid solubilization power was affected by the extent of digestion and was no longer a constant during the dissolution process. The time-dependent colloid solubilization power, $C_{eq, D,micelles}^{D}$, was thus expressed as a function of the digestion kinetics as the following. Using the Equation 8, the molar solubilization capacity of micelles $\chi$ is function of the amount of FA produced by the lipolysis:}

$$\chi = \frac{C_{eq, D,aq}^{D} - C_{eq, D,water}^{D}}{C_{surf} - cmc}$$

(25)

where: $cmc =$ critical micelle concentration (mmol/cm$^3$),

$C_{surf} =$ time-dependent molar concentration of total surfactants BS, PL, and FA (mmol/cm$^3$).

We assume that as digestion products partition into bile salt/phospholipid (BS/PL) micelles, micelle composition changes, while the number of micelles remains constant. The total surfactant concentration, $C_{surf}$ (mmol/cm$^3$) is expressed as:

$$C_{surf} = C_{BS} + C_{PL} + C_{FA,aq}$$

(26)

where: $C_{BS} =$ BS concentration in aqueous solution (mmol/cm$^3$),

$C_{PL} =$ PL concentration in aqueous solution (mmol/cm$^3$),

$C_{FA,aq} =$ FA concentration produced during lipolysis (mmol/cm$^3$).
Using the Equation 25, the micelle-water partition coefficient, $K_{m/w}$, is expressed as a function of the amount of surfactant that is associated with micelles and $\chi$:

$$
K_{m/w} = \frac{C_{eq,D,micelle}}{C_{eq,D,water}} = \frac{C_{eq,D,aq} - C_{eq,D,water}}{C_{eq,D,water}} = \chi \left( \frac{C_{eq,D,water} - cmc}{C_{eq,D,water}} \right)
$$  \hspace{1cm} (27)

where: $C_{eq,D,micelle} =$ equilibrium drug solubility in micelles based on the total aqueous volume (mg/cm$^3$),

$C_{eq,D,water} =$ equilibrium drug solubility in the water phase (mg/cm$^3$).

Micelle molar solubilization capacity, $\chi$ is a constant and can be experimentally determined, as well as the drug solubility at equilibrium, $C_{eq,D,water}$. Critical micelle concentration, $cmc$, is also a constant [1].

$C_{D,aq}'$, the hypothetical aqueous concentration of the solute in equilibrium with compound concentration inside emulsion droplet (Equation 23), is defined by the oil-aqueous phase partition coefficient, $K_{em/aq}$, as:

$$
K_{em/aq} = \frac{C_{eq,D,em}}{C_{eq,D,aq}} = \frac{C_{eq,D,em}}{K_{m/w} + C_{eq,D,water}} = \frac{C_{D,em}}{C_{D,aq}}
$$  \hspace{1cm} (28)

where: $C_{eq,D,em} =$ equilibrium solubility concentration of drug in oil emulsions based on emulsion volume (mg/cm$^3$),

$C_{eq,D,aq} =$ equilibrium solubility concentration of drug in the aqueous phase based on the aqueous volume (mg/cm$^3$),

$K_{m/w} =$ micelle-water partition coefficient.

Using the Equation 28, the hypothetical drug concentration in aqueous phase $C_{D,aq}'$ can be calculated using values for $K_{em/aq}$ and time dependent drug concentration.
inside emulsion droplets, \( C_{D,em} \). During the digestion process, we assumed that digestion products leaving oil droplets were incorporated into micelles, and that the solubilization capacity of aqueous phase, \( C^{eq}_{D,aq} \), increased consequently. Therefore, \( K_{em/aq} \) changes over time as a result of its dependency upon \( C^{eq}_{D,aq} \), and it can be determined by combining the Equations 27 and 28.

### 4.2.2 Estimation of the diffusion coefficients

The diffusion coefficient of the model drug TB, \( D_D \), was estimated using Wilke-Chang equation [174]:

\[
D_{AB} = 7.4 \times 10^{-8} \frac{\sqrt{\Psi_B MW_B}}{\eta_B V_A T^{0.6}}
\]

(29)

where: \( D_{AB} \) = diffusivity of compound A in solvent B (cm\(^2\)/s),

\( \Psi_B \) = constant expressing solvent/solvent interactions

(2.6 for water),

\( T \) = temperature (K),

\( \eta_B \) = viscosity of solvent B (cP),

\( MW_B \) = molecular weight of solvent B (g/mol),

\( V_A \) = molar volume of compound A.

According to Equation 29, the diffusion coefficient of TB, \( D_D \), was 8.3743*10\(^{-6}\) cm\(^2\)/s. The diffusion coefficient of mixed micelles, \( D_m \), was estimated using the Stokes-Einstein equation [175]:

\[
D_{AB} = \frac{kT}{6\pi\eta_B r_A}
\]

(30)

where: \( D_{AB} \) = diffusivity of compound A in solvent B (cm\(^2\)/s),
$k = \text{Boltzman constant } (1.3806 \times 10^{-23}\, \text{m}^2\, \text{kg}\, \text{s}^{-2}\, \text{K}^{-1})$,

$T = \text{temperature (K)}$,

$\eta_B = \text{viscosity of solvent B (Pa}\ast\text{s})$,

$r_A = \text{hydrodynamic radius of the particle (m)}$.

According to Equation 30, the diffusion coefficient of micelles, $D_{m}$, was $6.5578 \times 10^{-11}\, \text{cm}^2/\text{s}$. In both diffusivity estimations, the viscosity of the solvent $\eta_B$ was assumed to be equal to the water value at 37°C (0.00069244 Pa*s, or 0.69244 cP) and the temperature was fixed at the physiological value of 310°K.

### 4.3 Drug absorption and drug metabolism

Kinetic expression describing the rate of drug absorption (mg/sec) across the intestinal membrane was defined by the following first order relationship:

$$\frac{dm_{D,\text{abs}}}{dt} = P_{\text{eff}} A_{\text{int}} C_{D,aq}$$

(31)

where: $P_{\text{eff}} = \text{effective intestinal permeability (cm/sec)}$,

$A_{\text{int}} = \text{surface area of the intestine (cm}^2)$,

$C_{D,aq} = \text{drug concentration in the intestinal aqueous phase (mg/cm}^2)$.

In order to predict drug levels in blood plasma (bioavailability) from the rate of drug absorption and to allow comparisons to \textit{in vivo} data, a proper pharmacokinetic model was incorporated. Consequently, Equation 31 was expressed in terms of blood drug concentration $C_{D,\text{plasma}}$ and drug elimination taking place found in the circulatory system, as:

$$\frac{dC_{D,\text{plasma}}}{dt} = \frac{P_{\text{eff}} A_{\text{int}} C_{D,aq}}{V_{\text{plasma}}} - \frac{0.693}{t^{1/2}} C_{D,\text{plasma}}$$

(32)
where: \( V_{\text{plasma}} \) = total volume of plasma in the body (cm\(^3\)),
\[
t_{1/2} = \text{drug elimination time (sec)}.\]

In the case of low water solubility compounds, solubility in the intestinal fluid is often increased by bile micelles. In this study, the effective concentration available for permeation in the intestinal fluid (\( C_{D,aq} \)) was considered in both the following cases: i) as the sum of the concentrations of free molecules and the bile micelle bound molecules; ii) as only free drug fraction in the water phase of GI fluids.

4.4 **Integrated model to predict oral absorption in the presence of lipids**

The expressions derived for lipid digestion (Equation 16), drug dissolution (Equation 24), drug partition (Equations 27 and 28), and drug absorption (Equation 32) were combined in an integrated system-based model in order to predict the influence of ingested lipids on the overall oral absorption of compounds. Differential equations for the above main processes, mass balance, and expressions for predicting kinetic parameters were incorporated into a MATLAB® code and solved by using a built-in ordinary differential equation solver, ODE45, in MATLAB® by means of the Runge-Kutta numerical solution technique. \[159\]. The program inputs were physical and chemical properties of the drug as well as assumed properties of the GI environment in post-prandial conditions (Table 8). The program outputs were the amount of absorbed drug over time, as well as the concentration of drug present in the lumen, either free drug in the aqueous phase or associated with lipid-based particles. Therefore, the resulting final model simulated all the fundamental processes occurring in the GI tract when a compound is orally co-administered with fat-rich food. Data collected from *in vitro*
measurements, as well as those eventually obtained from *in vivo* experiment, were critically compared to *in silico* simulations in order to validate the model predictions.
5.0 Results and Discussion

Experimental investigations were performed to explore the influence of lipids on *in vitro* lipid digestion kinetics, formation and evolution of colloidal particles, drug dissolution and distribution between phases related to the lipid digestion, and intestinal drug permeability. The model drug TB (Figure 8) was selected based on its physical chemical properties, and carried out for all the aforementioned studies. Mathematical models were developed to describe the fundamental processes involved in oral drug absorption – namely lipid digestion kinetics, drug dissolution and partitioning, intestinal drug permeability – in the presence of food associated lipids. Finally, models’ predictions were compared to and validated based upon the experimental findings.

5.1 Feasibility of the proposed *in vitro* lipolysis models

Intermediate phases produced during lipid digestion can play a key role in the overall drug absorption process. However, traditional *in vitro* dissolution tests don’t generally take into account the complex drug interactions and trafficking issues related to the parallel digestion of lipids and dynamic formation of colloidal structures while lipolysis proceeds. Therefore, *in vitro* lipolysis has been considered an appropriate tool to understand the influence of lipid digestion products on drug dissolution, partitioning with colloidal phases, and ultimate absorption during the lipid digestion process. Previous *in vitro* digestion models have been used mainly to probe drug solubilisation in the aqueous phase during the progress of enzymatic hydrolysis of lipid-based formulation (250-500 mg of lipid substrate). Increasing the lipid concentration, this approach can be used to study the effect of ingested lipids with food on oral absorption of compounds.
In order to simulate the digestion process within the GI tract, *in vitro* lipolysis models have been employed, whose compositions have been updated considering information available in literature concerning *in vivo* conditions. A detailed experimental setup concerning the selected systems is described in Section 3.1 and shown in Figure 6. Briefly, *in vitro* lipid digestion models are comprised of a biorelevant medium, mimicking GI conditions in the fed state. The relevant lipolytic enzyme (pancreatic lipase) and lipid substrates were added in order to initiate the digestion experiment, always performed under continuous magnetic stirring and at the constant temperature of 37°C. The extent of lipid digestion was monitored indirectly by the volume of base (NaOH) added during the experiment.

The updated composition of the biorelevant medium for intestine in fed state is shown in Table 4 and described in details in section 5.1.1. Briefly, the medium was prepared in maleate buffer, in which NaTDC and lecithin were added to represent human bile components. The solution pH value was set to 6.5 to reflect the average duodenal pH in the fed state (Table 2). Many buffer solutions have been used in previous *in vitro* lipolysis experiments, as discussed in the Chapter 2 (Table 3). Among those, with a $\text{pK}_a$ of 6.27 [176] the maleate buffer can achieve an appropriate buffer capacity at the pH value under consideration. In addition, a retardant action of the maleic acid against the rancidity process of fats and oils has been reported [98, 177]. Although sodium taurodeoxycholate (NaTDC) is not the main component of human bile, it has been selected to represent the bile salt mixture due to its good solubility at the target pH. In addition, it has been reported that the type of the BS used has a limited effect on the digestion process [33]. However, there is no complete agreement on this point [96], and
some researchers have preferred using a mixture of BS reflecting physicochemical ratios between components [87, 94, 95, 99]. Lecithin from egg yolk can be used to represent endogenous PL. The system NaTDC/Lecithin as a human bile model has been proposed by multiple researchers [99, 101-105], but in our proposed medium the ratio BS/PL is 3:1. This value is closer to the average ratio (2.5:1) calculated from in vivo studies (Table 2) than the BS/PL ratios (from 3.3:1 to 5:1) commonly used in media for dissolution tests and in vitro digestion models (Table 3). Hay et al. found similar ratios between BS and PL (3:1) in bile extracted from human gallbladders [178].

Soybean oil (C18, long chain TG) was chosen as a main lipid substrate, although it has been shown that homogenized meals, artificial liquid meals, and milk are the most suitable media to simulate GI contents in fed state [91, 98, 179-184]. However, those systems are quite complex: they contain not only lipids but also proteins and carbohydrates. Proteins and carbohydrates can affect drug dissolution and absorption through several interactions [16]. These additional components would make it extremely difficult to understand the relation between digestion progression, lipids and colloidal phases present, and drug interactions. Therefore, for initial studies of these phenomena, the proposed biorelevant medium for fed intestine contains only lipids. Soybean oil was selected as a model for the lipid components of the daily food intake, because ingested lipids are composed of 95% long-chain TG [28]. Oil concentration was fixed at 50 mM, based on the average lipid intake per meal (50g/meal) [28]. Since the proposed in vitro lipolysis model simulated the lipid digestion in the intestine, in order to take into account the action of stomach lipase on ingested lipids, another lipid substrate was also selected. This was made of a mixture of TG, MG, and FA as the mixture of partially digested TGs.
in output from the stomach. The composition of the medium for the intestine in fed state is shown in Table 4. The lipid mixture composition has been calculated considering that the net enzymatic hydrolysis of each TG molecule produces in total two FA plus one MG (Figure 7), and the lingual and gastric lipases are responsible for 30% of the total lipolysis [29]. The pancreatin extract, containing intestinal lipase and colipase, was prepared from porcine pancreatic powder following the procedure proposed by Sek et al. [101]. The final enzymatic activity was 1000 TBU/ml solution, in agreement with the range of 800-1800 TBU/ml solution as reported for the post-prandial state [43, 185].

5.1.1 Characterization of biorelevant media

In order to evaluate if the proposed in vitro systems reflected the physiological properties concerning GI fluids, several investigations of their physical chemical properties were carried out. Buffer capacity, and critical micellar concentration (cmc) of the simulated intestinal fluids were determined according to the experimental plan in section 3.1.1.1. A solution has a buffer capacity of 1 when one equivalent of strong acid or alkali is required to change the pH value of 1 L by one pH unit [186]. It can be determined by titration of 1 L of sample using NaOH or HCl 1 M. Since it was not possible to prepare 1 L of biorelevant medium according to the previous definition, the buffer capacity has been determined by titration of smaller volumes of medium, in a 10-30 ml range. The different volumes of the biorelevant medium, prepared including lipid mixture previously defined, have been titrated using NaOH 1 M, keeping temperature constant at 37°C. Since a linear relationship between the buffer capacity and the volume of the medium used has been determined, the buffer capacity of 1 L of solution has been calculated by extrapolation. The biorelevant medium containing the lipid mixtures has a
buffer capacity of 29.3. This value falls into the range 28-30 for human intestinal fluids in fed states reported in literature [72]. Following the same procedure, the buffer capacity of a simple maleate buffer solution has been also determined, which is 20.3. Therefore, the presence of NaTDC, lecithin, and the lipid mixture contained in the biorelevant medium increased the buffer capacity up to a more physiological value for the fed intestinal state.

The cmc is the concentration at which amphiphilic molecules form micelles in solution. The cmc of the solutions containing sodium taurodeoxycholate (NaTDC) and lecithin in maleate buffer have been investigated at pH 6.5 and temperature of 37°C. No lipids were added during these experiments, since the investigation focused on cmc of endogenous micelles, which are natural components of the human bile in the absence of food. Briefly, three sets of NaTDC/Lecithin solutions (molar ratio α (NaTDC) = 1, 0.75, 0.50) were prepared and analyzed using two independent methods, tensiometric technique and a spectroscopic (UV-VIS absorption) technique, as described in section 3.1.1.2. The total surfactant concentration ranges from 0.1 mM to 8 mM for each set. The experimental results are presented in Figure 12, and the determined cmc values are summarized in Table 6. The two techniques used have shown a good agreement in detecting the cmc points except for the set of solutions at α (NaTDC) = 0.75. Further investigations are in progress to clarify the observed disagreement for α (NaTDC) = 0.75. However, the cmcs determined are in good agreement with literature values concerning analogous binary systems of bile salts/phospholipids, which reported cmc below 3 mM.
Table 6: Summary of the cmc values of NATDC/lecithin system investigated at different molar ratios.

<table>
<thead>
<tr>
<th></th>
<th>Tensiometric method</th>
<th>Spectroscopic method</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ (NaTDC) = 1</td>
<td>0.98 mM</td>
<td>0.77 mM</td>
</tr>
<tr>
<td>$\alpha$ (NaTDC) = 0.75</td>
<td>1.84 mM</td>
<td>0.44 mM</td>
</tr>
<tr>
<td>$\alpha$ (NaTDC) = 0.50</td>
<td>0.91 mM</td>
<td>0.81 mM</td>
</tr>
</tbody>
</table>

Figure 12: Determination of the cmc in solutions containing NATDC/lecithin prepared at molar ratios $\alpha$. In the tensiometric method, the concentration at which the surface tension shows a break is considered the cmc at that particular molar ratio. In the spectroscopic method, the concentration at which the maximum wavelength of absorbance for Rhodamine 6G (dye) shows a shift is considered the cmc at that particular molar ratio.
5.1.2 Sensitivity of digestion kinetics

Despite of a common experimental procedure, previous *in vitro* lipolysis models present many differences in the composition of biorelevant media used, such as pH, buffer type and concentration, ionic strength, type and quantity of lipid substrates (Table 3). Those experimental parameters are known to affect the enzymatic activity of the lipase, and consequently the rate of lipolysis. Further, the high variability among the proposed *in vitro* lipid digestion models may make difficult compare results obtained from different studies. In order to evaluate the sensitivity of *in vitro* lipolysis, experiments were conducted in which pH, calcium concentration, and type of substrate were varied, and the resulting change in digestion kinetics was assessed.

Briefly, the lipid digestion experiments were initiated by adding lipase/colipase enzymes and a lipid substrate – simple TG or partially digested lipids (lipid mixture). The extent of lipid digestion was monitored indirectly by recording the volume of NaOH added during the experiment to titrate the FA formed from the TG hydrolysis.

In the quantitative assessment of system sensitivity to experimental parameters, *in vitro* lipolysis experiments were performed according to $2^4$ multifactor designs shown in Table 5. Statistical analysis was carried out by Minitab software to determine the significance of each factor.

**Effects of pH and calcium concentration** - *In vitro* lipolysis of soybean oil has been performed in maleate buffer (Triz-ma 100 mM, CaCl$_2$*H$_2$O 5 mM, NaCl 65 mM, NaN$_3$ 3 mM, and NaOH) at two different pH values, 7.5 and 6.5 for 50 minutes, keeping the temperature constant at 37°C. Experiments have been repeated increasing the concentration of calcium to 10 mM at both pH values. At pH 7.5, the rate of lipolysis,
reported as amount of FA produced, was higher than that detected at pH 6.5 (Figure 13). This trend was observed at calcium concentration of 5 mM (‘a’ and ‘(1)’ profiles) and at 10 mM (‘ab’ and ‘b’ profiles) as well, due to the fact that the lipase enzyme is more active in vitro at pH values around 8. However, pH 6.5 was closer to physiological pH values (5.8-6.0) reported for human intestines in fed states. Experiments performed keeping constant all experimental conditions except of calcium concentration have shown that the rate of lipolysis increased as the calcium concentration increased. This trend was more evident at pH 7.5 (Figure 13, B) that at pH 6.5 (Figure 13, A). Calcium ions present in solution might be responsible for removing FA from the oil-enzyme interface, where FA acted as inhibitors for the lipase action. Therefore, a higher calcium concentration might lead to activate the lipase in the presence of lipolysis products.

**Effects of type of substrate** - *In vitro* lipolysis has been performed in biorelevant medium (NaTDC 12 mM, lecithin 4 mM in Triz-ma 100 mM, CaCl$_2$•H$_2$O 10 mM, NaCl 65 mM, NaN3 3 mM, and NaOH up to target pH) for 50 minutes, keeping the temperature constant at 37°C. Experiments have been performed at two different pH values, 7.5 and 6.5, and using two different substrates, such as soybean oil 35 mM and lipid mixture (soybean oil 35 mM, sodium oleate 30 mM, glyceryl monooleate 15 mM). At pH 6.5, the rate of lipolysis of the lipid mixture increased during the first 5 minutes, but then went to plateau until the end of experiments (‘bd’ and ‘d’ profiles). Unlikely, the rate of lipolysis of soybean oil continued to increase constantly over time (profiles without ‘d’). The products of lipolysis (sodium oleate and glyceryl monooleate) contained in the lipid mixture seemed to inhibit sooner the lipolysis reaction when they were added at the beginning of the experiment as lipid mixture. This trend was not
observed at pH 7.5 (‘abd’ and ‘ad’ profiles), probably because the more basic pH might have compensated and removed the inhibition action of FA.

**Presence of bile micelles** - Experiments have been repeated using soybean oil in maleate buffer only (not containing NaTDC and lecithin) at the same experimental conditions described above. In maleate buffer, the rates of lipolysis appeared to be lower than those observed in analogues experiments performed in biorelevant medium, supporting the fact that the presence of NaTDC and lecithin increased the rate of lipolysis by interacting with fatty acids produced during the digestion process [8].

In summary, the rate of FA (mmol/min) produced increased with: pH (from 6.5 to 7.5), calcium concentration (from 5 mM to 10 mM), and concentration of micelle-forming species. It decreased when a lipid mixture was used instead of soybean oil.

The impact of various experimental factors on lipid digestion was investigated also statistically over 50 minutes of enzymatic reaction. Statistical analysis performed on the extent of lipolysis, expressed as total fatty acid produced after 50 minutes of digestion, showed that pH was significant at $\alpha = 0.05$, while other factors were not statistically significant. However, the lipolysis profiles clearly changed as a function of different treatments, in particular during the first 10 minutes of digestion (Figure 13), suggesting that the experimental factors studied are more relevant in the initial part of lipolysis.
5.2 Characterization of the colloidal structures formed during in vitro lipolysis

The complex colloidal particles formed by interactions between bile components and lipolysis products seem to play a key role in dissolution and absorption of
compounds orally delivered. Investigation of colloidal particles’ morphology and composition are needed to enable modeling mathematically their role during the oral drug absorption. A preliminary characterization of these colloidal structures has been performed using i) quasi-elastic dynamic light scattering (DLS) technique coupled with \textit{in vitro} lipolysis, and ii) small angle neutron scattering (SANS) method.

5.2.1 Determination of size and Zeta-potential

The dynamic evolution of the size of colloidal particles formed during the \textit{in vitro} lipolysis experiments was monitored by DLS as described in section 3.2.1. Briefly, two sets of experiments were conducted by introducing respectively soybean oil or lipid mixture into the biorelevant medium at pH 6.5 and at 37°C. Samples were taken at defined times, up to 50 minutes. Digestion was then stopped with the addition of an enzyme inhibitor (4-bromobenzeneboronic acid 0.2 g/ml in methanol [114]), and samples were analyzed for particle size and zeta potential.

The biorelevant medium before addition of any lipid substrates contained particles, probably mixed micelles, with a hydrodynamic diameter less than 10 nm (Figure 14, A). This result was in agreement with previous \textit{in vivo} studies concerning human intestinal fluids in pre-digestion conditions [35]. During the digestion of soybean oil, detected particles suddenly increased to 100 nm in the early stage of digestion, after 5 minutes the addition of lipase and soybean oil (Figure 14, B). Then, it was observed a further increase in particle size as the lipid digestion proceeded, followed by a fluctuation in size for the rest of lipolysis (figure 15, A). This trend was observed also for the digestion of the lipid mixture (Figure 15, B). In this case, however, the size of particles was even bigger before the beginning of the digestion process (time 0), and then it
slightly decreased after 50 minutes. These observations might indicate that vesicles, formed during early stages of the enzymatic hydrolysis of lipids, are dynamic structures in continuous evolution as they interact with increasing amount of lipid digestion products. Similar hypothesis was also proposed by Fatouros et al. based on Cryo-TEM observations of in vitro lipolysis samples [110, 114].

![Particles size distribution](image)

**Figure 14: Particles size distribution A) in biorelevant medium in the absence of lipids, and B) after 5 minutes of the beginning of in vitro lipolysis.**

Zeta potential measurements of lipid emulsions were carried out on the fashion as described above. Results ranged from approximately +5 mV to −70 mV and confirmed the stability of the systems (Figure 16). However, Zeta potential values showed high variability between trials, and a complex trend over the digestion time of both soybean oil and lipid mixtures. Additional studies are ongoing to relate digestion kinetics and resulting colloidal phases to size and zeta potential data obtained.
5.2.2 Examination of lipid-based colloids by means of SANS

Ingested lipids, typically originating from food, can impact the extent of absorption of compounds in several ways. These effects originate, in large part, from lipid-based colloidal structures formed in the GI tract during the lipid digestion. In order to thoroughly characterize these colloidal structures and their changes in function of ingested lipids over time, biorelevant media simulating intestinal conditions in the fed state were investigated by means of SANS. Three sets of investigation were performed, concerning simulated GI conditions before and during the digestion process. These samples have been prepared and analyzed accordingly to the experimental conditions described in section 3.2.2. Briefly, samples containing no triolein simulated pre-digestion conditions, samples prepared at different concentrations of triolein represented different

Figure 15: Evolution of particles mean diameters in samples collected during \textit{in vitro} lipolysis of A) soybean oil and B) lipid mixture.

Figure 16: Zeta potential values recorded during \textit{in vitro} lipolysis of A) soybean oil, and B) lipid mixture.
stages of the lipid digestion process, and sample containing triolein and lipase performed a dynamic lipid digestion process.

SANS profiles of samples simulating pre-digestion conditions showed the presence of colloidal particles - mixed micelles of bile salt and lecithin - having ellipsoidal shapes with gyration radius Rg of 2.85 nm. These findings were in agreement with our previous DLS investigations, which have detected micelles with a diameter less than 10 nm in pre-digested samples, as well as DLS studies on micelles in human intestinal fluids [35].

Samples containing different amount of triolein showed the presence of two types of colloidal particles of different size (Figure 17). Mixed micelles were still detected at high scattering angle values (Q > 0.01), while bigger particles of oil droplets were also

![Figure 17: SANS profiles, reported in logarithmic scale, of samples containing triolein at different concentrations, which simulate diverse stages of the lipolysis process.](image-url)
present (low scattering angle values, $Q < 0.01$). From this preliminary analysis, we provided evidence that mixed micelles and oil droplets coexisted under the conditions investigated, while other techniques employed previously (DLS) failed in resolving the size of particles having such heterogeneous size distributions.

Additional SANS investigations concerning colloidal particles evolution during in vitro simulation of lipolysis processes was performed. In vitro digestion of 50 mM of triolein was performed over 60 minutes as described in case of soybean oil (Section 3.1.2). Upon data reduction and fitting analysis, the data showed that there was a regular increment in the gyration radius $R_g$ of micelles over the digestion time (Figure 18). These finding seemed to support the hypothesis of solubilization of digestion products (e.g. FA) into the fed state mixed micelles. However, further analysis of the collected SANS data will be performed in order to establish the nature of micelles/oil droplets interactions and the evolution of vesicles during digestion, conditional to obtain access to SANS facilities.

![Figure 18: Dynamic evolution of the gyration radius of micelles during the in vitro lipolysis shows a regular increment over time.](image-url)
5.3 TB dissolution and solubility in the presence of lipolysis

Lipid digestion kinetics, drug solubility and drug dissolution kinetics for ingested food lipids system (Figure 9) were studied experimentally. Simulations describing drug transport in presence of lipid digestion were run and validated using experimental data.

5.3.1 Calculation of digestion kinetic constants

The digestion kinetic constants for lipolysis of 50 mM soybean oil in the fed state biorelevant medium were determined according to Equation 16 as $k_{dig} = 3.6 \pm 0.2 \times 10^{-9}$ mmol/cm$^2$s and $k_{inh} = 4.3 \pm 0.3 \times 10^{-4}$ 1/s, based on experimental measurement of digestion kinetics (Figure 13). The excellent model fit (Equation 16) supports the theoretical description of digestion rate as proportional to emulsion droplet surface area and inhibited at a level proportional to the concentration of fatty acids produced.

Figure 19: Experimental digestion profile (data points) of soybean oil in the fed state biorelevant medium – measured during the dissolution experiment of TB – agreed well with the proposed digestion kinetics model (continuous line).
5.3.2 Drug dissolution without lipids

Dissolution kinetics and solubility values of the model drug TB were measured in maleate buffer, and in fed state biorelevant medium by means of HPLC (Figure 14). Significant (8 fold) improvement in solubility was observed for TB in fed state biorelevant medium relative to maleate buffer due to the presence of mixed micelle-forming species, NaTDC and lecithin (Table 7). Improvement in dissolution kinetics was also evident from maleate buffer to fed state biorelevant medium, as shown by the steeper slope of the initial part of the dissolution curve in medium containing micelles.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Solubility (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maleate buffer</td>
<td>0.020 ± 0.001</td>
</tr>
<tr>
<td>Fed state biorelevant medium</td>
<td>0.145 ± 0.006</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>217.8 ± 2.5</td>
</tr>
</tbody>
</table>

5.3.3 Drug dissolution in the presence of lipids

In dissolution experiments coupled with in vitro lipid digestion, dissolution kinetics of the model drug TB was determined as the digestion of 50 mM soybean oil - representing the lipid intake – proceeded (Figure 15). The fed state biorelevant medium enriched by ingested lipids showed an approximately 4-fold further enhancement in solubilization power after 3 hours of dissolution-digestion, due to the high solubility of TB in soybean oil (Table 7) and enhanced solubilization capacity of micelles. During lipolysis, TB concentration in solution (aqueous and oil phases) rose gradually over a time frame of approximately an hour, as opposed to the rapid increase to approximately
70% of the equilibrium solubility value in the case of dissolution experiments in fed state biorelevant medium in the absence of lipid digestion (Figure 15). Simulations were run to test the ability of the proposed model to describe drug dissolution in the presence of simulated intestinal fluid and lipid digestion. Parameters used in simulations, determined as described above, are shown in Table 8. Comparisons of simulation predictions with experimental dissolution profiles are (Figures 14 and 15) indicate favorable prediction of impact of biorelevant media and lipid digestion on drug dissolution kinetics.

Kinetic constants calculated separately for each process, lipid digestion and drug partitioning in the absence of digestive enzyme were used to solve the developed mathematical model considering both processing occurring simultaneously in order to predict their synergistic effect on dissolve drug concentration over time during digestion. Input parameters to the developed model are given in Table 8.
Figure 20: Experimental dissolution profiles and simulations of the model drug TB in maleate buffer and in the fed state biorelevant medium (n=3 for each dissolving medium).

Figure 21: Dissolution profiles of the model drug TB in the fed state biorelevant medium and during the digestion of 50 mM of soybean oil (n=3 for each dissolving medium).
Table 8: Input parameters to the developed models

<table>
<thead>
<tr>
<th>Input parameters</th>
<th>Drug dissolution from solid dosage form</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physiological parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Bile salt concentration</td>
<td>$12 \times 10^{-3}$ mmol/ml</td>
</tr>
<tr>
<td>Phospholipid concentration</td>
<td>$4 \times 10^{-3}$ mmol/ml</td>
</tr>
<tr>
<td>Critical micelle concentration [1]</td>
<td>$1.6 \times 10^{-3}$ mmol/ml</td>
</tr>
<tr>
<td><strong>Oil and drug parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Mean droplet diameter of oil emulsions [5]</td>
<td>386 nm</td>
</tr>
<tr>
<td>Molecular weight of TB</td>
<td>276.35 mg/mmol</td>
</tr>
<tr>
<td>Number of digestible FA per mole of oil [8]</td>
<td>2</td>
</tr>
<tr>
<td>Initial concentration of TB solution volume</td>
<td>0.003618 mmol/ml</td>
</tr>
<tr>
<td>Initial volume ratio of oil in intestinal lumen</td>
<td>4.8:100</td>
</tr>
<tr>
<td>(Molar) solubilization of TB in BS/PL micelles</td>
<td>0.2115 mmol/ml</td>
</tr>
<tr>
<td>Solubility of TB in maleate buffer</td>
<td>$7.2 \times 10^{-5}$ mmol/ml</td>
</tr>
<tr>
<td>Solubility of TB in fed biorelevant medium</td>
<td>$4.5 \times 10^{-4}$ mmol/ml</td>
</tr>
<tr>
<td>Solubility of TB in soybean oil</td>
<td>0.788 mmol/ml</td>
</tr>
<tr>
<td>Stationary diffusion layer, $h^1$</td>
<td>20 μm</td>
</tr>
<tr>
<td>Particle size of TB$^2$</td>
<td>20 μm</td>
</tr>
<tr>
<td><strong>Kinetic parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Diffusion coefficient of TB, $D_d$</td>
<td>$8.3743 \times 10^{-10}$ m$^2$/s</td>
</tr>
<tr>
<td>Diffusion coefficient of micelles, $D_m$</td>
<td>$6.5578 \times 10^{-11}$ m$^2$/s</td>
</tr>
<tr>
<td>Oil digestion kinetic constant, $k_{dig}$</td>
<td>$3.6 \times 10^{9}$ mmol/cm$^2$/s</td>
</tr>
<tr>
<td>Oil digestion inhibition kinetic constant, $k_{inh}$</td>
<td>$4.3 \times 10^{-4}$ 1/s</td>
</tr>
<tr>
<td>TB uptake constant into oil, $P_{rel}$</td>
<td>$3.52 \times 10^{-8}$ cm/s</td>
</tr>
</tbody>
</table>

Notes: 1: assumed to be equal to initial particles’ radius [9]; 2: estimated based on particles’ separation by controlled sieve mesh; all the other parameters were calculated as explained in the text.
In the drug dissolution with concurrent lipid digestion study, we investigated the effects of ingested food lipids and the lipid digestion process on drug transport in a simulated intestinal lumen. The presence of lipids and their digestion products alter greatly the intestinal lumen composition, which consequently influences compounds’ solubility, dissolution kinetics and partitioning among phases in the GI fluids. The effects of dynamic lipid digestion on drug transport were investigated. For the in vitro food lipids system, the digestion rate was described using an equation that relates it to the total droplet surface area available to the enzyme and takes into account the lipase inhibition due to the accumulation of FA at the oil/water interface. FA accumulation at the oil/water interface is related to the degree of their solubility in the aqueous phase. FA having short and medium chains were reported to not inhibit or to inhibit less the lipase than long chain FA [8]. Similarly, presence of higher concentration of bile salt/phospholipids in the aqueous phase may decrease the level of FA accumulation at the interface by increasing the solubilization capacity of the medium. In addition, FA inhibition action described here mainly characterizes the in vitro digestion systems, while it is expected to be less relevant in vivo where FA are continuously removed by absorption. Based on this equation, digestion rate constants $k_{dig}$ were calculated as $3.6 \times 10^{-9}$ mmol/sec*cm$^2$ and $4.7 \times 10^{-9}$ mmol/sec*cm$^2$. It should be noted that measured digestion profiles were based on titration of FA, and it has recently been reported that titration of FA can lead to underestimation of extent of digestion due to only a portion of the FA being ionized [187]. Thus, a more rigorous analysis of digestion rate may affect calculated digestion rate constants.
Our investigations regarding the dissolution rates of TB showed that the dissolution enhancement did not follow the same trend as solubility. The dissolution kinetics was faster in the fed state biorelevant medium compared to maleate buffer, following the same trend as in solubility. During food lipid digestion, there was an approximately 4-fold enhancement in solubility, comparing equilibrium solubility in fed state medium to drug dissolved at the conclusion of combined dissolution and digestion experiments. However, during food lipid digestion, the drug dissolution rate from solid dosage form was not enhanced to the same extent as solubility. The initial drug dissolution rates (first 5 minutes of dissolution experiment) were comparable in the presence and the absence of the digestion process (0.14 mmol/min*ml vs. 0.12 mmol/min*ml respectively). However, the overall drug dissolution in the presence of digestion was more gradual over a longer period of time than without digestion, despite the observed increased solubility (approximately 4-fold) due to the presence of oil droplets and lipid digestion products. This was in agreement with previous studies showing that the solubility of lipophilic drugs in simulated intestinal fluids increased proportionally to an increased content of surfactants, while the dissolution rates did not increase proportionally [70, 172]. The lack of a direct proportionality between the enhancement in solubility and the enhancement in dissolution kinetics after food intake was also confirmed by a study [188] that employed real human intestinal fluids obtained under fed conditions. Thus, the present observations support the in vivo relevance of including in vitro lipolysis models in drug dissolution tests. The lack of significant improvement in dissolution kinetics of TB observed during the lipid digestion process might be related to the relative kinetics of partitioning between the dissolved drug and
soybean oil droplets, and associated impact on concentration of drug in the aqueous solution, and thus the driving force for dissolution. Further investigations carried out by means of EPR revealed that indeed the transport of dissolved TB from the fed state biorelevant medium into soybean oil droplets occurred over a time scale similar to that of drug dissolution (3 hours) (Section 5.3.3, Figures 15 and 16). These results support the necessity of modifying the classical Noyes-Whitney equation in order to include explicitly: i) the role of colloidal particles in mass transport, and ii) the partitioning process between dissolved drug and oil droplets. Our results related to food lipids system indicate strong influences of the presence of lipids and lipid digestion on drug transport. During food lipid digestion, the drug dissolution rate from solid dosage form did not increase at the same extent as solubility (approximately 4-fold enhancement), as noted above.

Drug solubilization/release into aqueous phase during in vitro lipid digestion was previously studied using static [187, 189, 190] and dynamic lipolysis models [164, 191]. The most conventional way of measuring drug partitioning during digestion so far has involved sampling during in vitro digestion, centrifugation of collected samples in order to separate aqueous phase from the oil formulation phase, and quantification of drug concentration in the aqueous phase [164]. While this method has proven to give, in many instances, reliable information about the degree of drug release and partitioning and possible performance of the specific drug delivery system, it may result in misinterpretation of time profiles of this dynamic process since the time spent during centrifugation has been as much as 135 minutes. In our study, EPR was used as a non-invasive, online method to monitor model drug distribution in different phases. EPR
Spectroscopy offers advantages of a non-invasive, real-time method for analysis of the amount of compound in each phase (water, micelles, and oil formulation) during in vitro digestion, and is thus a powerful tool to monitor drug delivery processes [109, 192].

Recently, Sugano [61] summarized the effects of fed state intestinal conditions on oral drug absorption via interactions with bile micelles. In particular, the interactions between compounds and bile micelles present in the GI fluids were proposed as the theoretical basis for food effects on co-administered compounds, supporting the central role of micelles in overall oral absorption. Our modeling approach included lipids and dynamic lipid digestion products interacting with endogenous colloidal particles and drug compounds in addition to bile micelles. Furthermore, the model aimed to unify the mechanism behind the observed effects of ingested lipids on two processes - drug dissolution and drug release – based upon fundamental principles of mass transport across oil-water-micelle interfaces as lipolysis proceeds. The significance of predicting drug transport between different colloidal phases present during lipid digestion lies in the assumption that simultaneous absorption occurs in vivo, and that this absorption process is driven by aqueous drug concentration. Although overall drug absorptive flux has been related to total drug concentration [193], multiple literature reports indicate that drug absorption is driven by the concentration of drug in the aqueous phase [192-194].

Several studies [155, 175, 195] have shown that the classic Noyes-Whitney equation might have to be modified when the dissolution of solid compound takes place in solutions containing solubilizing agents. Previously, we have demonstrated that a model considering micelle-drug partitioning as a pseudo-equilibrium process and an unstirred boundary layer surrounding dissolving particles across which drugs and
micelles diffuse could effectively describe drug dissolution in simulated intestinal fluids [172]. However, this experimental study and modeling of dissolution of solid compounds did not include the presence of lipid emulsions and the lipid digestion process. In this study, therefore, ingested lipids and the lipolysis process were included in order to more closely mimic the \textit{in vivo} dynamic conditions of the GI fluids after lipid intake. The proposed model presented herein took into account the mass transport of the model drug TB between the aqueous phase – containing micelles – and the oil droplets during oil digestion [173, 196].

Close comparison between simulations of drug dissolution during digestion and experimental results (Figures 15 and 16) supports the validity of expressions for kinetic processes for the ingested lipids system. In addition, several assumptions made solving the model such as increased aqueous solubilization directly related to free fatty acid concentration, and shrinkage of emulsion droplet size proportional to the amount of fatty acid leaving droplets, were supported by the reasonable accuracy of simulation predictions. However, coalescence during digestion of lipid-based systems was reported previously [8]. In cases where oil droplet flocculation and coalescence is prominent during digestion, the model assumption that droplets decrease in size proportional to fatty acid leaving the droplets may not be valid. Lipid digestion was also associated with the formation of several liquid crystalline phases at the water-lipid interface at different stages of the lipolysis [197]. Liquid crystalline formation was also correlated with the type of the oil digested (long chain vs medium or short chain triglyceride) [100]. Our model was tested using a single long chain triglyceride, soybean oil. It should be noted that based on the lipid type, modification on the droplets surface structure (liquid crystal
phases) might occur during digestion, in which case model validity might potentially change. Furthermore, we assumed that the enhanced drug solubilization observed during the lipid digestion was directly proportional to the surfactants’ concentration. Simple micelle inclusion of FA may be an over-simplification of the more complex colloidal system formation during intestinal lipolysis. However, lacking insight into how colloidal species evolve over time, a first approximation was used to model mathematically the enhancement in solubilization capacity of such colloidal systems due to digestion. Increased solubilization capacity of aqueous phase during digestion in some cases was linked to a supersaturation phenomenon, [198] where an initial supersaturation status is followed by drug precipitation. At the drug load employed in this study, we did not observe a supersaturation as suggested by the lack of precipitate in samples upon centrifugation. Therefore, we did not consider supersaturation and precipitation processes in the presented model. These results substantiate the concept that mechanistic studies based on physiologically relevant in vitro experiments can provide a better prediction of drug dissolution in the presence of ingested lipids; they can ultimately be combined with permeability studies and pharmacokinetic models, enabling prediction of the overall impact on drug absorption and bioavailability.

5.4 Drug tracking and partition by electron paramagnetic spectroscopy (EPR)

The complex colloidal particles formed by interactions between bile components and lipolysis products seem to play a key role in drug dissolution process. In order to understand the kinetics of drug partition between phases (aqueous, micellar, and oil phases) formed before and during in vitro digestion, in vitro lipolysis experiments were coupled with EPR. This technique has the capability of detecting and quantifying radicals
that partition in phases having different polarity. The spin probe TEMPOL benzoate (TB), shown in Figure 8 was selected as a model for poorly water-soluble moderately lipophilic drug with an octanol/water partition coefficient (log P) of 2.46. In lipolysis experiments coupled with EPR, TB was added to the biorelevant medium before the beginning of the lipolysis process and tracked between phases before and as the digestion did proceed. EPR spectra of TB in separate environments (maleate buffer, biorelevant medium, soybean oil) were also acquired in order to obtain simulation parameters for resolving EPR multi-component spectra of pre and post-lipolysis conditions. Simulation of the EPR spectra was performed by means of Multicomponent, a LabVIEW program for fitting multi-component EPR spectra of nitroxide spin probes [166]. Experimental conditions and line shape fitting method are explained in details in Section 3.3.2.4.

5.4.1 EPR spectra of TB acquired in separate environments/solutions

In an effort to perform online, quantitative, non-invasive tracking of drug partitioning between aqueous and colloidal phases, electron paramagnetic resonance (EPR) was used to track the selected model drug, the spin probe TB. Properties of the probe’s microenvironment, such as microviscosity [199] and micropolarity [200], determine the shape of the EPR spectrum, thereby providing a useful method to study model drug distribution in multi-component systems [201]. In order to perform multicomponent analysis of complex spectra collected during in vitro digestion that reflect probe distribution in three different phases (oil, micelles, buffer), EPR spectra of TB in the separate environments were recorded in the absence of digestive enzyme (Figure 22). Differences in peak to peak distances (a_N), positions (g_iso) and peak widths among EPR spectra, especially evident in the third peak in Figure 22, enable resolution of
the different components of the spectrum by multicomponent lineshape fitting. In turn, the relative scaling factors of the components in the fitted spectrum give information on the probe distribution in the different environments (oil vs. vesicles and micelles vs. buffer). Lineshape fitting was first carried out on single component spectra of TB in buffer and in soybean oil. The dynamic and magnetic parameters obtained from these fit were then fixed in two component analysis of fed state intestinal fluids, which containing a micellar and an aqueous phases. The dynamic and magnetic parameter values obtained are in agreement with previously reported values [201]. The EPR spectra of TB in maleate buffer (Figure 22, A) show hyperfine coupling constants of 16.91 G, which is typical for polar media [201]. Due to the low viscosity of buffer, the amplitude and the line width of the three EPR lines are almost equal. In the more viscous soybean oil (Figure 22, C), the lines broaden, and the hyperfine coupling constant is smaller (15.49-15.68 G), indicating an apolar environment. The EPR spectra shows three lines of decreasing height as one goes from low to high field: This effect is characteristic of anisotropic motion of the spin probe, i.e. when the probe does not have free mobility in all directions. Similar to the spectrum in soybean oil, the EPR spectrum of TB in mixed micelles (Figure 22, B) shows three peaks of decreasing height and decreased mobility due to incorporation into/association with mixed micelles. EPR spectrum of TB in micelles also show a second component on the third peak, specifically evident in fed state biorelevant medium, arising from the distribution of the spin probe TB between buffer and micellar phases. Therefore, spectra of TB in micellar solutions (fasted and fed) were simulated with two components: a micelle component (x on Figure 22) with a coupling constant $a_N$ of 16.16 and 16.27 G for fed and fasted states respectively, and buffer
component (y on figure) with a coupling constant $a_N$ of 16.88-16.91 G. The determined hyperfine coupling constants of TB in mixed micelles lie between the polar values of maleate buffer and the nonpolar values of the oil phases, indicating an intermediate polarity.

![Graph showing EPR spectra and table of estimated constants](image)

**Figure 22: EPR spectra of TB dissolved in A) maleate buffer, B) biorelevant medium containing micelles, C) soybean oil, and D) relative quantification of their hyperfine coupling constant $a_N$.**

Satisfactory fits could only be obtained by including a small amount of Heisenberg spin exchange, which accounts for broadening due to the presence of oxygen in the sample, and any residual broadening due to probe-probe collisions at the concentrations used. Table 9 summarizes the estimated isotropic $^{14}$N hyperfine coupling constant ($a_N$), rotational diffusion constant ($R$), isotropic g-value and Heisenberg spin exchange constant ($\omega_{ss}$) for TB in each environment via fitting. Magnetic and rotational diffusion constant parameters for buffer and soybean oil were estimated for TB in separate experiments and fitting simulations, whereas parameters for TB in micelles were estimated by simulating the spectrum of TB in simulated intestinal fluid (probe...
distribution in micelles and buffer), while keeping tensor values for the buffer component constant. These parameters were used as initial values while fitting spectra collected during the course of in vitro soybean digestion.

<table>
<thead>
<tr>
<th></th>
<th>Maleate Buffer</th>
<th>Soybean Oil</th>
<th>Fed State Micelle</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_N/\gamma_e$ (G)</td>
<td>16.91±0.06</td>
<td>15.42±0.03</td>
<td>16.16±0.11</td>
</tr>
<tr>
<td>average g</td>
<td>2.0058±0.0012</td>
<td>2.0058±0.00005</td>
<td>2.0055±0.0001</td>
</tr>
<tr>
<td>log$_{10}$ R·sec</td>
<td>9.61±0.04</td>
<td>8.69±0.10</td>
<td>8.55±0.09</td>
</tr>
<tr>
<td>log$<em>{10}$ $\omega</em>{ss}$·sec</td>
<td>6.43±0.09</td>
<td>7.01±0.13</td>
<td>7.00±0.15</td>
</tr>
</tbody>
</table>

**5.4.2 EPR analysis of TB in different micelle concentration solutions**

In fasted state biorelevant medium (5 mM NaTDC and 1.25 mM lecithin), 37% of TB is located in the micellar phase. In the biorelevant medium simulating fed state conditions (12 mM NaTDC and 4 mM lecithin) 30% of the model drug TB is located in the aqueous phase and 70% is incorporated in the mixed micelles. We also investigated the effect of varying bile salt/phospholipid concentrations in biorelevant media on TB partitioning (Figure 23). At a concentration of 6 mM NaTDC and 2 mM Lecithin 50% of TB molecules were located in the mixed micelles, while this fraction increased to approximately 85% when increasing the concentration of two surfactants up to 60 mM and 20 mM, respectively. Micelle samples prepared using bile extract in fed state conditions (16 mM), did not show an evident hydrophobic X component on the third peak, although samples contained bile components in concentration (16 mM) comparable
to the fed state biorelevant medium used in this study (NaTDC 12 mM and 4 mM lecithin). In this medium, approximately 50% of TB was associated with mixed micelles.

**Figure 23:** EPR spectra of TB dissolved in micelle solutions prepared in
A) NaTDC 60mM/Lecithin 20 mM,
B) NaTDC 12mM/Lecithin 4 mM,
C) NaTDC 6mM/Lecithin 2 mM,
and D) bile extract 16mM.

### 5.4.3 Drug partitioning in the absence of lipid digestion

Drug partitioning of TB in the absence of digestion was investigated over time in solutions simulating food lipid ingestion, where the TB is initially located outside of food lipid emulsions. Upon the addition of soybean oil into the fed state medium, there was a significant change in the distribution of the model drug TB among phases over time. Before the addition of soybean oil into the fed state biorelevant medium, 70% of the
dissolved drug compound TB was associated with micelles. After the addition of soybean oil to this medium, EPR spectra of pre-lipolysis samples showed an increasing distribution of TB into the oil and micellar phases within 180 minutes (denoted by X in the Figure 24). The EPR spectra recorded in the absence of digestion represented complex spectra containing up to three different components (aqueous, oil, micellar), which were resolved using fitting parameters reported in Table 9. After the addition of soybean oil to this medium, EPR spectra of pre-lipolysis samples showed an increasing distribution of TB into the hydrophobic phases (oil and micelles) over 180 minutes (denoted by X in the Figure 24). The amount of the model drug TB in the oil phase increased up to 32%, while the amount of TB associated with mixed micelles was approximately 55% after 180 minutes (Figure 25).

![Figure 24: EPR spectra of TB dissolved in the fed state biorelevant medium containing soybean oil in the absence of digestion. “x” indicates the peak location of hydrophobic phases, “y” indicates the hydrophilic phases.](image_url)
Drug partitioning during in vitro lipid digestion

The distribution of TB was investigated in the presence of the lipolysis of soybean oil using an in vitro system simulating food lipid ingestion. The lipid digestion kinetics was investigated in two different experimental conditions: i) During the simultaneous dissolution of the solid compound TB; and ii) After the complete dissolution of the model drug TB in the fed state biorelevant medium, as detailed explained in Section 2.3. Digestion of soybean oil reached a plateau after 80 minutes with approximately 25% of oil digested (Figure 19). During the dissolution of solid TB and the concurrent enzymatic hydrolysis of soybean oil, EPR spectra results indicated a predominant partitioning of the spin probe TB into the colloidal phases over 3 hours of in vitro digestion (Figure 26, A). Multi-component fitting analysis of the recorded EPR spectra allowed quantitative determination of the amount of the spin probe TB in each phase over the time of digestion experiments (Figure 26, A). In the early stage of the drug dissolution and the simultaneous lipid digestion processes (15 minutes) the model drug TB distribution in oil, colloidal and buffer phases was approximately: 46%, 44% and 4%, respectively. Over the course of 180 minutes of in vitro digestion, there was an increase in the relative

Figure 25: Distribution of the model drug TB in different phases in the absence of digestion.
percentage of TB in the soybean oil and in the colloidal phase, and a decrease in the buffer. After 180 minutes of lipolysis, the distribution of TB was approximately 28%, 67% and 6% respectively (Figure 27, A).

The EPR spectra recorded during the lipid digestion of soybean oil, which was performed in the fed state biorelevant medium containing the completely dissolved TB, are showed in Figure 26, B. Before the beginning of the digestion process, the model drug TB was mainly located outside the oil phase (approximately 87%). Over the course of 90 minutes in vitro digestion, there was an increase in the relative percentage of TB in the soybean oil and in the colloidal phase, and a decrease in the buffer. After 90 minutes of lipolysis, the distribution of TB was approximately 28%, 67% and 6% respectively (Figure 27, B).

In the present study, we investigated the effects of ingested lipids and the lipid digestion process on drug partitioning and transport among phases in simulated intestinal fluids by means of EPR spectroscopy. We considered food-associated lipids in order to simulate food effects on drug dissolution and drug partitioning in the GI fluids. The formation of different phases and colloidal entities associated to the lipid digestion has been largely investigated with a variety of techniques, such as dynamic light scattering [202], small angle X-ray scattering [111], Cryo-TEM [110, 114], and size-exclusion chromatography [99]. However, few studies have employed EPR spectroscopy coupled with in vitro lipolysis systems in order to investigate in real time the drug fate during the digestion process. The most conventional way of measuring drug partitioning during digestion so far has involved sampling during in vitro digestion, centrifugation of collected samples in order to separate aqueous phase from the formulation phase, and
quantification of drug concentration in the aqueous phase [164]. While this method has proven to give, in many instances, reliable information about the degree of drug release and partitioning and possible performance of the specific drug delivery system, it may result in misinterpretation of time profiles of this dynamic process since the time spent during centrifugation has been as much as 135 minutes. In our study, EPR was used as a
non-invasive, online method to monitor model drug distribution in different phases. EPR spectroscopy offers advantages of a non-invasive, real time method for analysis of the amount of compound in each phase (water, micelles, and formulation) during \textit{in vitro} digestion, and is thus a powerful tool to monitor drug delivery processes [109, 192]. We

Figure 27: Distribution of the model drug TB in different phases present during the \textit{in vitro} digestion of food lipids performed A) during the solid model drug dissolution process, and B) after complete dissolution of TB in the fed state biorelevant medium.
performed multicomponent analysis on EPR spectra collected during drug partitioning in the presence of coexisting multiple phases (buffer, micelles, and lipid) based on magnetic and dynamic parameters of TB determined in separate phases.

Single component spectra of TB in buffer, in soybean oil and in two-component fasted and fed state intestinal fluids (micelles and buffer) are in agreement with what is previously reported [201]. Hyperfine coupling constant, $a_N$, estimated for TB in each species decreased in the following order in correlation with their polarity as expected: buffer, micelles, and soybean oil as also reported previously [201]. The quantification of two-component EPR spectra revealed that the distribution of TB in the micellar and the water phases was directly affected by the concentration of bile components employed (Figure 23). Higher concentration of BS and PL led to higher amount of model drug located into micelles, in agreement with previous studies reporting an increase of poor water-soluble drug solubility in the aqueous phase in the presence of increasing amount of bile salts [63, 112]. The type of bile components used to prepare the simulated intestinal fluids also had influence on drug partitioning into micelles, when the same total concentration of bile components was employed. In fed state samples prepared with NaTDC 12 mM and lecithin 4 mM (Figure 23, B), approximately ~70% of TB was associated with micelles, while in samples containing bile extract 16 mM (Figure 23, D) only ~50% of TB was located in micellar phase. Furthermore, EPR spectra appeared qualitatively different in shape; line width and line height on the third peak (Figure 23, B vs. D). These dissimilarities might be due to the relative amount of bile compounds (such as lecithin vs. bile salts) in the bile extract.
Using magnetic and dynamic parameters obtained from single and two component spectra, three component spectra were resolved to quantify samples where TB is distributed in three phases; buffer, micelle and soybean oil. Three component spectra were resolved successfully by including a small amount of Heisenberg spin exchange, which accounts for broadening due to the presence of oxygen in the sample, and any residual broadening due to probe-probe collisions at the concentrations used. Another parameter obtained via simulations was R, which gives information on microviscosity of phases and rotation of the molecule within phases. Rotation of TB was fastest in buffer as expected and slowest in micelle phase.

Our investigations regarding the partitioning of TB among phases in the absence of digestion (Figures 24) showed the transport of dissolved TB from the fed state biorelevant medium into soybean oil droplets occurred over 3 hours, a time scale comparable to that of drug digestion and drug dissolution. These results supported the necessity of considering explicitly the partitioning process between dissolved drug and oil droplets in an effort to develop models describing drug dissolution and drug release in the presence of lipid digestion.

This real time dynamic, quantitative information can be used to mathematically model and predict the effect of lipids on drug partitioning in the intestinal lumen. Predictive capability on this important step in overall drug absorption - drug release/dissolution - could ultimately serve as an essential component of an overall systems based model predicting bioavailability of a drug administered with lipids.
5.5 Intestinal drug permeability studies

In order to investigate transport proprieties of the model drug TB in the presence of ingested lipids in the human small intestine, cell cultures mimicking more closely physiological conditions (co-cultures of Caco-2 and HT29-MTX cells) were developed and employed in drug permeability studies. Drug permeability results obtained by means of co-cultures were compared to permeability values estimated with the relative monocultures of Caco-2 and HT29-MTX cells.

5.5.1 Characterization of co-cultures of Caco-2/HT29-MTX cells

The HT29-MTX cells, cultured on 12 transwell plates for 21 days, appeared elongated and smaller in size than the Caco-2 cells (Figure 28). Monocultures of HT29-MTX and co-cultures of Caco-2/HT29-MTX cells grown for 21 days on a permeable membrane developed a macroscopically visible, shiny mucus layer that covered the entire apical monolayer surfaces. The presence of mucus was demonstrated by means of Alcian Blue staining, which is specific for acidic mucus components. After the same staining treatments, no Alcian Blue could be detected on the Caco-2 monocultures (Figure 29).

Figure 28: Monolayers of Caco-2 cells (left) and of HT29-MTX cells (right) cultured for 21 days on permeable membranes showed cells of different size and morphology.
At lower HT29-MTX ratios, there were clusters of HT29-MTX cells surrounded by CaCo-2 cells (Figure 30). Overall, the number of HT29-MTX cells in the 21 day old monolayers increased with increasing the initial HT29-MTX seeding ratios, as previously reported elsewhere [203].

**Figure 29:** Monolayers of Caco-2 cells (left) and of HT29-MTX cells (right) cultured for 21 days on permeable membranes and stained by means of Alcian blue, which revealed the presence of acidic mucus components covering the HT29-MTX cells (right).

**Figure 30:** Co-cultures of Caco-2 and HT29-MTX cells at seeding ratios A) 90/10, B) 75/25, and C) 50/50, showed an amount of HT29-MTX cells proportional to the initial seeding ratios after 21 days of culture.
5.5.2 Monolayers integrity and development of tight junctions

The development of tight junctions of cell monolayers was followed by means of TEER measurements acquired on the 7th, 14th, and 21st days after seeding. Monocultures of HT29-MTX cells and co-cultures of Caco-2 and HT29-MTX showed higher TEER values than Caco-2 cell monocultures starting from the 2nd week after seeding (Figure 31). Therefore, the addition of HT29-MTX mucus-producing cells was responsible for the formation of tighter junctions between cells.

![Figure 31: Comparison of TEER measurements, acquired over 21 days of culture, in Caco-2 monocultures, HT29-MTX monocultures, and Caco-2/HT29-MTX co-cultures.](image)

5.5.3 Cytotoxicity tests

The toxicity of the model drug TB - dosed in HBSS at two different pH values, 7.4 and 6.5 - and the toxicity of the post-prandial simulated intestinal fluids was investigated on Caco-2 cell monolayers, HT29-MTX cell monolayers, and Caco-2/HT29-MTX co-cultures by means of the cell counting kit – 8 (cck-8) cytotoxicity assay (Section 3.4.2). Eight wells were used to test each solution. Wells incubated only with complete
growth medium were used as a positive control, assumed to give 100% of cell viability, while wells incubated with 1% sodium dodecyl sulfate (SDS) solution was employed as a negative control, given 0% cell viability. Cell viability of each tested solution was then expressed in percent with respect to the positive control.

The doses of TB employed in this study were limited by the low aqueous solubility of the drug, being 0.02 mg/ml in maleate buffer (Table 7), and expected to be comparable in HBSS. The model drug TB resulted to be not cytotoxic at the aforementioned doses on all type of cell monolayers used (Figure 32).

The greater toxicity effects were observed after dosing the fed state intestinal fluids, in particular on Caco-2 cell monocultures. Caco-2 cells showed 0% viability as the monolayers were completely damaged and detached from the bottom of the wells after

![Cell viability tests](image)

**Figure 32:** Cell viability tests performed on Caco-2 monocultures, HT29-MTX monocultures, and Caco-2/HT29-MTX co-cultures showed no toxic effects for the model drug TB. A reduced toxic action of fed state simulated intestinal fluids was observed in the presence of the mucus layer.
the exposition to the fed state biorelevant medium. The bile components seemed to be the major responsible in toxicity, as shown by comparison to 100% viability after dosing maleate buffer alone. However, the toxicity of simulated intestinal fluids was contained on HT29-MTX cell monocultures and Caco-2/HT29-MTX cell co-cultures, which showed ~ 60% and 50% viability respectively (Figure 32). These findings seemed to confirm the protective function of the mucus layer that covered these cell monolayers against toxic actions of bile components.

5.5.4 Permeability experiments using TB as a lipophilic model drug

Drug transport studies were performed on Caco-2 monocultures, HT29-MTX monocultures, and Caco-2/HT29-MTX co-cultures, grown on permeable supports for at least 21 days. The model drug TB was dosed in HBSS at pH 7.4, in HBSS at pH 6.5, and in fed state biorelevant medium on the donor compartment of each type of cultures. The apparent permeability coefficient $P_{app}$ (cm/sec) was calculated from cumulative mass transported-time profiles assuming simple first order absorption process and sink conditions at the receptor side, according to Equation 15, and they are presented in Figure 33.

The permeability of TB in Caco-2 monocultures was decreased as compared to HT29-MTX monocultures and Caco-2/HT29-MTX co-cultures, when the drug was dosed in HBSS at either 7.4 or 6.5 pH values. However, the permeability of TB was significantly increased in the presence of fed state simulated intestinal fluids in the donor compartment. We did not make any assumptions regarding the mechanism of transport occurring in the in vitro cell-based systems. Therefore, $P_{app}$ (cm/sec) of the model compound TB was calculated in the following cases: i) only the free fraction of drug in
intestinal lumen was absorbed across the intestinal wall; ii) the drug dissolved in the aqueous phase (the free fraction and the micelle-associated fraction) was absorbed across the intestinal wall.

5.6 Simulation results for absorbed drug

Simulations were run to predict drug concentrations profiles in plasma after absorption and elimination over time upon oral co-administration of the model drug TB with and without 50 mM of soybean oil. We ran simulations in both the following cases: i) only the free fraction of drug in intestinal lumen was absorbed across the intestinal wall; ii) the drug dissolved in the aqueous phase (the free fraction and the micelle-associated fraction) was absorbed across the intestinal wall. We selected two drug dose levels, 200 mg and 400 mg. In regard of the small intestinal emptying time,
physiologically, 50% of the contents of small intestine empty in 3-6 hours, depending whether fasted or fed state [204]. We assumed that after 3 hours all content in fasted state intestinal lumen was emptied, while we selected 6 hours for emptying time in the fed state.

Drug plasma profiles were simulated in function of variation in drug dose, presence or absence of co-administered food lipids, and type of transport across intestinal wall. Figures 34 and 35 demonstrated the effect of the amount of TB dosed orally in fasted and fed states respectively. As expected, the low water solubility of the solid drug TB limited its dissolution in fasted state in the absence of ingested lipids. Consequently, the drug plasma profiles did not vary much between 200 mg and 400 mg dose, suggesting that drug concentration in plasma was not proportional to the initial doses beyond a certain value. On the contrary, in case of post-prandial conditions, an initial higher drug dose (400 mg vs. 200 mg) did show to have a strong impact in drug plasma profiles, enhancing the concentration of TB in plasma of over 20 times at the maximum of the drug plasma curves. This effect was probably correlated to the increased overall aqueous drug concentration in the intestinal lumen over time in the presence of the lipid digestion process.
Drug plasma profiles were also simulated in case of different driving forces involved in drug permeability across the intestinal mucosa (Figure 36). When only the free fraction of TB (portion of drug not associated to colloidal structures) in the intestinal
fluids was considered available for absorption, the maximum drug concentration in plasma resulted to be approximately 0.1 µg/ml, 20 times lower than simulations ran considering that the total aqueous drug concentration was available for absorption. The plasma levels in case of free fraction of TB were almost comparable to the ones obtained in the fasted state simulations. Furthermore, as noted for solid drug TB dissolving in fasted state, the initial drug dose showed to have much less impact on drug plasma level variations than simulation results obtained when the total drug concentration (in the aqueous medium was considered (Figure 35).

![Graph showing TB concentration in plasma (µg/ml) against time (hr) for different doses and state conditions](image)

**Figure 36:** Simulations of TB pharmacokinetics profiles obtained in fed state conditions (drug co-administered with food lipids) in case of the only free drug fraction was considered available for absorption.
6.0 Conclusions

6.1 Design and characterization of in vitro lipolysis systems

The assessment of biorelevant media able to accurately represent the gastrointestinal contents is crucial to investigation of the impact of ingested lipids, and their digestion products, on dissolution and absorption of co-administered drugs and delivery agents. The objective of this study was twofold. First, design and characterize biorelevant in vitro lipid digestion models able to mimic fundamental features of human intestinal contents in post-prandial conditions, such as physical-chemical properties of GI fluids and morphology of the intermediate phases of the lipid digestion. Media compositions were mainly based on in vivo experiments that examined GI tract contents in the fed states and critically compared to previous in vitro lipolysis models. In vitro lipolysis profiles were constructed by basic titration method, while DLS and SANS were employed to investigate different phases present throughout digestion. The proposed simulated GI fluids had all the main colloidal structures (emulsion droplets, vesicles, micelles) that characterize human intestinal contents in fed state. Second, assess the sensitivity of the proposed in vitro lipolysis models with respect to experimental parameters - pH, calcium concentration, medium composition and type of substrate – known to affect the enzymatic activity of the lipase, and consequently the rate of lipolysis. The quantitative assessment of system sensitivity to experimental parameters and the resulting change in digestion kinetics were statistically evaluated based on a $2^4$ multifactor design.

In conclusion, we revised and discussed the composition of suitable biorelevant media for in vitro lipolysis models in light of known psychochemical properties from
investigations of *in vivo* intestinal fluids. We effectively evaluated the relationships between various experimental parameters on the sensitivity of the *in vivo* lipid digestion process using a balance $2^4$ design. The pH of the medium was the most important factor that influenced the release of FA after 50 minutes of the *in vitro* lipid digestion due to known effects on the *in vitro* activity of the lipases. This study has important implications for designing and testing *in vitro* models that involve lipid digestion.

### 6.2 System-based models predicting oral drug absorption co-administered with food lipids

A system-based model was developed in order to describe mass transport proprieties of a solid compound administered orally with food lipids in the GI tract. The following main kinetic processes involved in the overall drug absorption were considered in building the model: lipid digestion, drug dissolution from a solid dosage form, drug partitioning into colloidal species, and drug absorption. Simulation results for solid drug dissolution in simulated intestinal fluids with concurrent food lipids digestion were compared to experimental results obtained by means of HPLC and EPR during *in vitro* lipid digestion combined with *in vitro* drug dissolution tests. Good agreement between simulation results and experimental findings supported the validity of assumptions made in model development. The model was further employed into a one compartment pharmacokinetic model in order to predict plasma concentration profiles of absorbed drug. Simulation results first were used to compare the extent of drug absorption in the fasted and fed states. Therefore, the pharmacokinetics profiles were computed when TB is taken orally as a solid form in the absence and the presence of food lipids respectively, and the level of enhancement in oral bioavailability of a compound co-administered with
ingested lipids was assessed. Results demonstrated a great enhancement in drug plasma profiles in post-prandial conditions. Furthermore, simulations showed a strong dependence of level of enhancement of drug plasma concentration on initial drug dose in post-prandial conditions, whereas there was no dependence on initial drug dose in the fasted state.

Pharmacokinetics profiles of the model compound TB were also simulated considering different driving forces involved in drug permeability across the intestinal mucosa wall. When only the free fraction of TB (portion of drug not associated to colloidal structures) in the intestinal fluids was considered available for absorption, the maximum drug concentration in plasma was 20 times lower than simulations ran considering that the total aqueous drug concentration was available for absorption. Furthermore, in this case the initial drug dose showed to have much less impact on drug plasma level variations than simulation results obtained when the total drug concentration in the aqueous medium was considered.

We believe that the significance of the experimental and theoretical framework resulted from this project could be high. The proposed system-based model will enable future studies considering more complex food composition and comprehensive lipid trafficking, which can be used to design strategies to address food-related issues (i.e. obesity) and to provide food intake guidance for general population healthcare. Pharmaceutical scientists and medical professionals can use developed models to provide guidance for dosing to maximize oral absorption. Healthcare companies will be able to rationally design lipid-based formulations to enable successful delivery.
7.0 Recommendations

The modeling proposed is simple: this is by design to first test the ability of a simple model to predict overall lipid effects. Therefore, it might not capture completely the complex nature of the GI absorption.

Firstly, *in vivo* studies of the model drug TB co-administered with food lipids are highly recommended for validation of the presented model simulations concerning oral drug bioavailability. In case of eventual discrepancies between *in vitro* and *in vivo* measurements, the model will be re-assessed for key processes using comparisons to *in vivo* measurements. Complexity might be also incorporated in future works, such as extending the model across a broader range of drug compounds, and including in the model other food components. Studying drugs with a range of different hydrophobicity might help further generalize these results and might strengthen the overall mechanistic model to optimize drug absorption in the presence of food lipids. At last, taking into account interactions between orally administered drugs and all the components of a meal (proteins, sugars, etc.) could provide a comprehensive mechanistic understanding of how food alters effectiveness of orally delivered compounds.

Simulations presented here were constructed considering alternative driving forces involved in drug permeability across the intestinal mucosa wall. However there is literature supporting both scenarios: i) only the free fraction of drug in intestinal lumen is available for absorption across the intestinal wall; ii) the total drug dissolved in the aqueous phase (the free fraction and the micelle-associated fraction) can be absorbed across the intestinal mucosa. Therefore, the subject is still controversial. In addition, the contribution of the lymphatic drug transport was not investigated in the present study, and
consequently it was not included in the model as a co-existing drug transport route in the circulatory system. Further *in vitro* investigation and *in vivo* validations of compound absorption associated with lipid digestion products/mixed micelles and of intracellular lipid trafficking are recommended. They might shed light into contribution of the absorption mechanisms (both intestinal absorption and alteration of lymphatic transport) on overall impact of ingested lipids first, and food later, on oral drug bioavailability.
8.0 Nomenclature

\( A_{em} \)  Total oil emulsion surface area, cm\(^2\)

\( A_{int} \)  Surface area of intestine, cm\(^2\)

\( BS \)  Bile salts

\( C_{FA,aq} \)  FA concentration in aqueous media, mmols/cm\(^3\)

\( C_{D,oil} \)  Drug concentration in oil, mg/cm\(^3\)

\( C_{D,aq} \)  Drug concentration in the aqueous phase (water and micelles), mg/cm\(^3\)

\( C_{D,em} \)  Drug concentration inside oil droplets, mg/cm\(^3\)

\( C_{eq,D,em} \)  Drug solubility in oil emulsion droplets based on emulsion volume, mg/cm\(^3\)

\( C_{eq,D,micelle} \)  Drug solubility in micelles, mg/cm\(^3\)

\( C_{D,micelle} \)  Drug concentration in micelles per aqueous medium, mg/cm\(^3\)

\( C_{D,water} \)  Free drug concentration in the total volume, mg/cm\(^3\)

\( C_{eq,D,water} \)  Drug solubility in water, mg/cm\(^3\)

\( C'_{D,aq} \)  Hypothetical aqueous drug concentration, mg/ in equilibrium with the drug concentration inside oil droplets \( C_{D,em} \), mg/cm\(^3\)

\( cmc \)  Critical micellar concentration

\( D \)  Diameter of oil droplet over time, cm

\( D_0 \)  Initial diameter of an oil droplet, cm

\( D_m \)  Micelle diffusion coefficient in water, cm\(^2/s\)

\( D_s \)  Drug diffusion coefficient in water, cm\(^2/s\)

\( DG \)  Diglycerides

\( EPR \)  Electron paramagnetic resonance

\( FA \)  Fatty acids

\( GI \)  Gastrointestinal
Stationary diffusion layer around a dissolving particle, cm

Oil emulsion-aqueous phase partition coefficient, unitless

Micelle-water partition coefficient, unitless

Digestion rate constant, mols/s*cm²

Inhibition digestion rate constant, 1/s

Initial mass of drug dosed, mg

Drug absorbed across intestinal membrane, mols

Initial digestible FA in oil emulsion droplets, mols

Free FA in aqueous solution, mols

Remaining digestible FA in oil emulsion droplets, mols

Mass of undissolved drug, mg

Monoglycerides

Number of moles FA produced per mole of oil

Drug permeability across oil/water interphase, cm/s

Apparent drug permeability across intestinal membrane, cm/s

Phospholipids

Surface area of N dissolving drug particles, cm²

Small-angle neutron scattering

Tempol Benzoate

Triglycerides

Volume of aqueous phase, cm³

Volume of oil emulsions, cm³

Initial volume of oil emulsions, cm³
\( V_{\text{plasma}} \) \quad \text{Total volume of plasma in the body, cm}^3

Greek

\( \rho \) \quad \text{Drug density, mg/cm}^2

\( \chi \) \quad \text{Molar solubilization capacity of micelles at certain concentration}
9.0 References

1. Spivak, W., et al., Spectrophotometric determination of the critical micellar concentration of bile salts using bilirubin monoglucuronide as a micellar probe. Utility of derivative spectroscopy. (0264-6021 (Print)).


