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Release of Resin-bound Ferulic Acid During an *In Vitro* Digestion Simulation and Antioxidant Activity Evaluation

MASTER DISSERTATION

Gonçalo Nuno Gouveia Martins
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
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ORIENTADORA
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Esta dissertação foi desenvolvida no grupo de Produtos Naturais do Centro de Química da Madeira (CQM), sob a orientação da Professora Doutora Paula Cristina Machado Ferreira Castilho. Foi apresentada à Universidade da Madeira, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Aplicada.

Gonçalo Nuno Gouveia Martins

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Funchal, Madeira - Portugal

"A química não se pode eliminar, pelo simples facto que se encontra em tudo o que nos rodeia e em nós. Está à nossa volta nos fenómenos naturais indispensáveis à vida, como a fotossíntese, e nos produtos artificiais de importância primária para a nossa civilização […]. Está em nós porque o homem "funciona" ou "não funciona" através de reacções químicas. A concepção, o crescimento e a morte são processos químicos, ainda que muito complexos. […] Portanto, abolir a química quereria dizer não só abolir as adulterações alimentares e poluição, mas também abolir os combustíveis, os fármacos, os fertilizantes, as matérias plásticas, os semicondutores, os detergentes, ou seja, todos os benefícios que, dum modo quase inconsciente, usufruímos todos os dias; e quereria também dizer abolir as plantas, os animais, e o próprio homem. Quereria dizer abolir tudo, porque tudo […] é química."

Vincenzo Balzani,

Boletim da Sociedade Portuguesa de Química,

Série II, Volume 68, 1998, p. 10

Acknowledgments

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Finally, I would like to thank my colleagues of CQM and all my friends for their support.

Abstract

Ferulic acid is one of the most abundant hydroxycinnamic acids in Nature, with impact in human health whereas an antioxidant protection might be implicated. It has uses in cosmetics and food industry. It can be found in various foodstuffs, but mostly present etherified to lignins or esterified to carbohydrates or sterols, which hinders its absorption by the organism during the digestive process. The free form of ferulic acid is rapidly absorbed from the stomach, jejunum and, in a much lesser extent, from the ileum, in a pH dependent process.

Since the ileum is particularly susceptible to inflammation and oxidative stress it is important to ensure that ferulic acid is able to exert its action in that part of the body without being absorbed from the stomach. A possible way to increase ferulic acid's bioavailability to the ileum is by immobilization in solid matrixes resistant to low pH but not neutral or basic conditions.

In the present work, the adsorption of ferulic acid onto the polystyrene adsorbent resin Lewatit® VP OC 1064 MD PH was studied, and a loading of 144 mg/g dry resin was obtained.

To evaluate the release of the resin-bound ferulic acid, an *in vitro* "digestion" was performed with simulated gastrointestinal juices. The intestinal step was the most relevant with a release of 13-35 % of FA from the loaded resin.

After each step of the in vitro digestion simulation, the antioxidant activity of ferulic acid was evaluated using the DPPH radical scavenging assay and all samples successfully maintained antioxidant activity throughout the digestive process.

The confirmation of the incorporation of ferulic acid onto the resin was made by ATR-FTIR spectroscopy and morphological analysis was made by SEM. The quantification of ferulic acid in solution was performed by HPLC-DAD throughout the entire work.

This work showed that the free form of ferulic acid can be delivered in the intestine, after immobilization of solid matrixes, maintaining its antioxidant activity. This study is probably the first on this subject with these materials and methods.

Keywords: Ferulic acid; resin; immobilization; digestion; antioxidant.

Resumo

O ácido ferúlico é um dos ácidos hidroxicinâmicos mais abundantes na Natureza, podendo ter um impacto benéfico na saúde humana, em processos que poderão ter por base o seu potencial antioxidante. Actualmente as suas aplicações passam pelas indústrias cosmética e alimentar. Pode ser encontrado em várias fontes alimentares, onde a sua forma eterificada a ligninas ou esterificada a carbohidratos ou esteróis é muito comum, o que dificulta a sua absorção pelo organismo durante o processo digestivo. A forma livre, por outro lado, é rapidamente absorbida no estômago, jejuno e, em menor extensão, no íleo, num processo dependente do pH.

Visto que o íleo é particularmente suspectível a inflamação e *stress* oxidativo a entrega da forma livre do ácido ferúlico neste segmento do tracto gastrointestinal deve ser assegurada, sem que seja absorvido no estômago.

Uma forma de aumentar a biodisponibilidade do ácido ferúlico é por imobilização em matrizes sólidas que sejam resistentes ao meio ácido do estômago, mas que garantam a sua entrega em meios neutros ou alcalinos. Neste trabalho, a imobilização do ácido ferúlico foi feita por adsorção na resina adsorvente de polistireno Lewatit® VP OC 1064 MD PH, tendo sido obtida uma incorporação de 144 mg/g resina seca.

Através de uma simulação *in vitro* da digestão, a libertação do ácido ferúlico da resina foi avaliada. Efectivamente, o meio intestinal proporcionou a maior libertação de ácido ferúlico, sendo que 13-35 % do ácido ferúlico incorporado na resina foi libertado nestas condições.

Foram recolhidas amostras após cada passo da digestão e as suas actividades antioxidantes foram aferidas com recurso ao teste do DPPH, tendo sido observado que todas as amostras apresentaram actividade antioxidante após a digestão.

A confirmação da incorporação do ácido ferúlico na resina foi feita por espectroscopia ATR-FTIR e foram realizadas análises físicas e químicas por SEM. Durante o decorrer do trabalho, a quantificação do ácido ferúlico foi feita por HPLC-DAD.

Este trabalho demonstrou que a forma livre do ácido ferúlico pode ser entregue no intestino, após imobilização em matrizes sólidas, mantendo a sua actividade antioxidante. Este é possivelmente o primeiro estudo sobre este assunto com estes materiais e nestas condições.

Palavras-chave: Ácido ferúlico; resina; imobilização; digestão; antioxidante.

List of Oral Communications

Martins, G, Castilho PC. Delivery of Ferulic Acid During an *In Vitro* Digestion Simulation. 5th CQM Annual Meeting, Funchal, Madeira. 01-03 of February 2018. ISBN: 978-989-54090-0-6.

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Abbreviations

AA	Antioxidant Activity
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared
BCS	Biopharmaceutics Classification System
CAT	Catalase
DPPH	2,2-diphenyl-1-picrylhydrazyl
DVB	Divinylbenzene
EDS	Energy-Dispersive X-ray Spectroscopy
FA	Ferulic acid
HCA(s)	Hydroxycinnamic acid(s)
HMG-CoA	Hydroxymethylglutaryl coenzyme A
HPLC-DAD	High Performance Liquid Chromatography with Diode-Array Detection
IRE	Internal Reflection Element
IVIVC	*In vivo-in vitro* correlation
Lewatit	Lewatit® VP OC 1064 MD PH
L-Phe	L-phenylalanine
L-Tyr	L-tyrosine
qe	Amount of compound adsorbed for unit mass of adsorbent (mg/g)
qr	Amount of ferulic acid released from the resin (mg/g)
ROS	Reactive oxygen species
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SOD	Superoxide dismutase
UV	Ultraviolet
uv-vis	Ultraviolet-visible

I. Introduction

1. Secondary metabolites as bioactive compounds – the case of ferulic acid

1.1. Introduction – secondary metabolites

In order to ensure that organisms can survive and reproduce, they need certain compounds. Apart from energy in the form of ATP (adenosine triphosphate), molecules such as nucleic acids, proteins, carbohydrates, and lipids are essential to vital processes, like respiration and photosynthesis, and can be found in all organisms. For this reason, they are referred to as *primary metabolites*, and the set of synthetic pathways associated with their production is called the *primary metabolism*, since they remain practically unaltered from organism to organism.[1,2]

However, there are certain metabolic pathways that are not present in all life-forms, and, consequently, their products cannot be found ubiquitously, like the previous ones. These *secondary metabolites* are a wide range of compounds, with a rich variety of chemical structures, whose functions are not yet known in all cases, but it is assumed they play a role in the survival of their producer. Although they were once regarded simply as waste products of the primary metabolism, they seem to be important for protection - for instance from ultraviolet (UV) light or against predators; while others are thought to be involved in reproduction, as attractors, for example. Their production is dependent on a balance between synthesis, storage, and degradation, and their metabolism is often associated with growth and morphological changes.[2–4]

Plants represent an interesting case for the study of these molecules because they have a very developed *secondary metabolism*, being able to store large amounts of these metabolites, whereas other organisms need to acquire them in their diet, even consuming plants for this purpose. P. M. Dewick (2002) even states that “it is thus fairly obvious that the human diet could be both unpalatable and remarkably dangerous if all plants, animals, and fungi produced the same range of compounds”.[1] Although secondary metabolites have relevant roles in the life cycle of the organisms which produce them, they have different roles in the organisms that consume them. When it comes to human usage, consumption may be through ingestion (diet), external (skin) application, inhalation, or other forms. [4]

Differences in their biosynthesis allows for their general characterization in three major groups, according to A. Crozier et al. (2006), namely “(i) flavonoids and allied phenolic and polyphenolic compounds, (ii) terpenoids and (iii) nitrogen-containing

alkaloids and sulphur-containing compounds".[4] For the interest of this work, only a class of the first group, the hydroxycinnamic substances, and its consumption through ingestion will be discussed in further detail.

1.2. Hydroxycinnamic acids

The hydroxycinnamic acids (HCAs) are phenylpropanoids: they show a chain of three carbon as substituents in a benzene ring but have an OH group in the *para* position; and were the compounds studied in this work. The name "hydroxycinnamic acid" comes from the hydroxylation of cinnamic acid, which is their precursor and their general structure can be found in Figure 1.

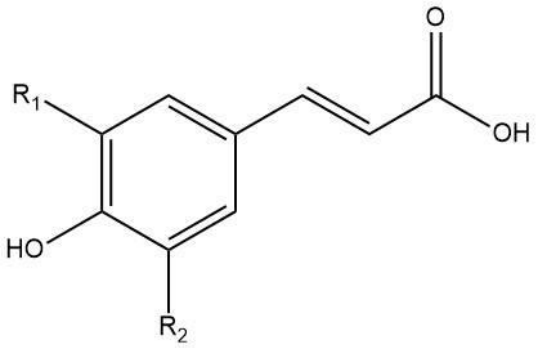


Figure 1 - General structure of a hydroxycinnamic acid. R_1, R_2 = H; OH; OCH_3.

1.2.1. Biosynthesis

HCAs are produced in the phenylpropanoid pathway (Figure 2) and derive mostly from L-phenylalanine (L-Phe), but also from L-tyrosine (L-Tyr). These aromatic amino acids are only produced by plants, bacteria, and fungi, and provide the C6-C3 backbone common to all phenylpropanoids.

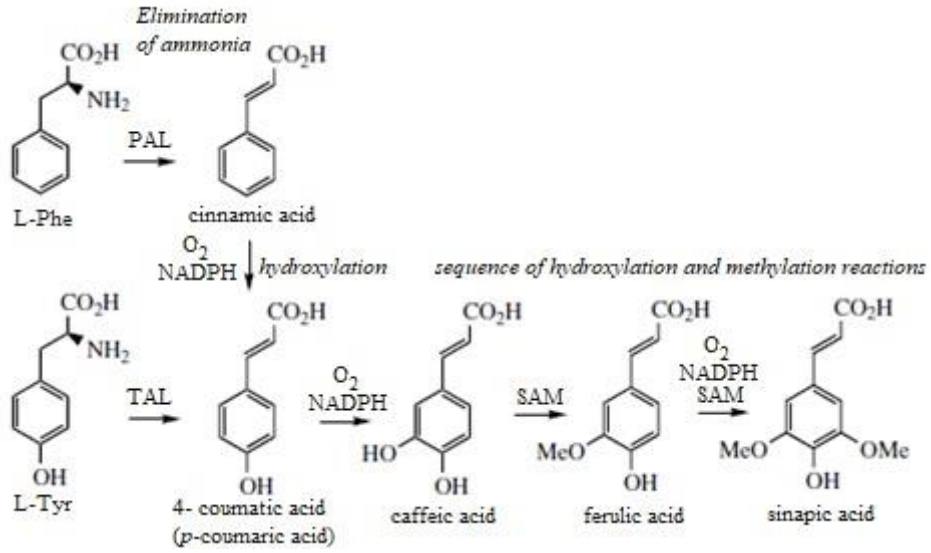


Figure 2 - The formation of *p*-coumaric, caffeic, ferulic and sinapic acids. Adapted from Dewick, M. (2002).[1]

The first step of these compounds' synthesis is the production of *p*-coumaric acid, from either one of two forms: the hydroxylation of cinnamic acid, after deamination of L-Phe by the action of L-phenylalanine ammonia lyase (PAL); or by deamination of L-Tyr by L-tyrosine ammonia lyase (TAL). Consecutive hydroxylations (in a NADPH-depending reaction with molecular oxygen) and methylations (by SAM, S-adenosylmethionine), caffeic, ferulic, and sinapic acids are obtained from *p*-coumaric acid. [1–4]

The most common hydroxycinnamates are the esters of these four compounds and quinic acid (Figure 3). These esters are collectively referred to as "chlorogenic acids" even though that name is usually attributed to the most abundant of them: 5-*O*-caffeoylquinic acid; and the lack of distinction in the literature between the group and the latter compound can be both tiresome and confusing at times. Additionally, esterification with shikimic, tartaric, malic, and malonic acids is also common. Actually, HCAs are not usually found in the free form in most vegetable matrices since they are able to bind to saccharides and lignin through ester and ether linkages. [5,6]

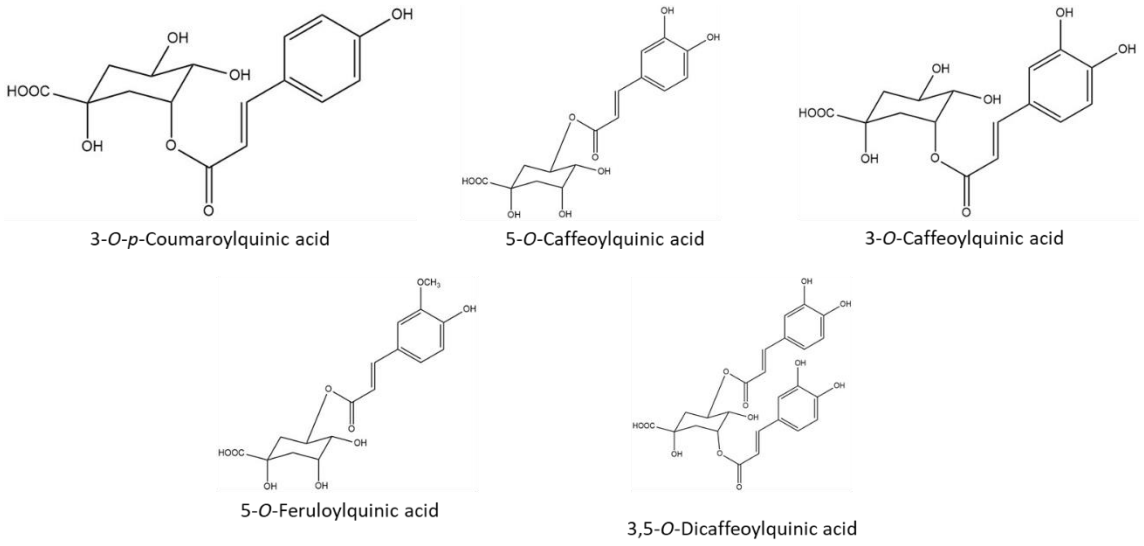


Figure 3 – Structures of some chlorogenic acids.[5]

Among all HCAs, ferulic acid was the most important in this work, and for this we will discuss deeper its properties.

1.2.2. Ferulic acid

Ferulic acid[1] (FA), or 4-hydroxy-3-methoxycinnamic acid (Figure 4), is one of the most abundant HCAs in Nature. It was first isolated in 1866 by Hlasiwetz and Barth, from

[1] Its chemical and physical properties are described in Table S1 of the Supplementary Information.

the plant *Ferula foetida* (Apiaceae family) and chemically synthesized in 1925 via condensation of vanillin with malonic acid, in an amine-catalysed reaction. By the 1970's its antioxidant potential was already reported.[7] However, in 1992, E. Graf commented on the fact there was a lack of study and publications on this topic in the 126 years since the molecule's isolation, an issue that has changed dramatically since then. Nowadays, FA is a well-known and well-studied compound, with many applications in the industry and as a phytochemical.[8]

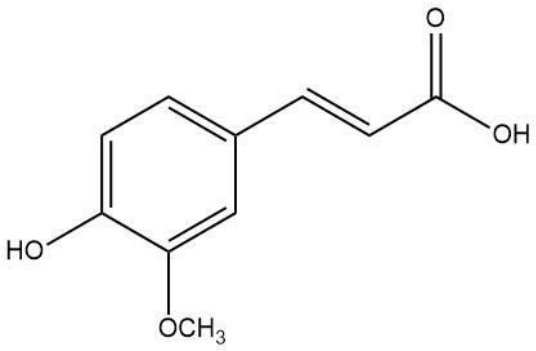


Figure 4 - Structure of ferulic acid.

1.2.2.1. Occurrence

As stated previously, ingestion of secondary metabolites through dietary intake is one of the most significant forms of human consumption of these substances. As such, it is important to consider the distribution of FA in products present in human nourishment.

FA can be found throughout the plant kingdom as a ubiquitous component of plants' tissues, particularly as a constituent of their cells' walls. Therefore, it is only natural it is widely present in foodstuffs, namely grains, fruits, and vegetables, but it can also be found in beverages such as coffee and beer. Table 1 shows some examples of the amounts of FA detected in different sources, as reported in a review by Zhao, Z. and Moghadasian, M. H. (2008). [7]

Table 1 - FA (mg/100 g of fresh weight) present in different foodstuffs. Adapted from Zhao, Z. and Moghadasian, M. H. (2008). [7]

Source		FA (mg/100 g of fresh weight)
Grains	Refined corn bran	2610-3300
	Barley extract	1358–2293
	Soft and hard wheat bran	1351–1456
Fruits	Grapefruit	10.7–11.6
	Orange	9.2-9.9
	Banana	5.4
Vegetables	Bamboo shoots	243.6
	Water dropwort	7.3-34
	Eggplant	7.3-35
Commercial foods and beverages	Sugar-beet pulp	800
	Popcorn	313
	Coffee	9.1-14.3
	Beer	0.24-0.9

As Table 1 shows, grains are the richest source of FA in human dietary products. Among different types of grains (Table 2), FA stands out as the major HCA.

Table 2 - HCAs' content in different grains (mg/kg of fresh weight). Legend: 1 = flour; 2 = grits; 3 = flakes; nd = not detected. Adapted from Shahidi, F. and Chandrasekara, A. (2015). [9]

HCA	Wheat[1]	Rye[1]	Corn[1]	Millet[2]	Barley[1]	Oat[3]	Brown rice
Ferulic	890	860	380	260	250	250	240
Sinapic	63	120	57	nd	11	55	20
p-coumaric	37	41	31	18	40	nd	76
Caffeic	37	10	26	1.1	1.7	3.1	nd

Furthermore, although FA can be present in large quantities in its free form, mostly in the *trans*-isomeric form, in some vegetables, such as burdock, water dropwort, and eggplant, the conjugated form, usually through esterification, is predominant. FA conjugates with a wide range of chemical species like hydroxyl acids (e. g. quinic acid), saccharides (e. g. arabinose residues, glucose), and others, but can also be found as

oligomers (e. g. 8-8-diferulic acid, Figure 5). The free form can be obtained by alkaline hydrolysis. [5,7,10]

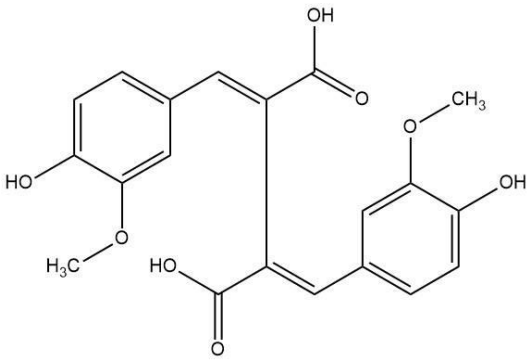


Figure 5 – Structure of 8-8-diferulic acid.

The high quantities of FA found in grains come from the conjugated form. These conjugated forms can be soluble or insoluble, and the latter represents the majority of FA (Table 3).

Table 3 – Content of soluble and insoluble FA in different cereals. Legend: 1 = µg/g grain; 2 = µg/g dry weight; 3 = µg/g defatted meal; a = Soluble FA includes both the free and conjugated soluble forms. Adapted from Shahidi, F. and Chandrasekara, A. (2015). [9]

Cereal type	Soluble FA[a]	Insoluble FA	Total
Soft wheat, choptank[1]	39	560	599
Soft wheat, VA97W-024[1]	41	521	562
Soft Wheat, SS560[1]	41	527	568
Soft Wheat, vigoro tribute[1]	49	407	456
White corn[2]	13	1193	1206
Yellow corn[2]	21	1009	1030
Red corn[2]	19	1284	1303
Blue corn[2]	21	1279	1300
Kodo millet[3]	365	1844	2209
Finger millet[3]	27	331	358
Foxtail millet[3]	225	631	856
Proso millet[3]	112	332	444
Little millet[3]	164	185	349
Pearl millet[3]	176	637	813

The review by Silva, E. and Batista, R. (2017) reports an extensive list of compounds with a feruloyl moiety, such as flavonoids, lignans, nitrogen-containing

compounds, saccharides, terpenoids, oligomers, and miscellaneous compounds, found from 1990 to 2015 in different families of plants and their described bioactivities. [11]

1.2.2.2. Bioactivity, potential uses and applications

Currently, the uses of FA are mainly based on its antioxidant activity (AA), which in turn is responsible for other applications, as antimicrobial or UV-protector. It is an approved food preservative in Japan, USA, and Europe, and in China it is used in the form of sodium ferulate against cardiovascular and cerebrovascular diseases.[7,12] From the various applications, areas such as food, cosmetic and pharmaceutic industries present the most uses for FA (Figure 6).

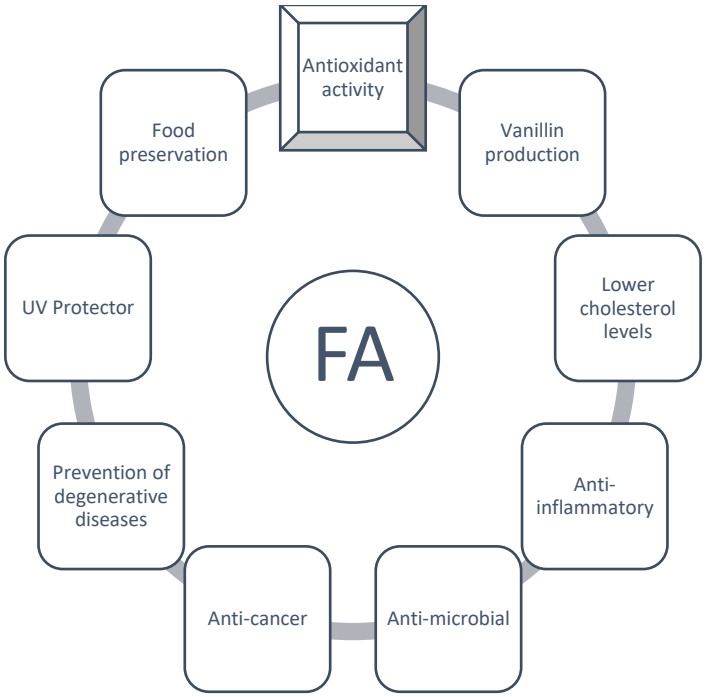
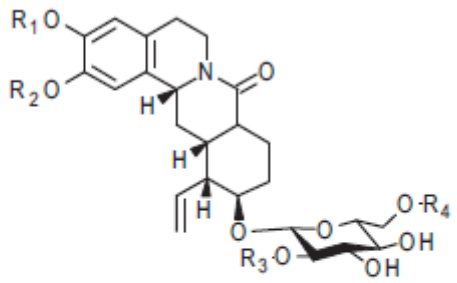


Figure 6 - Bioactivities and applications of FA.

A. Chemotaxonomical marker

Some families of plants only produce certain types of feruloyl conjugated molecules and, for that reason, FA can be used as a chemotaxonomical marker. As an example, the Alangiceae, Amaranthaceae and Annonaceae families only produce the feruloyl moiety in nitrogen-containing compounds (Figure 7). In the review by Silva, E and Batista, R. (2017) there is an extensive description of compounds, found in plants from 1990 to 2015, containing a feruloyl moiety, and other examples of the potential use of FA in chemotaxonomy are given. [11]



127 $R_1 = R_2 = R_4 = H$; R_3 = feruloyl
128 $R_1 = CH_3$; $R_2 = R_4 = H$; R_3 = feruloyl
129 $R_1 = R_4 = H$; $R_2 = CH_3$; R_3 = feruloyl
130 $R_1 = R_2 = R_3 = H$; R_4 = feruloyl

Figure 7 - Nitrogen-containing compounds found in the Alangiceae plant family bearing a feruloyl moiety. Adapted from Silva, E. and Batista, R. (2017).[11]

B. Antimicrobial activity

Studies have showed that FA exhibits antimicrobial activity against viruses, such as HIV and the influenza viruses, bacteria, and yeast cells. For example, it is thought that FA diminishes the release and action of the p24 antigen, a protein present in the HIV's virus capsid, inhibiting its replication.[13] The antibacterial activity against both gram-positive and gram-negative bacteria, e.g. human intestinal microflora - *Escherichia Coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and others - is thought to happen *via* the inhibition of arylamine N-acetyltransferase – an enzyme that catalyses the transference of an acetyl group from acetylcoenzyme A to a xenobiotic acceptor[14].

C. Food preservation and other applications

As mentioned earlier, FA has many uses in the food industry, for instance as food preservative. It is used as an additive in several products mainly for the prevention of lipid and protein oxidation, but also to provide protection from radiation, and to prevent microbial contamination. The addition of FA in food is advantageous because of its antioxidant properties and stability to both temperature and pH.[12] Additionally, FA can interact with other antioxidants present in food, such as Vitamins C and E, and synergically prevent oxidation.[15]

Additionally, this versatile molecule can be used as a cross-linking agent with different kinds of molecules, namely carbohydrates such as arabinoxylans, but also proteins, producing gels from low viscosity substances.

Moreover, FA is present in functional foods, specifically in sports foods, for the stimulation of hormone secretion.[12]

D. Production of vanillin

FA is a known precursor on the synthesis of vanillin. Vanillin is an important compound used in different fields, as a flavouring agent in food and beverages, in cosmetics for the production of perfumes, in cleaning products, to alter the flavour and aroma of medicines in the pharmaceutical industry, and, for example, as a staining agent in analytical chemistry, in thin-layer-chromatography. The conversion of FA into vanillin is mostly done by biosynthesis, using enzymes from yeast, bacteria and fungi.[10,12]

E. Antioxidant Activity

When oxygen reacts with reduced compounds such as carbohydrates and lipids for the production of energy, an oxidation reaction occurs. This leads to the formation of reactive oxygen and nitrogen species (ROS and RNS), in a normal process that is important for the maintenance of biological systems as ROS[2] can serve as cell-signalling molecules and take part in immune response against micro-organisms and in phagocytosis. However, high concentrations of these molecules result in oxidative stress, since they can attack and damage biomolecules like proteins and DNA, causing cell death and disease.

Factors influencing ROS formation can be both endogenous – mitochondrial oxidative metabolism and inflammation - and exogenous: UV light, smoking, diet and pharmaceuticals; so, organisms developed antioxidant systems to help maintain healthy levels of ROS. Antioxidant compounds such as glutathione, vitamins C, A, and E, as well as enzymes like catalase (CAT), superoxide dismutase (SOD), and others, help prevent oxidation.[11] According to Shahidi, F. (2015) “antioxidants may be defined as substances that, when present in food, delay, control, or inhibit oxidation and deterioration of food quality. In the body, antioxidants reduce the risk of degenerative diseases arising from oxidative stress”.[9]

FA shows AA in distinct mechanisms: as radical-scavenger, by UV-light absorption, regulating antioxidant systems, and inhibiting oxidant enzymes. Also, by

[2] As done by some authors, the term “ROS” will describe both ROS and RNS, since both are oxygenated compounds. [11]

anchoring in lipid bilayers of cells with the carboxylic acid end, FA can prevent lipid peroxidation.[16] The phenolic hydroxyl group, as well as the double bond in the aliphatic chain, account for its radical-scavenging efficiency, because FA can stabilize by resonance (Figure 7) after interacting with a radical species.

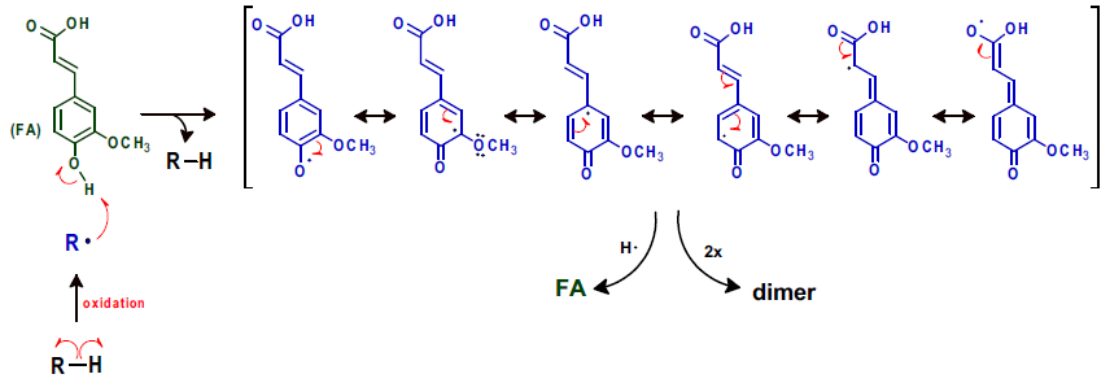


Figure 8 - Resonance stabilization of FA's phenoxyl radical, during radical-scavenging. Adapted from Silva, O. and Batista, R. (2017). [11]

Figure 8 shows how an unstable radical (R•) can be neutralized (R-H) by abstraction of a hydrogen atom from FA, which then stabilizes itself by resonating in different structures that are practically unreactive, stopping the radical chain reaction. These structures are so stable because the unpaired electron can delocalize through the entire molecule.[11] Later, the phenoxy radical can either regenerate or condensate and form dimers like curcumin (Figure 9), by reacting with yet another feruloyl radical.

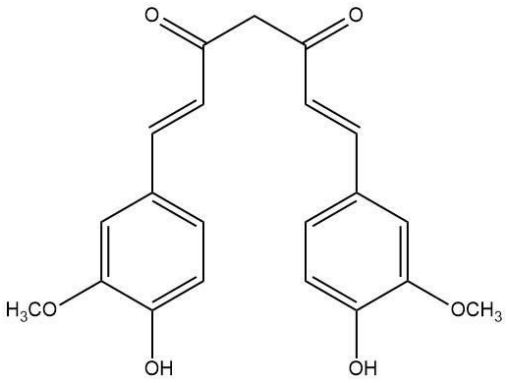


Figure 9 – Structure of curcumin.

Likewise, it synergically assists the action of other antioxidant systems, regenerating them or by up-regulating protein/genes responsible for cells' defence against oxidative stress such as the heme oxygenase/biliverdin reductase system, SOD, CAT, and heat shock proteins (e.g. Hsp 70). Additionally, FA can also inhibit the action of ROS generating enzymes, like tyrosinase, and down-regulates pathways involved in cell death, such as inducible nitric oxide synthase.[12,16,17]

Also, due to the presence of conjugated unsaturated bonds, FA can absorb UV light, thus protecting light-sensitive compounds from oxidation, reducing the amount of radiation received.[11] After absorbing UV radiation, a phenoxy radical is formed, and then *cis-trans* isomerization occurs. As before, these are stable radical species that stop the radical chain reaction.[8]

These antioxidant mechanisms clarify the use of FA in several applications, such as in food preservation, and are responsible in part for many of FA therapeutic activities.

F. Therapeutic activity

a. Cosmetics/Skin disorders

The UV-protection provided by FA is the main reason it is present in various skin lotions. It is well absorbed at acidic and neutral pH and prevents skin damage, hyperpigmentation from UV-caused erythema, and skin cancer. It can be associated with other antioxidant species such as vitamins C and E. It was also found that it helps the wound healing process in the skin of diabetic rats. [10,12,16,17]

b. Anti-cancer activity

The antioxidant capacity of FA is responsible for a number of different cytoprotective effects against cancer. By scavenging ROS and inducing the activity of detoxication and cytoprotective enzymes, SOD, CAT, vitamins A, C, and E, FA helps prevent lipid peroxidation and damage to DNA, protein and cell membranes. This effect was demonstrated in rat lymphocytes, HeLa (cervical cancer) and NCI-H460 (lung cancer) cells. When administered topically, FA can help prevent skin cancer, from damage caused by UV-rays, by absorbing radiation. It is also reported that it can inhibit telomerase activity in adenocarcinoma cells. [10,16,17]

c. Degenerative diseases

FA could also be relevant in prevention or cure of degenerative diseases such as Parkinson's and Alzheimer's diseases. These conditions are characterized by an excessive production of ROS and an impairment in antioxidant mechanisms, causing oxidative damage to proteins, RNA and tissues, resulting in neuronal dysfunction. Studies have suggested that FA can prevent oxidative damage to tissues, neutralize radical species, as well as regenerate antioxidants such as SOD and Glutathione. Studies in mice also showed that the administration of FA resulted in decreased neuroinflammation and oxidative stress on the brain, increasing the mice's cognitive activity. [13,16,17]

d. Anti-cholesterolemic activity

This bioactivity was reported by different studies where it was noted that administrating FA in rats resulted in the decrease of low density lipoprotein. Its ability of reducing cholesterol levels in the plasma of mice was even compared to that of clofibrate, a known substance used for lowering cholesterol and triglycerides in blood.[13] The mechanism behind this activity acts through competitive inhibition of hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase), an enzyme responsible for the most critical step in cholesterol synthesis' regulation.[12,17]

e. Anti-inflammatory activity

Studies have shown that FA can also act as an anti-inflammatory agent. FA has been identified as one of the bioactive components in plants used as anti-inflammatory drugs in Japanese medicines. It can inhibit the production and up-regulate the expression of pro- and anti-inflammatory cytokines, respectively. It has also been reported that it can aid the anti-inflammatory response in cases of chemical induced inflammation, such as in ulcerative colitis. Additionally, through the use of topical formulations containing FA, UV-B induced inflammation can be prevented.[12,16] Other studies suggest that FA possesses anti-depressant-like properties, related to its inhibitory action towards inflammatory agents in mice.[18]

f. Intestinal protection

The intestines are prone to oxidative damage from ROS and inflammation, resulting in conditions such as ischemia-reperfusion injury. Studies have found that although FA's radical scavenging efficiency is weaker than that of other antioxidants, it is capable of preventing increases in vascular permeability caused by oxidative damage, by auto-oxidation of lipids. Also, it is known that FA stays in circulation longer than other antioxidants such as ascorbic acid, thus its protective effect towards these kinds of injuries may be relevant. Furthermore, studies have shown that FA may be able to prevent inflammatory injury such as colitis, suggesting it can be useful for the treatment or prevention of conditions like Inflammatory Bowels Disease.[16,19]

1.2.2.3. Intake and pharmacokinetics

The bioactivity of phytochemicals depends not only on their properties, but also on a number of factors, including the amount present in food, their absorption by the body, delivery to target cells and tissues, and metabolization.[6]

Dietary intake

Zhao, Z. and Moghadasian, M. (2008) state in their review that the daily intake of FA from regular consumers of cereals, vegetables, fruits, coffee, and juices ranges from 150 to 250 mg. [7] However, the presence of the free and conjugated forms of FA in food influences the amount of compound absorbed.

Absorption

Briefly, the transportation of molecules through the intestinal epithelial cells can happen through different mechanisms, as depicted in Figure 10. The main types consist on the paracellular and the transcellular transports: [20]

- During paracellular transport, molecules pass through the intercellular junctions between epithelial cells.
- The transcellular transport, however is further divided in endocytosis, carrier-mediated transport, and passive diffusion.
    - Endocytosis is when a molecule is carried through the cell inside a vesicle.
    - Carrier-mediated transport (CMT) is the transport of solutes with the aid of protein carriers located on the cell's membrane. There are two kinds of CMT: when the transport is in the direction of the concentration gradient on both sides of the membrane (high concentration → low concentration) and no energy is needed, the process is called Facilitated Diffusion; the transport against concentration or electrical gradients, require energy and is designated as Active Transport.
    - The general transport mechanism for small lipophilic drugs is by Passive Diffusion through the intestinal cells' bilayer membrane, according to the concentration gradient.

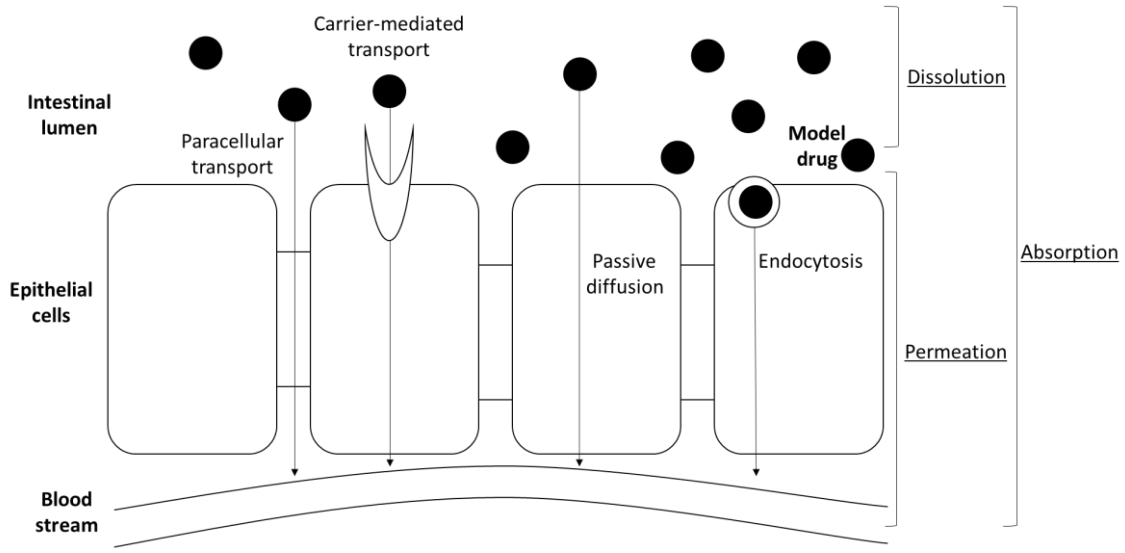


Figure 10 - Types of drug transport across the intestinal epithelial cells.

It is reported that FA can be efficiently absorbed, mainly from the stomach, but also from the small intestine, through different absorption mechanisms. Studies in rats have shown that only a small amount of the ingested dose (0.5-0.8 %) was detected in the faeces, proving the high absorption efficiency, and that the stomach is the main absorption site for FA, since only after 25 min of administration around 70 % of FA had been absorbed.[7]

The high absorption of FA in the stomach is mainly attributed to the low pH, because it allows the diffusion of FA from the food matrix[21] and, since its pKa ≈ 4[3], it allows the passive diffusion of the unionized form of FA through the gastric mucosa.

Although the amount of FA absorbed in the intestine is lower than in the stomach, passive diffusion is also the primarily mechanism of absorption of FA in the intestine, reportedly ≈ 90 % of the amount absorbed at this stage.[17] Additionally, experiments have also attributed the absorption mechanism to active transport, by monocarboxylic acid transporters (MCTs).[6]

Bioaccessibility is defined as the dose of a certain compound released from its matrix and found on the gastrointestinal tract. It should not be confused with bioavailability, as the latter comprises the notions of bioaccessibility, absorption, distribution, and bioactivity. It means that bioaccessibility ≥ bioavailability, as the absorption represents a limitation to the bioavailability of some compounds.[22]

In the case of FA, it has been suggested that the absorption is not a limiting step, however differences in its bioaccessibility from different food sources have greatly

[3] Table S1 in the Supplementary Information.

influences its bioavailability. For instance, the bioavailability of FA from cereals is very low (3 %), whereas from beer it is very high (19-98 %), what is explained by the prevalence of the conjugated form in cereals (Table 3).[22] Effectively, FA is mostly found in the conjugated form in food, hindering its absorption, as compared to that of the free form. [6]

Additionally, the conjugation of FA, particularly to sugars, lowers its absorption in the stomach, resulting in a slower absorption rate throughout the full extent of the gastrointestinal tract.[23] When FA is conjugated, there is a need for cleavage of the ester bond prior to its absorption. Several enzymes are responsible for this, namely feruloyl esterases present in the intestine. It is reported that microbial xylanases and esterases are of paramount importance in the hydrolysis of the ester bonds with polysaccharides. [6]

The conjugated forms of FA are not as well absorbed as its free-form, given the need for bond cleavage. Recent studies evaluate the simultaneous ingestion of bran-enriched cereals and lactic bacteria that can act as feruloyl esterases, both passing unchanged through the stomach and producing the release of the free form in the small intestine. Other approaches consist on the administration of larger amounts of the free FA through food supplements or incorporation in functional foods. The absorption of such form from the stomach is straightforward but this means that very little amounts would reach the intestine. Since it is well known that the intestinal mucosa is extremely sensitive to ROS, the beneficial AA of FA would be of particular interest at this point. Like so, in order to enhance the uptake during digestion, there is the need for strategies of extraction and delivery of the free compound, and this is the main goal of this work. One way to do this is immobilization by adsorption, which will be discussed in the next chapter.

Distribution

The form in which FA is present in food influences directly its plasma maximum concentration and peak time. Studies in humans showed that the free form (sodium ferulate, 4.3 µmol/Kg *per os*) takes around 24 min to achieve a C_{max} of 2-3 µM, with a half-time of 42 minutes, whereas when the conjugated form is ingested orally (wheat bran, 22.5 µmol/Kg), T_{max} was about 180 minutes, the C_{max} was 0.2 µM, and $t_{1/2}$ of 325 min.[17]

Serum albumin has been identified as the main carrier of FA, with the major site of distribution being the liver (50 %), while the remainder compound can be found in the kidneys, gastric mucosa, and the bloodstream. It was found that the conjugation after

absorption increased distribution in tissues, allowing FA to enter in the enterohepatic circulation. [13]

Metabolism

Conjugation is one of the first modifications to FA after absorption, occurring mainly in the liver, but also in the kidneys and in the intestinal mucosa, by the action of sulfotransferases (EC 2.8.2.1) and UDP glucuronosyl transferases (EC 2.4.1.17). Consequently, the unmodified compound accounts for 9-20 % of the total FA metabolites found, while the glucuronide and sulfoglucuronide forms represent the remainder 3-20 % and 60-90 %, respectively.[17] However, these conjugation reactions seem to be dose dependent, since a substantial amount of unconjugated FA was found in the plasma of rats, after administration of a high dose, suggesting that the enzymes may become saturated and so the unmodified compound is accumulated. Other derivatives found consist on dihydroferulic acid, vanillic acid, vanilloylglycine, and *m*-hydroxyphenilpropionic acid.[7]

Elimination

Regarding the elimination of FA and its derivatives, kidney excretion represents its major form. In humans, this may take 7h to 9h after administration, while in rats it is much faster. Also, the time necessary for elimination depends greatly on the form it is consumed – i.e. the excretion of the free form is 15 times quicker than that of the conjugated form, for instance, present in wheat bran. [13,17]

Bile excretion is another possibility, although it only accounts for 4-6 % of the ingested dose. It seems that this form of elimination requires high amounts of FA in circulation. This was noted after detection of FA and its metabolites in the faeces of rats after intraperitoneal administration. [7]

2. Polymeric resins

Polymeric resins are synthetic materials with a high degree of crosslinking. These structures can be made up of only one kind of monomer such as styrene or divinylbenzene (DVB) but copolymers are also common. Given their adsorbent properties, they have been used for decades for various applications in industry, mainly water treatment, by removal of organic compounds such as phenols, halogenated compounds, and pesticides, but also in purification of air, in column packing for chromatographic analysis, and others. In recent years, their use in the pharmaceutical and food industries is growing and more applications are being developed.

In terms of their physical properties, these macroreticular polymers consist of 0.25-0.85 mm spherical beads comprised of an agglomeration of several microspheres, allowing for a network of micropores to come together and make-up macroporous structures. The high degree of crosslinking accounts for a high surface area and mechanical endurance.

Regarding the chemistry, the unfunctionalized resins are hydrophobic, due to the presence of aromatic rings present in most of the polymers - this is the reason the main use of these materials is in the adsorption of organic compounds, especially those that are not very water soluble. Additionally, functionalization increases their hydrophilicity and selectivity, by the attachment of ionic groups on the benzene surface, producing ion-exchange resins. By reaction with sulfuric acid, for example, a $-SO_3^-H^+$ group is added, allowing for cation exchanges with the proton. Anion exchange resins can also be tailor made, by functionalization with ammonia, for instance.[24]

2.1. Polymeric resins in medicine and biomedical applications

Polymers have been used in the biomedical field, such as in dentistry, as part of biomedical devices, in tissue engineering, and even as bioactive compounds, for their low toxicity and enhanced selectivity, among other factors, replacing other drugs when these have limited therapeutic effect.

Resins have been and continue to be used as sequestrants of (undesired) compounds present in the body – e.g. for poison and drug detoxication; by hemoadsorption, a form of extracorporeal blood purification, that uses hemoperfusion cartridges/columns packed with adsorbent resins, such as Amberlite XAD-2[25] or HA330 (styrene-DVB),[26,27] for the removal of toxic compounds; or *per os*, for the treatment of certain conditions in the gastrointestinal tract such as intractable diarrhea by sequestration of bile acids,[28–30] in cases of hyperkalemia,[29] and others, since these

macrobeads are not absorbed from the gastrointestinal tract and are excreted in the faeces. This, in turn, allows for their use as drug carriers, showing high drug loading capacity and slow release rates, without being systemically absorbed. Adsorbent resins have been used for the delivery of proteins, genetic material, and small molecules,[29] while ion-exchange resins have been used as carriers, as taste masking and stabilizing agents, to help control the diffusion and release rates of drugs, and other applications.[31]

2.2. Adsorption of phenols by polymeric resins

Regarding the adsorption of phenolic compounds, studies performed with polymeric resins provide insight into this possibility. Using a non-ionic, hydrophobic DVB resin (XAD-16), Dávila-Guzman, N. E. et al. (2012) were able to obtain 133 mg FA/g, noting recoveries were pH-dependent, with the best result at pH 3, at which point the unionized form of FA was prevalent and, so, the interactions with the non-polar resin's matrix are stronger.[32] Conidi, C. et al. (2015) tested the adsorption of chlorogenic acids, using anionic, cationic, and neutral polystyrene resins (Lewatit S 6328 A, S 2328, and S 7968, respectively) and realized the latter was the more efficient, because of the hydrophobic interactions between the adsorbing materials and the solutes.[33]

The significance of the hydrophobic forces was also noted by Niederwieser, A., back in 1971, who stated "it is generally accepted that nonelectrostatic attraction from hydrophobic or van der Waals-London dispersion forces provides the driving forces for the binding of large ions to polymers. This is of importance especially if the solute is bearing a large hydrophobic group attached asymmetrically to the charged group". In that work, the adsorption of 2,4-dinitrophenyl onto a neutral polystyrene resin, Porapak Q, was being studied, however due to the low understanding of the mechanisms at work it was also stated that "much more data is necessary".[34]

This idea has since been confirmed, by works such as that of Ku, Y. and Lee, KC., in 2000, in which the adsorption efficacy was owed to the hydrophobic interactions. The authors realized that the degree of functionality on the benzene ring of phenols increased the compound's polarity and negatively influenced their adsorption onto XAD-4 (a polystyrene-DVB resin). It was also noted that this was also pH dependent, having higher adsorption capabilities in acidic environments due to the presence of molecular phenol, contrary to the anionic counterparts at higher pH. They also became conscious of the correlation between the adsorption degree and the octanol/water coefficient, once again emphasizing the importance of hydrophobic forces in the process.[35]

3. Simulated *in vitro* digestion

3.1. Absorption

Trying to predict how a model drug would be absorbed is a complex task that depends on many factors, both intrinsic to the organism and to the drug's properties. The main limiting steps are the dissolution/diffusion of the drug and their transport across the gastrointestinal membranes (Figure 10).[36] For this reason, drugs can be divided in four categories depending on their solubility and membrane permeability, according to the Biopharmaceutics Classification System (BCS):[37]

- Class I: High permeability and high solubility;
- Class II: High permeability and low solubility;
- Class III: Low permeability and high solubility, and
- Class IV: Low permeability and low solubility.

This information provides scientists with an insight into the *in vivo* behaviour of the studied drug. Most (poly)phenols are classified as Class II and IV,[38] with FA belonging to Class II,[39] implying that the critical step in its absorption is the diffusion from the carrier/delivery system in the gastrointestinal medium, not the transportation through the biological membrane – as already discussed previously, FA is well absorbed in its free form by the organism.

A compound's lipophilicity reflects its membrane permeability and is measured by the 1-octanol/water diffusion coefficient, *log D*. It is similar to the partition coefficient (*log P*), however, unlike the previous, *log D* takes into consideration the medium's pH (usually 7.4), since it uses a buffered solution, in order to account for the drug's ionization in biological fluids.[40] A compound with a $log\ D_{7.4}$ in the range of -0.5 and 2 is considered a good candidate for an orally administrated drug, showing a good balance between lipophilicity (permeability) and hydrophilicity (solubility).[36] FA's *log D* of 0.42[41] further supports the previous notion of the compound's permeability and helps explain why passive diffusion is its major absorption mechanism, when it is present in its free from, both in the stomach and in the intestines.

For all these reasons, without disregarding the complexity that absorption of drugs is and its influencing factors, such as the absence/presence of food along the gastrointestinal tract, the action of microflora, and others, the dissolution/diffusion of FA received greater focus in this work, in which FA was considered as a drug, not as part of

a food matrix, as the differences between the conjugated and free forms of FA were not studied. [36]

3.2. Release - *In vitro* digestion simulation

In vivo testing provides the most accurate results of the absorption of drugs administered in solid form. However, these methods are often time consuming and expensive. Therefore, *in vitro* digestion models were developed to describe/predict the *in vivo* behaviour of the studied compound, using cheap and fast procedures.[42]

These simulated digestion methods must accurately mimic the environment the drug would be subjected to while in the gastrointestinal tract, and so physiological conditions and parameters from each segment of the tract are taken into consideration, such as pH, temperature, residence time, chemical composition of the media and site-specific enzymes. The literature is filled with reports of different methods, with various degrees of complexity and variations: the number of gastrointestinal compartments simulated (mouth, stomach, small and large intestines), the composition of the media (salts, enzymes, surfactants) and the pH, among others. Additionally, some methods are designed to mimic the fasted conditions (absence of food) of the tract, while others study the fed conditions (presence of food), including substances such as milk for this purpose. These adjustments are made according to the goals of each study, whether it is to determine the dissolution rate of the drug, chemical or structural changes, and so forth. Also, depending on the BCS Class of the studied compound, different procedures can be employed, since Class II and IV drugs show greater sensitivity to the dissolution tests, given their lower solubility.[42,43]

In this work, a standard method was used to identify the gastrointestinal compartment of major release of resin-bound FA. Later, the AA of the released compound was evaluated in each step of the *in vitro* digestion, by the DPPH radical scavenging assay, to study the effect of digestion on the radical-scavenging properties of FA. More detail is provided regarding both *in vitro* procedures in the next chapter.

II. Methods and Experimental Procedures

1. Immobilization in macroporous resin

1.1. Objective

The main goal was to immobilize FA by adsorption into an inert material.

The starting point of this work was choosing an appropriate material for the adsorption of FA, which was previously chosen as the molecule of interest of this work, for its therapeutic activity.

Also, the goal was to optimize the experimental conditions that would influence the adsorption process and so increasing the amount of adsorbed drug, considering time and cost.

1.2. Methodology[4]

The methodology described is divided in the adsorption studies, the quantification using HPLC-DAD, and the physical and chemical characterization through ATR-FTIR spectroscopy, SEM and EDS.

1.2.1. Adsorption experiments

1.2.1.1. Selection and pre-treatment of the adsorbent

Several materials were evaluated as possible adsorbents for FA. Preliminary attempts to immobilize FA into several clays, such as halloysite (a tubular nanoclay) gave very poor results and these materials were abandoned. Previous studies in the lab showed that the adsorbent resin Lewatit® VP OC 1064 MD PH (henceforth Lewatit) had the necessary characteristics for the immobilization of secondary metabolites, specifically phenols, such as anthocyanins[44] and HCAs[45], from different sources, so it was chosen for this purpose.

This decision also took into consideration the description of the resin by LANXESS, Germany, who kindly provided the material. The manufacturer states[5] this resin is FDA approved and is described as a material suited for the purification/extraction of a wide variety of organic compounds, both natural or synthetic. The stability of the resin to a wide pH range was also relevant for this choice. In Table 4 it is possible to find some physico-chemical properties of Lewatit.[46]

[4] All the equipment and reagents used throughout the work are listed in Tables S2 and S3 of the Supplementary Information, respectively.
[5] See Figures S1 and S2 of the Supplementary Information.

Table 4 – Physical and chemical properties of the synthetic adsorbent resin Lewatit® VP OC 1064 MD PH. Adapted from LANXESS (2011). [46]

Matrix		Crosslinked polystyrene
Functional group		None
Ionic Form		Neutral
Structure		Porous beads
Appearance		White, opaque
Mean bead size		0.44 – 0.54 mm
Surface area		800 m^2/g
Pore volume		1.2 cm^2/g
Pore diameter (average)		5 – 10 nm; 50 – 100 Å
Water retention		50 – 60 %
Stability	pH	0 - 14
	Temperature	-20 °C – 120 °C

Resin preconditioning, as performed by Conidi, C. et al. (2015) [33], is important for the cleaning of impurities remaining from the synthesis of the adsorbent and consisted in consecutive washes with HCl 6 % and NaOH 4 %, and washing with distilled water between the application of the acid/base. This process was over as the cleaning waters' pH was close to that of distilled water. After this, water retention percentage was determined, in triplicate, using a moisture analyser, and was found to be 64 %.

1.2.1.2. Studying the influence of experimental parameters in the adsorption process

Several adsorption experiments were made, where different parameters were changed in order to study their effect on the adsorption capability of the resin over FA. In these assays, adsorbent dose, FA's initial concentration, temperature, and contact time were studied. This was achieved by changing each parameter separately and having the set of experimental conditions reported as follows, serving as a starting point:

- Resin dose: 10 mg/mL of solution;
- Initial FA concentration: 0.5 mg/mL;
- Temperature: room temperature (≈ 23 °C ± 2 °C);
- Contact time: 5 h;
- Solution's volume used: 3 mL.

As conclusions were drawn, those parameters were changed accordingly, so that the following experiment already featured the "best" conditions.

The adsorption assay's protocol had already been established in the lab, for the loading of compounds onto different adsorbent materials. They were performed, in triplicate, placing the resin in centrifuge tubes and adding the solution, thus starting the assay. During the experiments, samples were kept in the dark and with head-over-heels rotation, for appropriate mixing. A control in the absence of the resin was also made. [44]

At the end of the established contact time, mixtures were centrifuged for 20 minutes at 4000 rpm, and 10 °C. Supernatants were recovered and filtered through 0.45 µm cellulose acetate filters. When appropriate, the supernatants were diluted 10x with 0.1 % formic acid in ultrapure water, for compound quantitation by HPLC-DAD.

For the preparation of the FA solutions throughout the work, FA was dissolved in absolute ethanol and the final volume was made up with distilled water. Ethanol's volume used for dissolution changed according to the solution's final volume, so that its final concentration would never be greater than 2 %. When needed, solutions were placed in an ultrasonic bath (35 kHz, 200 W) to ease compounds' dissolution. Also, these solutions were not buffered because it was determined experimentally that after dissolution the pH was appropriate for these experiments (around 3.2), and to avoid effects from competitive adsorption between the HCAs and the buffer.[47]

A. Resin dose

Three different amounts of resin were used, keeping the solution's volume and concentration constant. The assayed doses were 5, 10, and 20 mg of resin per mL of FA solution.

It was determined that 5 mg of resin/mL of FA solution resulted in a more efficient adsorption process, so was this proportion was used throughout the rest of the work.

B. Ferulic acid initial concentration

FA solutions with 0.25, 0.5, and 1.0 mg/mL were prepared for these assays.

Although the increased concentration of 1.0 mg/mL resulted in a larger amount of loaded compound, the 0.5 mg/mL concentration was chosen. The low solubility of FA in water was a limitation when concentrations higher than 0.5 mg/mL were needed, so this decision was made.

C. Temperature

The former assays were all performed at room temperature (≈ 23 °C ± 2 °C), so this experiment was performed at 10 and 40 °C. The lowest temperature was achieved using a Dewar and a refrigerator and the highest by using a thermostatic bath. In both experiments the head-over-heels rotation was replaced with magnetic stirring.

Room temperature remained the best option since no relevant differences were observed in the amount of FA adsorbed to the resin in the other two cases.

D. Contact time

The contact time was also studied by performing the adsorption experiment for 2h and 5h. Previous studies using the same resin showed that the results obtained with only two hours were comparable to those obtained after five hours, so this hypothesis was tested.[44]

As expected, reducing the contact time did not result in a relevant loss of adsorbed FA, so the two hours were used for the rest of the work, lowering energy costs and time.

E. Kinetic study

The rate of FA's adsorption onto the resin was determined, measuring the amount of compound in solution in regular intervals up to five hours.

The quantities used in this assay were increased, with 250 mg of resin and 50 mL of a 0.5 mg/mL FA solution. The mixture was placed in a water bath at room temperature with magnetic stirring for five hours, during which 1 mL aliquots were withdrawn, at certain times. These samples were immediately filtered with 0.45 µm cellulose acetate filters and diluted with formic acid 0.1% for HPLC-DAD analysis. This experiment was performed in triplicate.

F. Optimised parameters

A final adsorption assay was performed, in triplicate, in larger quantities, combining the optimized parameters as follows:

- Resin dose: 5 mg/mL of FA solution;
- FA initial concentration: 0.5 mg/mL;
- Temperature: 23 °C ± 2 °C (room temperature);
- Contact time: 2h;
- Solution's volume used: 1000 mL.

After the adsorption, the loaded resin from the three assays was washed with distilled water, lyophilized, and mixed. The sample was kept in the dark at 10 °C.

1.2.1.3. Resin selectivity

FA was the main compound studied in this work, however, in a food matrix, several HCAs are usually present (Table 2). Therefore, the selectivity of the resin towards the most common HCAs was tested. This was done by an adsorption assay, evaluating the resin's efficacy in removing each of the five compounds from solution.

In order to mimic a somewhat real matrix, a mixture was prepared containing ferulic, *p*-coumaric, caffeic, sinapic, and chlorogenic (5-*O*-caffeoylquinic acid) acids. The preparation of these solutions and the experimental procedure for the adsorption studies were performed as previously stated, using the pre-established conditions.

1.2.2. HPLC-DAD compound quantification[6]

Throughout this work, compound detection and quantification were done using High Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD), in an instrument equipped with a binary pump, an autosampler, and a column compartment at 30 °C.

During the evaluation of the resin's selectivity, the chromatographic run and elution gradient were optimized, in order to achieve a good resolution between the peaks of the different HCAs (Figure 11). Table 5 shows the final conditions used in this assay, displaying the retention times of each compound, since they were previously determined

[6] The work performed directly in the HPLC-DAD equipment, from programming the chromatographic run's parameters, the maintenance and analyses setups, was made prominently by Vítor Spínola (PhD student), and occasionally with the help of Pedro Silva (PhD student).

while adjusting the chromatographic parameters. Calibration curves were prepared for all HCAs - Table S4 of the Supplementary Information.

Table 5 – Chromatographic conditions used for the study of the resin selectivity.

Stationary phase	Phenomenex Gemini C18 Column (5 µm, 250 x 3.0 mm i.d.)
Mobile phase	A: Formic acid in ultrapure water (0.1 %) B: Acetonitrile
Elution gradient	0 min: 20 % B 0 – 2 min: 20 % B 2 – 13 min: 25 % B 13 – 14 min: 20 % B 14 – 15 min: 20 % B
DAD acquisition wavelength	320 nm
Run time	15 min
Analysis temperature	30 °C
Injection volume	5 µL
Retention time	Chlorogenic acid: ≈ 7.5 min Caffeic acid: ≈ 8.9 min *p*-coumaric acid: ≈ 12.5 min Sinapic acid: ≈ 13.1 min Ferulic acid: ≈ 13.6 min

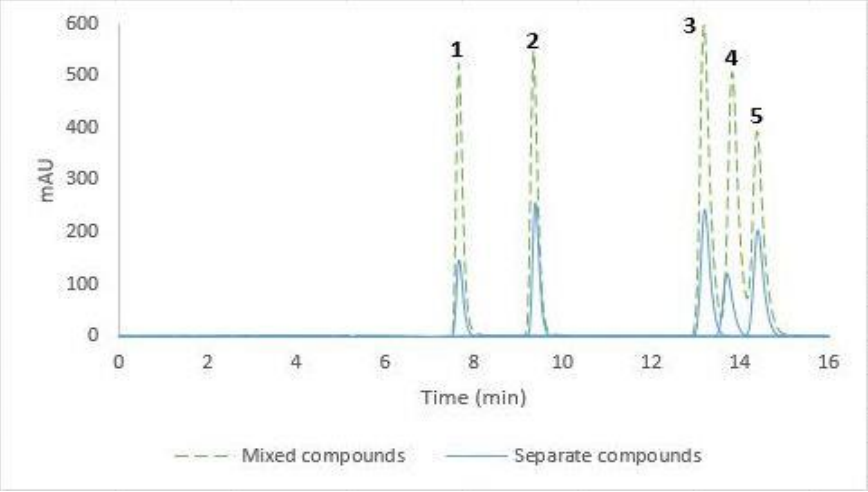


Figure 11 - Chromatograms of chlorogenic (1), caffeic (2), *p*-coumaric (3), sinapic (4), and ferulic (5) acids isolated in different solutions and in the same solution (dashed green line), determined by HPLC-DAD at 320 mn with the conditions described in Table 5.

However, for FA quantification, the chromatographic conditions were different from those described previously, in order to shorten the time of elution (Table 6).

Table 6 – HPLC-DAD parameters used for FA quantitation.

Stationary phase	Phenomenex Gemini C18 column (5 µm, 250 x 3.0 mm i.d.)
Mobile phase	A: Formic acid in ultrapure water (0.1 %) B: Acetonitrile
Elution gradient	0 min: 20 % B 0 – 1 min: 20 % B 1 – 2 min: 50 % B 2 – 8 min: 50 % B 8 – 9 min: 20 % B 9 – 10 min: 20 % B
DAD acquisition wavelength	320 nm
Run time	10 min
Analysis temperature	30 °C
Injection volume	5 µL
Retention time	Ferulic acid: ≈ 8 min

The chromatogram of FA obtained with these conditions is depicted in Figure 12.

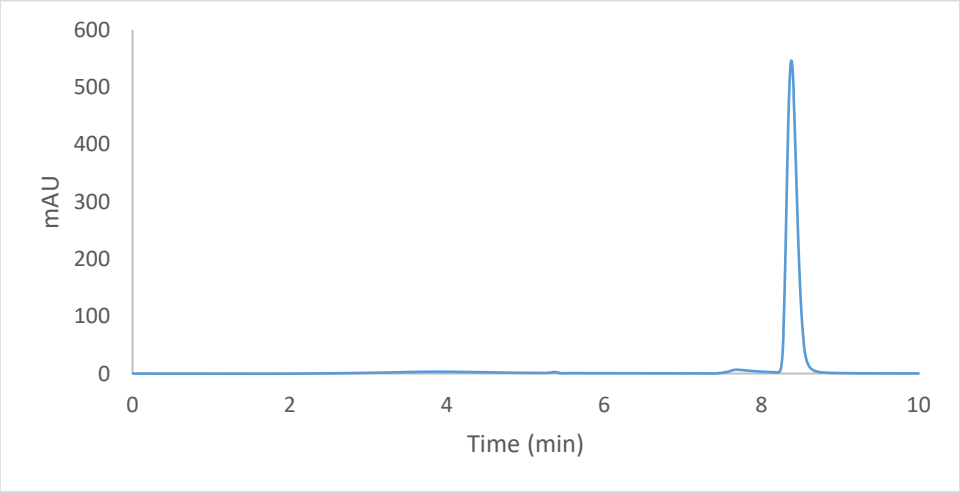


Figure 12 - Chromatogram of FA as determined by HPLC-DAD at 320 nm, with the conditions described inTable 6.

A new calibration curve (Figure 13) was prepared using standard FA solutions (1-100 mg/L). Also, the intra and interdays precision was determined, after analysing standard solutions of 5, 50 and 100 mg/L six times in non-consecutive days, by the relative standard deviation values, and were ≤ 5.3 % and ≤ 7.8 %, respectively.

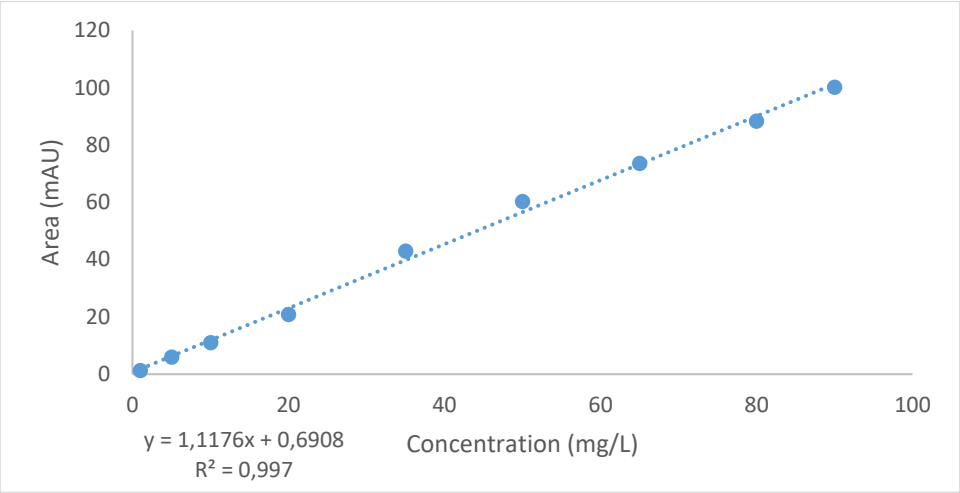


Figure 13 - Calibration curve obtained for FA by HPLC-DAD at 320 nm, using the conditions in Table 6.

1.2.3. Physical and chemical characterization

Although HPLC-DAD was used for the quantification of FA in solution after the adsorption process, the Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR), the Scanning Electron Microscopy (SEM), and the Energy-Dispersive X-ray Spectroscopy (EDS) techniques were used for the physical and chemical characterization of the samples and possible confirmation of the loading of FA into the Lewatit resin. An adsorption assay was made with the optimized conditions, after which the samples were washed with distilled water, lyophilized and analysed.

ATR-FTIR spectroscopy

The FTIR technique was deemed appropriate for the detection of FA in loaded resin samples due to the chemical groups difference between the carboxylic acid and the polystyrene resin. However, the ATR-FTIR was used instead of the KBr pellets method. The higher quality spectra, the lower analysis time - resulting from simple sample preparation (generally dehydration); and the non-destructive nature of the assay were factors taken into account, but the main reason was difficulties in sample preparation for the resin analysis with the KBr pellets method. Lewatit resin is too hard to grind manually to achieve the powder form necessary to prepare KBr pellets. Additionally, the mechanical grinding could lead to the release of FA from the resin. For these reasons, the KBr pellets technique was not used.

For the ATR experiments, samples were placed on the IRE (internal reflection element) and all spectra were obtained over the spectral range between 650 to 4000 cm^{-1},

acquiring 36 scans per spectrum. Analysed samples consisted on FA and dry loaded and unloaded resin. The standard FA spectra were collected using the powered sample, which had been left overnight in the desiccator, protected from light, before the analysis.

For the scanning of the resin spectra, a special sample holder (Figure 14) was used. This device made of acrylonitrile butadiene styrene (ABS) was kindly made and efficiently designed by Jorge Lopes (M. Eng.), in the Electronics and Telecommunications Laboratory of UMa, using a 3D-printer, at the author's request.

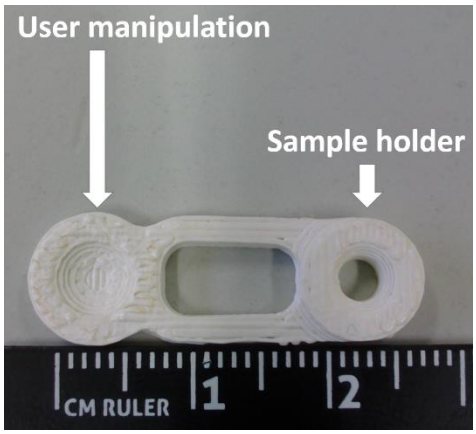


Figure 14 – Specially made sample holder for ATR-FTIR spectroscopy.

This piece was needed because the resin samples were difficult to place on the IRE due to their spherical shape and light weight. The sample holder allowed for the proper placement and fixation (of a higher amount) of the spherical samples atop the 1.5 mm IRE (Figure 15).

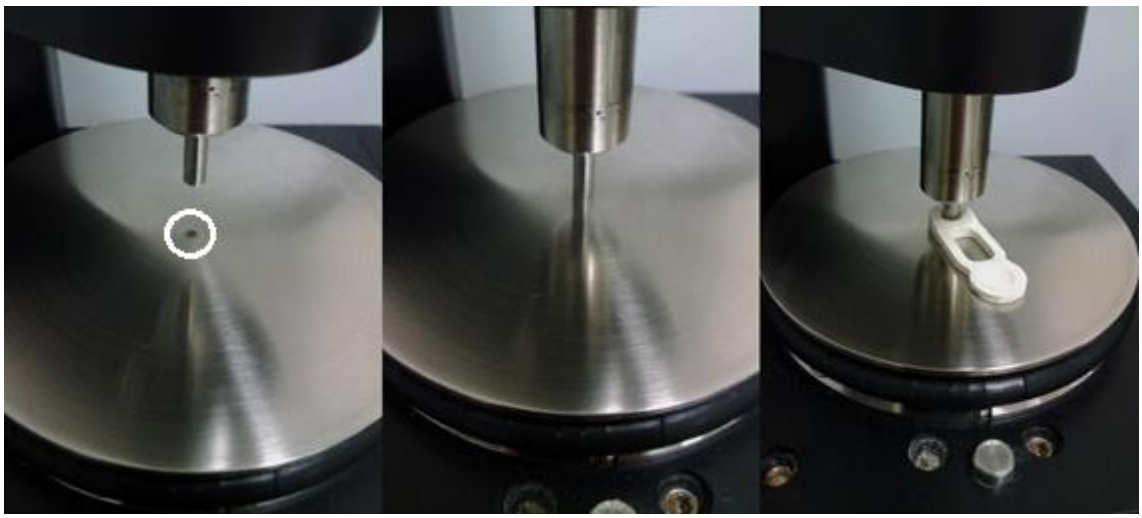


Figure 15 - Left: detail of the 1.5 mm IRE (the black spot inside the white circumference); centre: scanning without the sample holder; right: scanning with the sample holder.

SEM's morphological and elemental analysis[7]

The microscopic analysis was meant to identify possible changes in the resin's shape and dimensions as the result of the adsorption of FA. On the other hand, elemental identification assays were performed to confirm the loading of the drug inside the resin, so each analysis was performed for the unloaded and loaded resin.

First, the intact resin was observed in the microscope in both optic and electronic mode, using the conditions more appropriate for this kind of non-conductive samples, and size measurements were made.

Then, for the EDS elemental determination, both samples were broken into smaller pieces and placed on a double-sided copper tape to help lower the accumulation of charge on the resins. The X-ray spectra were collected, and the relative compositions were determined.

The same procedure was applied to the standard FA, whose molecular formula is known, and so it served as a control.

[7] All the work described in this part was done by Carla Miguel (M. Sc.) and Radenka Whiffen (PhD).

2. *In vitro* digestion simulation

With the *in vitro* digestion simulation, the primary objective was to evaluate the release degree of resin bound FA and determine the gastrointestinal site of major release, using HPLC-DAD for molecule quantification.

The digestion assays would also allow the comparison between the stability of the adsorbed FA and the freeform compound (in solution), when subjected to pH shifts and in the presence of gastrointestinal enzymes.

Although microflora is known as an essential part of the digestive process, its effect on the digested samples was not studied in this work.

2.1. Methods

2.1.1. Samples

Samples of loaded resin were prepared according to the experimental conditions established earlier. They were lyophilized prior to the assays and kept in the dark, in the cold.

A FA (1 mg/mL) standard solution was prepared as previously for the adsorption studies. This concentration was chosen having in mind the dilution that would take place during the *in vitro* digestion, so FA could be quantified with HPLC-DAD.

2.1.2. *In vitro* digestion assays

The method used was originally developed by Flores, FP et al. (2014) [48] and previously used in our lab[49]. The composition of the juices, found in Table 7, mimics that of each of the simulated gastrointestinal compartments, with site specific salts, enzymes, and pH. Two sets of juices were prepared, with and without the addition of adjuncts (enzymes/bile salts), so the latter could serve as a control. These compounds were mixed and dissolved in distilled water, and the pH was adjusted using NaOH 1M and HCl 1M. All stock solutions were kept at 10 °C.

Table 7 – Composition of simulated gastrointestinal juices. Adapted from Flores, P. F. et al. (2014) [48]

Composition		Salivary juice	Gastric juice	Duodenal juice	Bile juice
Main components	NaCl (mg/mL)	0.117	5.504	14.024	10.58
	KCl (mg/mL)	0.149	1.648	1.128	0.752
	$NaHCO_3$ (mg/mL)	2.1	-	6.776	11.57
	NaH_2PO_4 (mg/mL)	-	0.532	-	-
	$CaCl_2 \cdot 2H_2O$ (mg/mL)		0.798		
	NH_4Cl (mg/mL)		0.612		
	KH_2PO_4 (mg/mL)		-	0.16	
	$MgCl_2$ (mg/mL)			0.1	
	Urea (mg/mL)	0.4	0.17	0.2	0.5
	Concentrated HCl (mL)	-	6.5	0.180	0.150
Adjuncts	α-Amylase (mg/mL)	2	-	-	-
	Mucin (mg/mL)	1	6		
	Pepsin (mg/mL)	-	5		
	Pancreatin (mg/mL)		-	18	
	Lipase (mg/mL)			3	
	Bile salts (mg/mL)			-	60
pH		6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

The procedure was performed according to Flores, FP et al. (2014) [48], with modifications. Samples were placed in 50 mL Falcon tubes, in a water bath at 37 °C, with shaking at 500 rpm, in the dark, and then gastrointestinal juices were added.

To recover solid and liquid samples after each stage of the *in vitro* digestion, several experiments were conducted with various incubation times. The same amount of sample was digested with:

- 6 mL salivary juice (5 min. incubation) – Salivary step
- 6 mL salivary + 12 mL gastric juices (5 min. + 2 hours incubation) – Gastric step
- 6 mL salivary + 12 mL gastric + 12 mL duodenal & 6 mL bile juices (5 min. + 2 hours + 2 hours incubation) – Intestinal step/complete digestion

Digested mixtures were centrifuged at 4000 rpm for 20 minutes at 10 °C, after which the supernatant was removed, filtered and diluted 10x with formic acid (0.1 % in ultrapure water), for HPLC analysis. Lewatit's digested samples were freeze-dried and after that were kept in the freezer (4 °C). All these assays were done in triplicate.

A schematic representation of the procedure is shown in Figure 16.

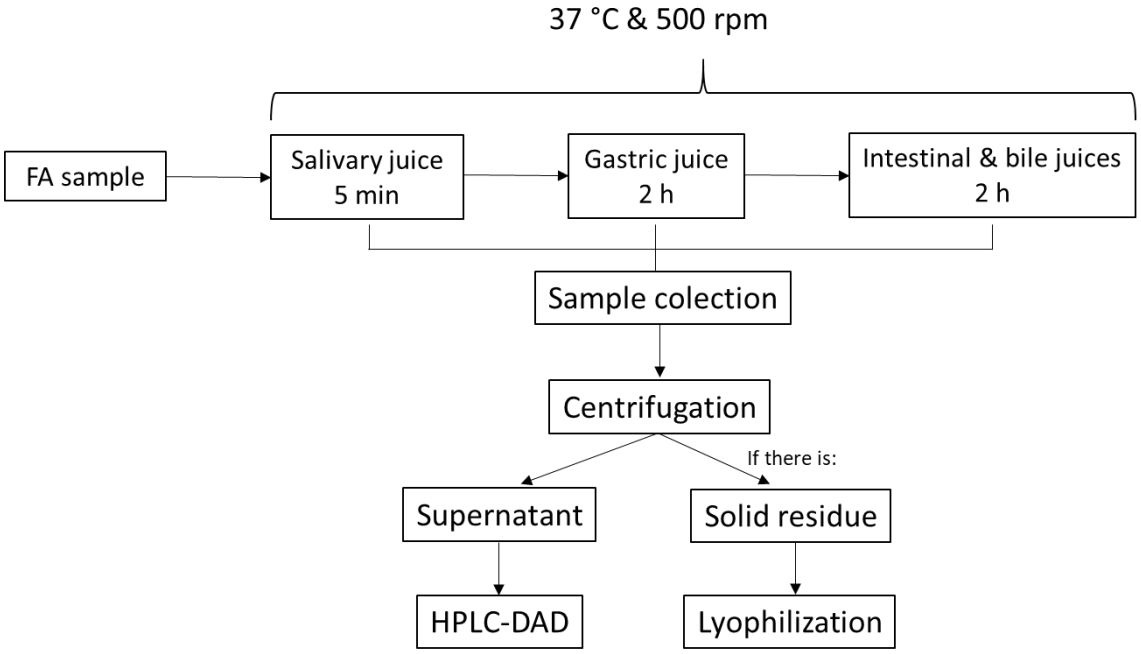


Figure 16 - Schematics of the *in vitro* simulated digestion procedure.

Solid samples' digestion

Loaded resin was digested, using 250 mg of solid sample, instead of the 3 g suggested by Flores, FP et al. (2014) [48]. This adaptation was necessary because on the attempt to perform the assay using 3 g of resin and 6 mL of salivary juice it was realized that the fluid barely covered the resin and the assay was experimentally impossible. Flores, FP et al. used whey protein and gum arabic microcapsules, much smaller and easily dispersed, so this proportion was not a limitation.

Since the aim is the oral administration of free FA bound to the resin, there is a practical issue with the swallowing of the spheres. This issue was solved by enclosing samples in gelatine capsules – *Capsulas 00 incoloras*, Fagron, Spain.[8] This type of material is commonly used for the oral delivery of drugs. The matrix is regarded as nontoxic and is biodegradable, dissolving in the gastrointestinal media. The preparation

[8] These capsules were kindly provided by Sara Morna, Master in Pharmaceutics, of *Farmácia Morna*, Funchal, who confirmed this was a possible way to orally administer these samples.

of the loaded capsules is simple, and diverse kinds of materials can be loaded into the gelatine shells, including a variety of solid samples, such as powders, and liquids.[50]

The amount of resin used in these assays was the same – 250 mg was weighted and then transferred to the inside of the capsules, as depicted in Figure 17. However, before performing the *in vitro* digestion, an experiment was made placing empty capsules in 50 mL Falcons, in the same temperature and shaking conditions as the rest of the samples and adding distilled water, in equal volumes to the juices, to assess their dissolution rate. It was noted that the time to fully dissolve it varied, meaning that in each experiment resin samples could come in contact with the digestion media at different times. After some consideration, adjustments were made to this particular experiment:

- Gastric juice was added immediately after the salivary juice – since capsules in real-life only remain for a few seconds in the mouth before being swallowed (with water), salivary juice was added but the incubation time was skipped and there was no sample collection in this stage;
- Sample collection only occurred at the end of the full digestion – the previous digestion assays, without the capsules, showed that there was little release of FA in the gastric environment. Bearing in mind this information and the dissolution experiments, it was considered pointless to recover samples at the gastric stage as well.

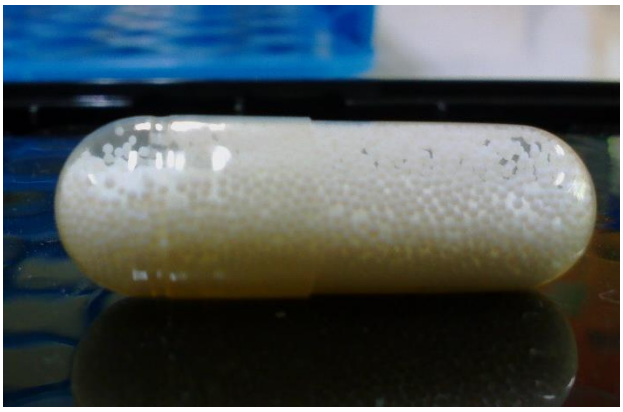


Figure 17 – Encapsulated loaded resin.

Liquid samples' digestion

A standard 1 mg/mL FA solution was digested, starting with 1 mL of solution and adding the gastrointestinal juices. Complete digestions were always performed in these assays, withdrawing 1 mL aliquots at the end of each stage, since there was no solid residue to recover.

3. Antioxidant activity evaluation

The aim of this part of the work was to evaluate and compare the AA of FA with and without immobilization, during its passage through the gastrointestinal tract. This assay had a more qualitative than quantitative nature. The DPPH radical scavenging assay allowed for a direct comparison between samples, in a simple and fast procedure.

DPPH assay modification

The DPPH radical scavenging assay is a colorimetric method based on the change in absorbance of a sample containing the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) at 515 nm. When the naturally purplish radical $DPPH^{\bullet}$ is in the presence of an H^+ donor antioxidant, the colour of the solution turns yellow, due to the reduction of the radical species, forming $DPPH_2$. The mechanism is described in the following equation, where PhOH = phenol.[51]

$$DPPH^{\bullet} + PhOH \rightarrow DPPH_2 + PhO^-$$

Although the reaction time is short (30 min), this process becomes time consuming when dealing with a large number of samples. The product of the reaction is light sensitive, so the absorbance must be quickly read to avoid further reduction of the radical. Consequently, many samples cannot be analysed at once, even with the automatic measurements in the uv-vis spectrometers. Additionally, in the case of the solid samples, both rotation and centrifugation were needed, further increasing the waiting time. For this reason, this method was adapted with the proper adjustments to a microplate reader format, in which the analysis is quite brief and larger amounts of samples can be examined at once.

For the adaptation of the DPPH assay into a microplate reader format, the spectra depicted in Figure 18 were collected before and after the reaction, using 3.5 mL of a 0.06 mM DPPH methanolic solution with and without the addition of 100 µL of 0.4, 0.6, and 1.0 mM Trolox[9] solutions.

[9] Trolox is a vitamin E (α-tocopherol) water-soluble derivative/analogue that is commonly used as a reference compound in antioxidant activity determination protocols and assays.[51]

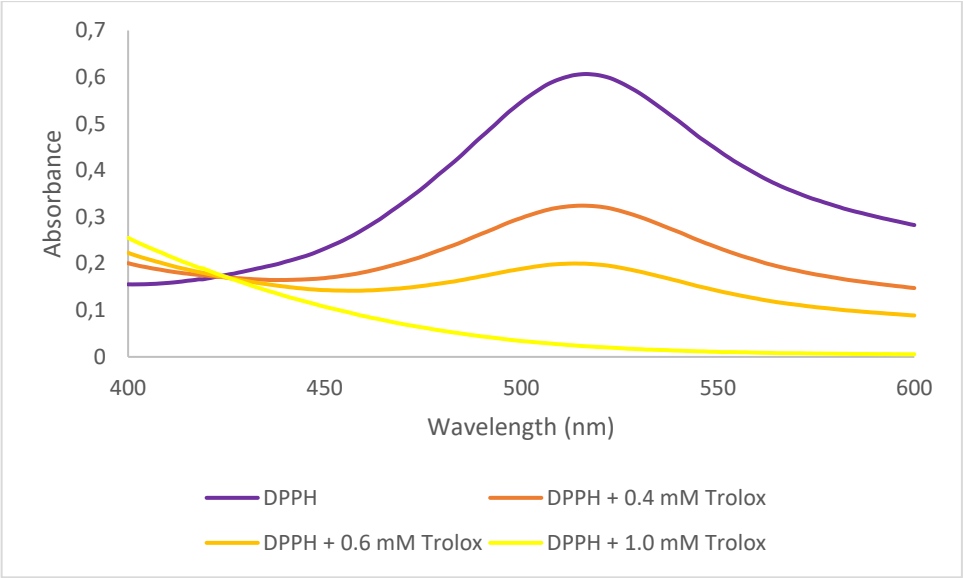


Figure 18 - UV-Vis spectra of DPPH and DPPH + Trolox.

It was observed that the maximum absorption wavelength of the DPPH solution was 516 nm and that the peak disappeared after the addition of Trolox. However, the microplate reader did not possess the filter for 516 nm or any other close wavelength - the nearest were 490 and 550 nm. Since DPPH shows an isosbestic point around 425 nm, 550 nm was considered the most appropriate wavelength to perform this assay, because it is further away from the isosbestic point and, so, the influence of the absorbance of the reduced form of DPPH is smaller. Also, it is important to state that FA has no absorbance in this region (Figure S3), so there is no interference during the analysis.

Afterwards, using the microplate reader, at 550 nm, a calibration curve was determined for Trolox, as depicted in Figure 19. As expected, there is a decrease in the absorbance as the concentration of the antioxidant increases, however after 1.0 mM there is a stabilization. A similar curve was prepared for FA and EC_{50} values (effective concentration required for lowering the amount of DPPH to 50 %) were determined for both compounds.[51] Trolox was more effective in the reduction of DPPH, with an EC_{50} of 0.6 mM, whereas for FA the value obtained was 1.2 mM. This is also noticeable in the plot of the amount of DPPH in solution versus the concentration of antioxidant, present in the Supplementary Information (Figure S4), by comparing the slopes obtained for both compounds.

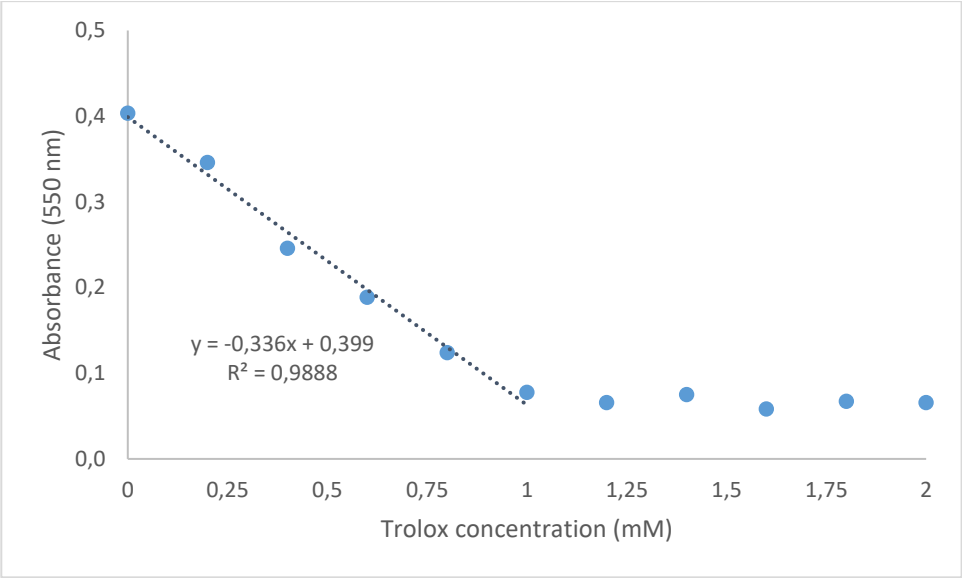


Figure 19 - Calibration curve for the absorbance at 550 nm versus the concentration of Trolox.

From the calibration curve's equation, the Trolox equivalents (eq.) of the analysed samples are calculated, for a better comparison of their AA.

Standard compounds

A calibration curve was determined using Trolox as a reference antioxidant. Trolox solutions were prepared in methanol with concentrations ranging from 0.2 to 2.0 mM. From these, 100 µL were transferred to centrifuge tubes and 3.5 mL of a 0.06 mM DPPH methanolic solution were added, starting the reaction, as performed by Gordon, M. H. et al. (2001) [52]. The experiment was done in triplicate and the tubes were left in the dark with head-over-heels rotation for 30 min. After the reaction time was complete, 350 µL aliquots were transferred to a 96-wells plate and the absorbance was read at 550 nm.

Similarly, a calibration curve for FA was determined in the concentration range of 50 to 350 mg/L, for EC50 calculation.

Samples

Several samples were analysed in this assay, besides the immobilized and dissolved FA, before and after the simulated digestion, as depicted in Figure 20.

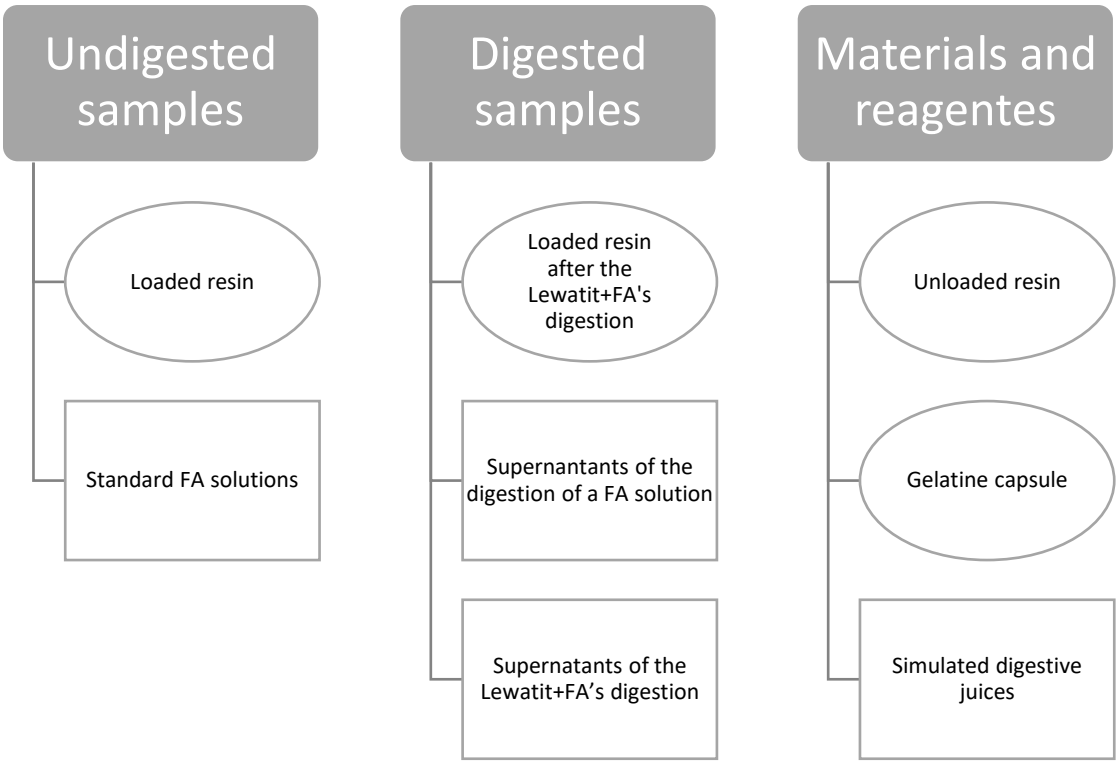


Figure 20 - Samples analysed in the DPPH assay. Circles and squares represent solid and liquid samples, respectively.

When assaying liquid samples, the method used was the same as for the standard compounds, adding 100 µL to 3.5 mL radical solution. However, in the case of solids an adaptation was made, according to a previous work and 1 mg was used, adding 3.5 mL of DPPH. All radical scavenging activity determinations were made in triplicate. [44]

III. Results and Discussion

1. Immobilization in macroporous resin

1.1. Studying the influence of experimental parameters in the adsorption process

The first experiments were conducted in conditions previously used in our laboratory for immobilization of other materials. After that, an attempt was made to optimize some of the parameters such as the ratio resin:FA (resin dose), FA's initial concentration, Temperature and contact time.

The results of the adsorption studies, are presented in mg/g resin dry weight (q_e), calculated from the following equation:

$$q_e = \frac{(C_0 - C_e) \times V}{m}$$

Where:

q_e = amount of compound adsorbed for unit mass of adsorbent (mg/g);

C_0 = initial concentration (mg/mL);

Ce = concentration at equilibrium (mg/mL);

V = volume of solution used (mL), and

m = mass of the Lewatit resin used (g, dry weight).

A. Resin dose

When the resin dose was studied it was noted that increasing the amount of adsorbent resulted in a decrease of FA adsorbed per unit mass of resin, nearly halving each time the amount of resin was doubled, as can be seen in Figure 21. Using 5 mg of resin for each mL of FA's solution was considered the most efficient proportion to achieve higher compound loading, and so it was used throughout the rest of the work.

Dávila-Guzman, N. E. et al. (2012) were able to obtain 133 mg FA/g, on XAD-16, a DVB resin, using a higher proportion (25 mg resin/ mL) and twice the initial concentration of FA. In contrast, in similar conditions in this work, a dose of 20 mg/mL on Lewatit was only able to achieve 36 mg/g. A higher amount of loaded FA was obtained using Lewatit (189 mg/g) with half the concentration of FA and a 25-fold decrease in the adsorbent dose. So it seems that the nature (structure) of the adsorbent is key to the adsorption process.[32]

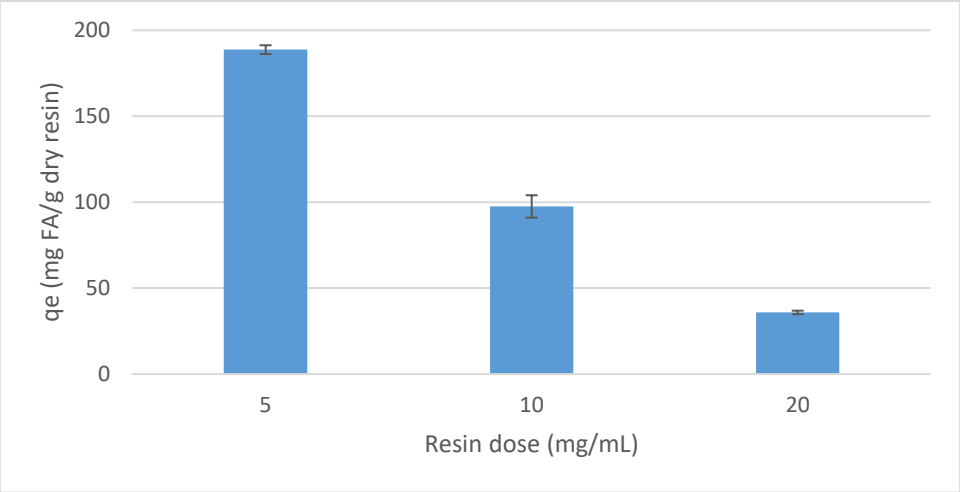


Figure 21 - Amount of FA (mg) adsorbed for mass unit (g) of dry resin, for different adsorbent doses. Values are presented as mean ± Standard Deviation (SD) (n = 3).

Regarding the decrease in amount of FA adsorbed as the resin dose is increased, this is a tendency commonly observed in the literature, and several possible explanations are provided. Although the number of active sites increases with the growing amount of adsorbent used, they remain unsaturated during the adsorption process, thus explaining why the *qe* value decreases.[53] Also, adsorption/desorption is a dynamic process and a good dispersion of the adsorbent translates in a higher adsorption. As the density of adsorbent dispersed in the solution increases there may be particle aggregation, leading to a decrease in the total surface area of the adsorbent[54] and overlapping of active sites.[55] Furthermore, enhanced particle interactions can cause the desorption of weakly adsorbed compounds.[54] This, combined with the fact that large pore diameters (5-10 nm for Lewatit) tend to, simultaneously, desorb molecules as they are being adsorbed, can help explain why the experiment with the smaller amount of resin was the one with the better results.[56]

In conclusion, less quantities of adsorbent result in higher amounts of adsorbed compound. Additionally, it would seem possible to keep decreasing the amount of resin used and continuously obtain higher amounts of loaded compound, at least for some extent, however this study was not performed.

B. Ferulic acid initial concentration

In the adsorption experiments with different initial concentrations of FA, the higher concentration resulted in a higher loading efficiency (Figure 22). The amount adsorbed roughly doubled, as the initial concentration was also doubled. The 0.5 mg/mL

was the standard concentration used and 189 mg/g was obtained, whereas using 1.0 mg/mL solutions, 312 mg/g was obtained.

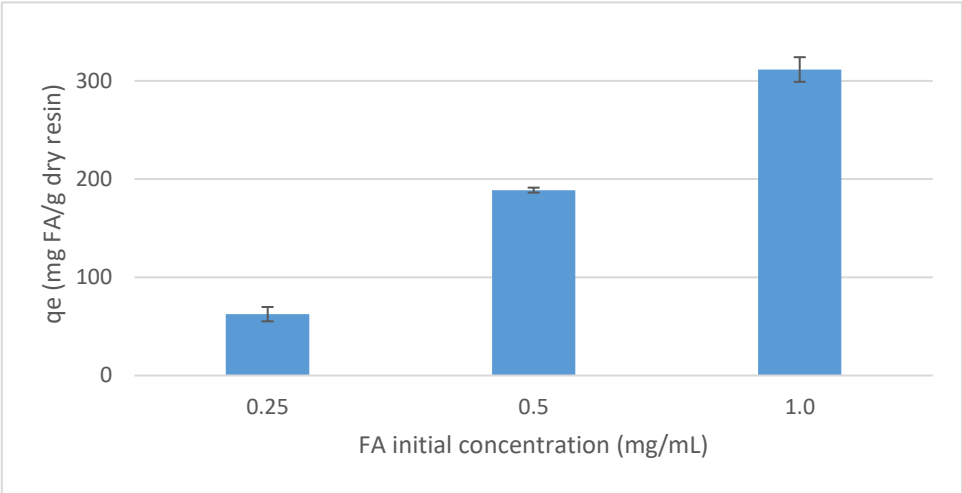


Figure 22 - Amount of FA adsorbed per gram of dry resin, using different initial concentrations. Values are presented as mean ± SD (n = 3).

Although, doubling the concentration from 0.5 mg/mL to 1.0 mg/mL saw a considerable increase in the amount of adsorbed compound, the preparation of concentrated solutions is a challenging task, and so, the previous concentration was used for the rest of the experiments. The dissolution of FA in water was a major issue throughout the entire work, given its low solubility, and for this reason, solutions were prepared using ethanol, as described previously, and were sonicated and heated when needed.

C. Temperature

The change in temperature (10-40 °C) did not result in relevant changes in the *qe* values, as they remained practically unchanged, 189-203 mg/g, in the range of 10-40 °C (Figure 23).

This was unexpected as temperature generally positively affects the adsorption process, lowering the viscosity of solutions and promoting the diffusion of molecules through the pores of the adsorbent and changing its adsorbing capacity.[57] Effectively, there was a very slight increase in the amount of loaded compound, but comparing all assays it can be seen that the increase in temperature has very little influence in the adsorption of FA in the conditions used in this work.

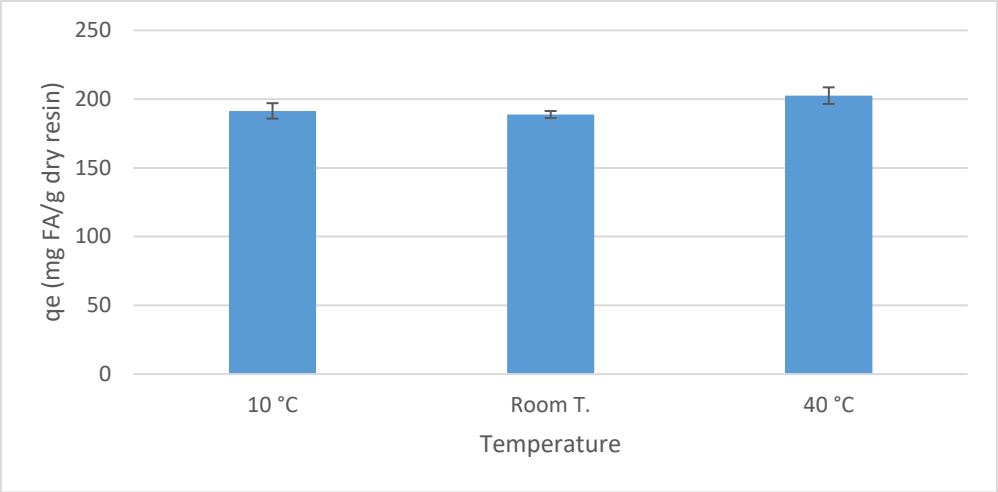


Figure 23 - Amount of FA adsorbed per gram of dry resin, at different temperatures. Values are presented as mean ± SD (n = 3).

This effect was also witnessed by Kammerer, D. et al. (2005), when increasing the temperature to 50 °C had no effect on the adsorption degree of anthocyanins into a styrene-DVB resin (XAD-16 HP),[58] and by Scordino, M. et al. (2003), while adsorbing hesperidin into an acrylic resin (EXA-31). These results may be attributed to desorption of molecules, but also to the fact that temperature lowers the surface tension of resins, increasing their wettability, and consequently their adsorbing capacity, however, if a resin is already wet there will be no change as the temperature increases. In fact, as part of the pre-condition of the resin, Lewatit was thoroughly washed with distilled water, and was not dried prior to its usage, so this is a strong possible explanation of the results.[59]

D. Contact time

The contact time was lowered for only 2 hours, in contrast to the 5 hours previously employed in first experiments. This test was done to assess if this reduction would result in a comparable amount of loaded compound. In fact, 156 mg/g were achieved at 2 hours and 189 mg/g at 5 hours.

Indeed, the reduced contact time did not produce a noteworthy loss in the amount of FA adsorbed (≈ 10 mg/h). Also, the 156 mg of FA/g obtained at 2 hours were higher than the 133 mg/g obtained by Dávila-Guzman, N. E. et al. (2012) at 2.5 hours.[32]

In retrospect, this study should had been completed with a wider range of contact times (e.g. 0.5, 1, 2, 3, 4, and 5 hours), but it was sufficient to conclude on lowering the contact time for only two hours for the rest of the work, and consequently the energetic costs of the process.

E. Kinetic study

The plot of the percentage of adsorption versus time was made to determine when the adsorption process of FA reached its equilibrium. This was evaluated as the point where there were no noteworthy changes in the percentage of adsorption in time, as plotted below.

From the experimental data, a model function was plotted, using the Solver tool in Microsoft Excel 2016.[10] A good correlation was obtained between the experimental and calculated values (R^2 = 0.9576) and a comparison of two can be found in Figure 24.

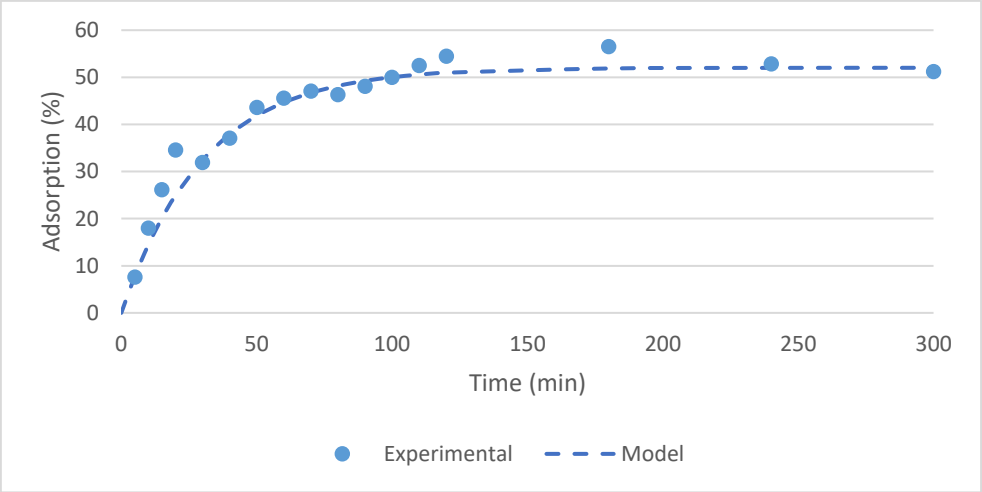


Figure 24 - Percentage of adsorption over time. Dots are the experimental results (average of three assays) and the dashed line is function that better fitted the present data.

A similar plot was obtained by Dávila-Guzman, N. E. et al. (2012),[32] and, in this work, the function was determined to have the equation: $y = 52.0 \times (1 - e^{-t/30.7})$, meaning that the system will evolve in time, as the percentage of adsorption stabilizes at 52.0 %. This happens because the function obtained is an equation of the type:

$$y = A_0 + A_1 \times (1 - e^{-t/B_1})$$

Where:

A_0 = Adsorption percentage at t = 0. In this case, A_0 = 0;

A_1 = Adsorption percentage at equilibrium, when t → +∞. A_1 = 52.0;

t = time (min); and

B_1 = A constant that represents the growth rate of the function in time. The bigger the value of B_1, more time is needed for the system to reach its equilibrium. B_1 = 30.7.

[10] These calculations were performed with the help of Professor Pedro Pires.

The plot obtained shows that during the first ≈ 60 minutes the system is evolving at a fast pace, due to the transport of molecules in the solution surrounding the resin (film diffusion), and at this point it starts to slow down, caused by intraparticle diffusion (diffusion of molecules inside the adsorbent), thus stabilizing the percentage of adsorption.[32]

It was determined that at 2 hours, the percentage of adsorption was 51.5, very close to the 52.0 %, at which the system is stable, confirming that the previous experiment, reducing the contact time is advisable and substantiated.

F. Optimised parameters

When the final adsorption was made, using the optimised parameters, 144 mg FA/g dry resin were obtained.

G. Final considerations/remarks

It is understood that this study is far from complete as there are still questions left unanswered, as stated throughout the discussion, with the adsorbent dose and regarding the contact time. Also, the influence of the pH was not investigated, since Dávila-Guzman, N. E. et al. (2012) state that the adsorption of FA into hydrophobic neutral resins is inadvisable in media with pH > 3, as there is deprotonation and hydrophobic interactions between the solute and the resin become weaker, as the compound's polarity increases.[32]

1.2. Resin selectivity

For this work, the loading of the resin was performed by adsorption of FA from its solution. Here, it was hypothesized the option of loading from a food matrix extract, which would save the step of FA purification. To test the resin's selectivity a mixture of the most common HCAs was used.

Figure 25 shows the results obtained from the adsorption experiment with a mixture of five HCAs, presented in mg HCA/g resin dry weight (q_e).

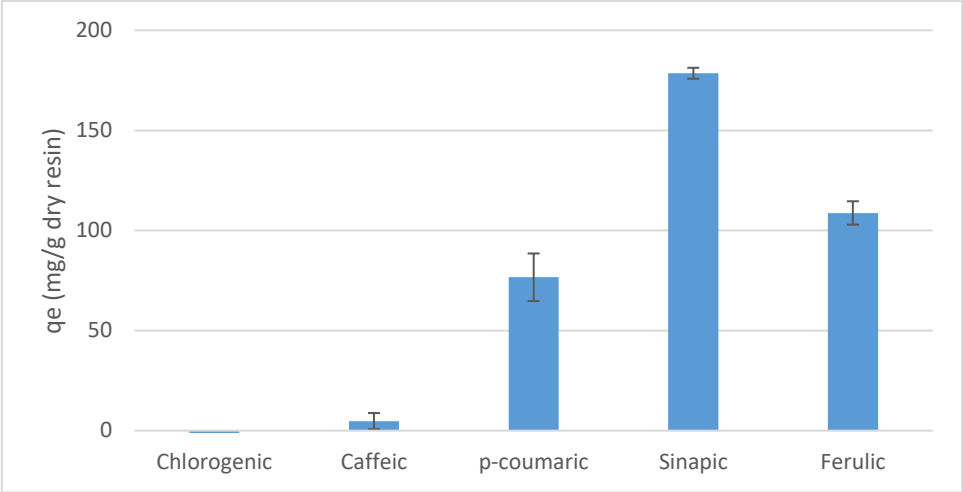


Figure 25 - Amount of each HCAs studied in this work adsorbed to the Lewatit resin (mg/g dry weight). Values are presented as mean ± SD of three assays.

Chlorogenic and caffeic acids were the least adsorbed compounds, in the conditions used in this work. In contrast, the remaining compounds were better adsorbed by the resin, obtaining 109 and 179 mg/g of ferulic and sinapic acids, respectively, loaded into Lewatit.

Since the solution used was a mixture of the five compounds, the results may suggest a competition for the active sites of the resin by the analytes. However, the small error bars suggest sinapic acid is the hydroxycinnamate with the highest affinity for the neutral polymer, probably because the hydrophobic interactions increase with the presence of the extra methyl groups in sinapic acid (Figure 2).

These results seem to be dependent of the compounds' hydrophobicity to some extent, i.e. the higher the hydrophobic nature of the acid, the higher the adsorption yield, as it is already known that hydrophobic forces are main interactions when dealing with adsorption on neutral non-polar resins, such as Lewatit. When analysing the chromatogram of the mixture in Figure 11 (Chapter II) and knowing that compounds 3-5 were the ones better adsorbed, one can see that this indeed results from their hydrophobic nature. The chromatography was made in reverse-phase (non-polar stationary phase and polar mobile phase) and so, the more hydrophilic compounds have the lowest retention times (compounds 1 and 2), what may explain why these had the smallest q_e.

Sinapic acid is the second most abundant HCAs in cereal (Table 2 in the Introduction) and adsorbs more into this resin. This competing effect should be taken in account if the resin is to be loaded from a food matrix rather than from pure compound solutions.

1.3. Physical and chemical characterization

In order to confirm the effective immobilization of FA in the resin, the ATR-FTIR, SEM, and EDS techniques were used.

1.3.1. ATR-FTIR Spectroscopy

ATR-FTIR Spectroscopy was used for the confirmation of the loading of FA on the polystyrene resin. Spectra of the resin prior and after the adsorption were collected, as well as spectra of pure FA, as depicted in Figure 26.

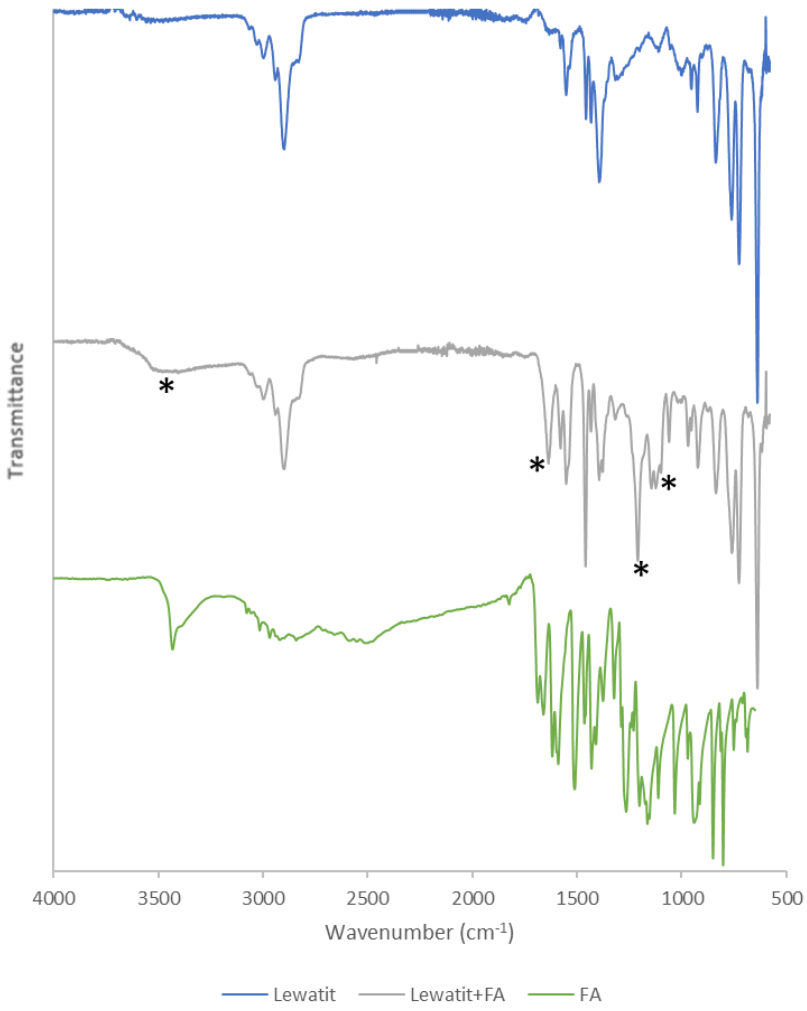


Figure 26 - ATR-FTIR spectra of the unloaded and loaded resin and FA. * = peaks attributed to FA.

The unloaded and loaded polystyrene resin's spectra were very similar, indicating no variations in the resin structure, although the presence of FA resulted in key differences, with the appearance of new peaks and an apparent increase of intensity of all peaks in the Lewatit+FA spectrum, since polystyrene and FA share some chemical groups.

The most relevant spectral change after the loading of the resin is the disappearance of the free OH band around 3500 cm^{-1} from the phenolic OH group of FA, and of the two broad bands (3100-2500 cm^{-1}) of dimerized carboxylic groups from the FA's spectrum, that later appeared as a wide band around 3500 cm^{-1} in the Lewatit+FA's spectrum. This shows that the interaction between the resin and FA is not a deposition of FA on the resin's surface.

The appearance of new peaks in the Lewatit+FA spectrum, at 1685, 1267, and 1184 cm^{-1}, result from the presence of oxygen containing functional groups of FA, C=O, C-O-C, and C-O, respectively. Also, peaks such as those at 1602, 1511, and 702 cm^{-1} seem to become more intense after the incorporation of FA into the resin, however some of FA's characteristic peaks seem to be overlapped by those of the resin itself.

In conclusion, it was possible to confirm the adsorption of FA, using the ATR-FTIR technique. There was little sample preparation and the peaks obtained were very resolved, indicating this tool can be used routinely for this kind of procedures. Additionally, the peaks on the resins' spectra were not very intense, with the lowest value having around 88 % Transmittance (values not shown). Although it is a limitation, it is also possible that increasing the amount of adsorbed FA will increase the intensity of its own characteristic peaks, and the confirmation of its immobilization would be more accurate, as the peaks would be more prominent.

1.3.2. SEM morphological and elemental analysis

The morphological analysis of the resin spheres before and after the adsorption of FA was made on the SEM, and the pictures captured are shown in Figure 27.

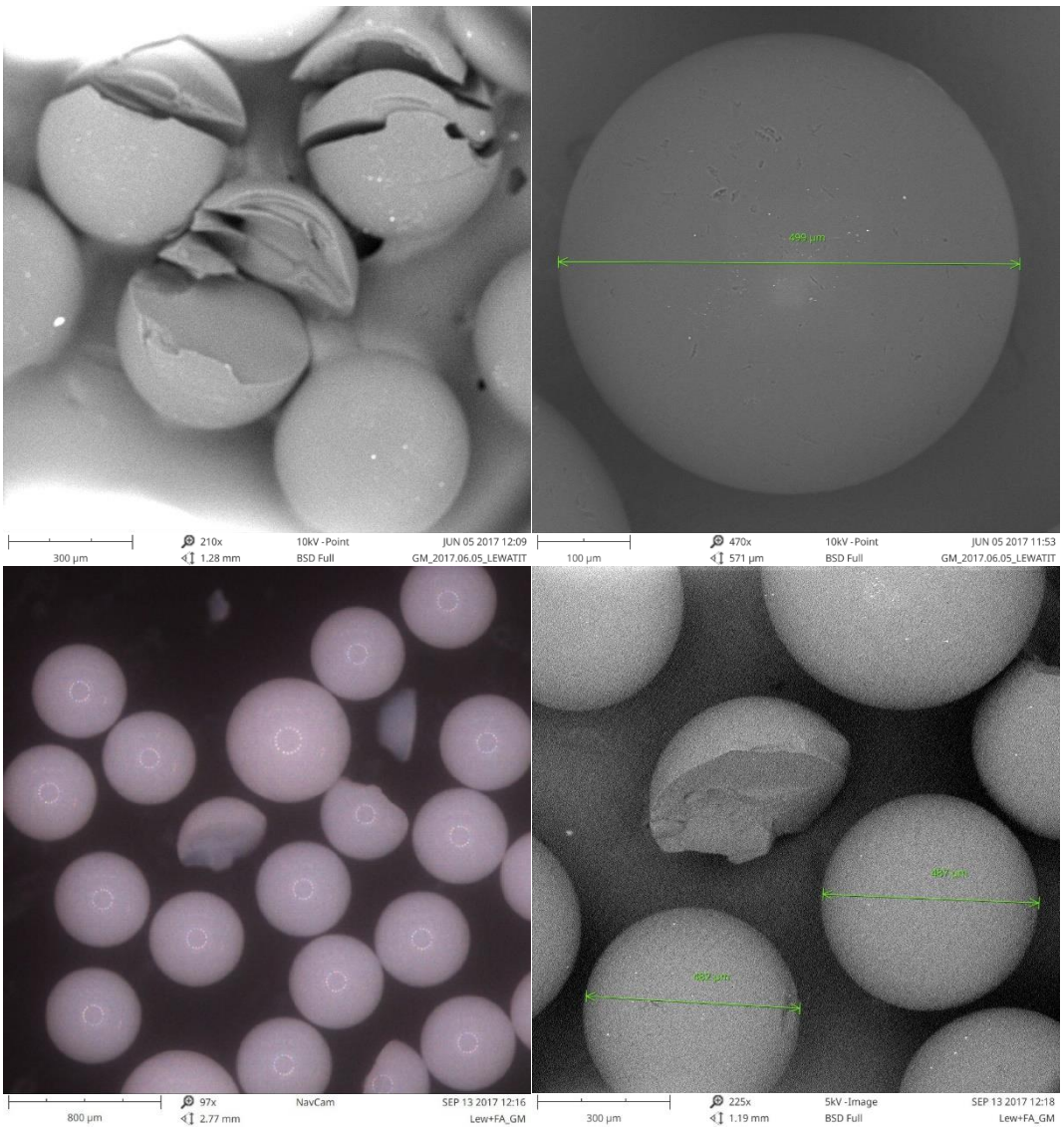


Figure 27 – Scanning electron micrographs of unloaded and loaded Lewatit resin. Upper line: general view of intact and broken unloaded resins (left) and detail with size measurement (right). Bottom line: optical visualization (left) and electronic visualization details with size measurements (right) of the FA loaded resin.

There were no observable differences in the physical properties between the resins, before and after the adsorption, both in terms of size and morphology. A homogenous size distribution was observed in both cases. Almost all spheres had about 500 µm in diameter, as evidenced in the pictures on the right, although some outliers were found, as seen in the bottom-left picture, with a size of 550 µm (measurement not shown). The surfaces of the resin were quite smooth and there was no change after the loading of FA.

Regarding the confirmation of the incorporation of FA onto the resin, it seemed appropriate to use the EDS technique, given that the polystyrene resin (Figure 28) has no oxygen in its composition, contrary to FA (Figure 4, Introduction). It was expected that

the elemental analysis suggested the resin was around 100 % carbon – hydrogen has no characteristic X-ray, so it is not detectable by this technique; whereas its loaded counterpart would have a lower percentage of carbon and oxygen would be detected.

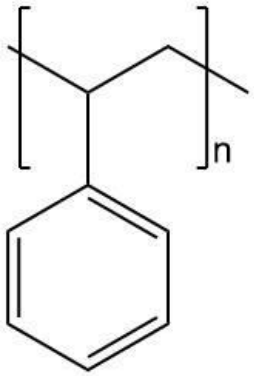


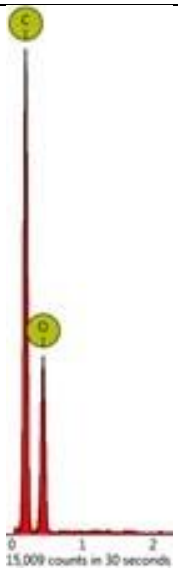
Figure 28 – Structure of polystyrene.

The obtained elemental concentrations are shown in Table 8. Surprisingly, both carbon and oxygen were detected in similar amounts in both resins, so the confirmation of the loading of FA was not successful by this technique. This can be due to water adsorbed on the samples, even though they were dried prior to the assays, causing an overestimation of oxygen, especially for unloaded Lewatit and FA itself. Additionally, the fact that the samples are not conductive may have influenced the analysis because the accumulation of charge seemed to affect the integrity of the samples and the analyses needed to be quick.

EDS analysis is semi-quantitative and when using the charge reduction sample holder, elements present in the atmosphere can be detected, as the vacuum is less intense. Also, the sample area analysed is very small, so it is possible the results are not representative of the whole sample, especially if dealing with materials that are not homogenous in their composition. Lastly, considering the real concentration of FA adsorbed to the resin (144 mg/g = 14.4 %), it simply is not possible to accurately determine any differences before and after the adsorption of FA. [11]

[11] Information provided by Pedro Prazeres from Phenom-World.

Table 8 – EDS results of the composition of unloaded and loaded Lewatit, and FA.

Sample	Lewatit	Lewatit+FA	FA
Spectrum			
Composition (atomic percentage)	61 % Carbon 39 % Oxygen	60 % Carbon 40 % Oxygen	26 % Carbon 74 % Oxygen

It was concluded that, using the EDS technique, it is possible to qualitatively detect elements such as carbon and oxygen, but their quantification is not accurate. Consequently, it cannot be used for the confirmation of the loading of FA onto the resin. However, a possible way to do this, would be to link FA with a heavier element, as a "marker", prior to the adsorption, that could then be detected and accurately quantified using EDS.

This being a study leading to a Master's degree, it was considered valuable to experiment as much as possible the available techniques that could confirm the immobilization of FA on the resin. Fluorescence Spectroscopy was also performed. However, both the resin and the acid exhibited fluorescence and a lot of optimizing work would be needed so any conclusions could be withdrawn, so this technique was left for future work and will not be further discussed.

2. *In vitro* digestion simulation

2.1. HPLC-DAD compound detection

As previously in the adsorption studies, FA detection and quantification was carried out with HPLC-DAD. Digested samples were complex mixtures of small organic molecules, salts, and enzymes, so this technique was useful in the selective determination of the amount of FA in the post-digestion media.

Figure 29 shows the chromatogram of FA obtained after a full digestion (mouth, stomach, and intestine, with adjuncts). It resembles perfectly to the one of the standard compound depicted in Figure 11 (Chapter II, section 1.2.2). This means that although there are many compounds in solution at this point, there is no change in the profile near the retention time of FA and no peak overlapping. Also, there are no other compounds resulting from the degradation of FA. Chromatograms of FA at other stages of the simulated digestion are present in the Supplementary Information (Figures S5-S7) and provide the same conclusions.

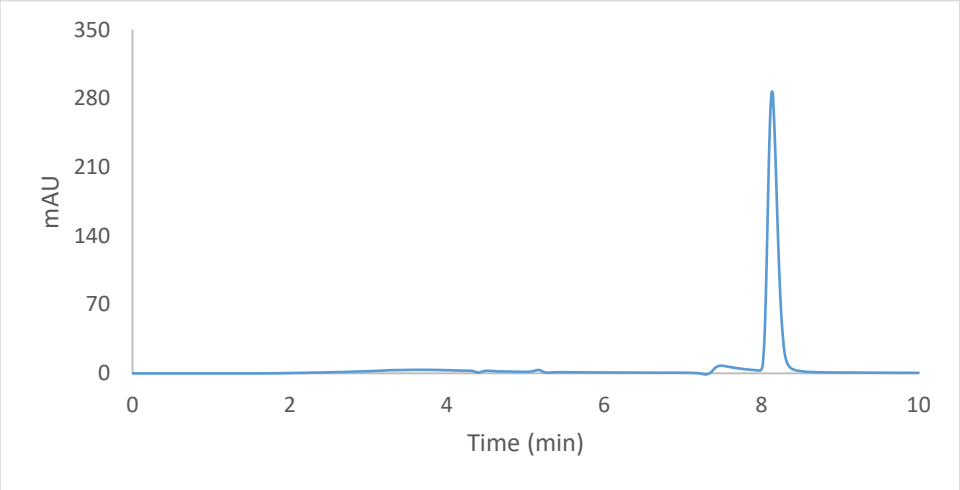


Figure 29 - Chromatogram of FA obtained at 320 nm by HPLC-DAD after a full digestion with adjuncts.

2.2. Liquid samples' digestion

A solution of FA (1 mg/mL) was digested as described in the previous chapter. This assay helped to understand what would happen to this compound while in the gastrointestinal media, i.e. if the expected decrease in concentration resulted from the dilution with the simulated juices or if there was some degradation during the process.

The aliquots withdrawn at the three stages of the digestion were analysed by HPLC-DAD and FA's concentration was determined, for both digestion methods (with and without adjuncts). These results were compared with the predicted values of the

amount of FA present at each point, calculated by successively applying the dilution equation. The concentrations determined for the digested samples should be close (equal or lower) to the calculated values.

$$C_1 \times V_1 = C_2 \times V_2$$

For the salivary step, C_1 = initial concentration (mg/mL); V_1 = initial volume (mL); C_2 = concentration to be determined in the salivary step (mg/mL); and V_2 = final volume (mL), after the addition of the salivary juice.

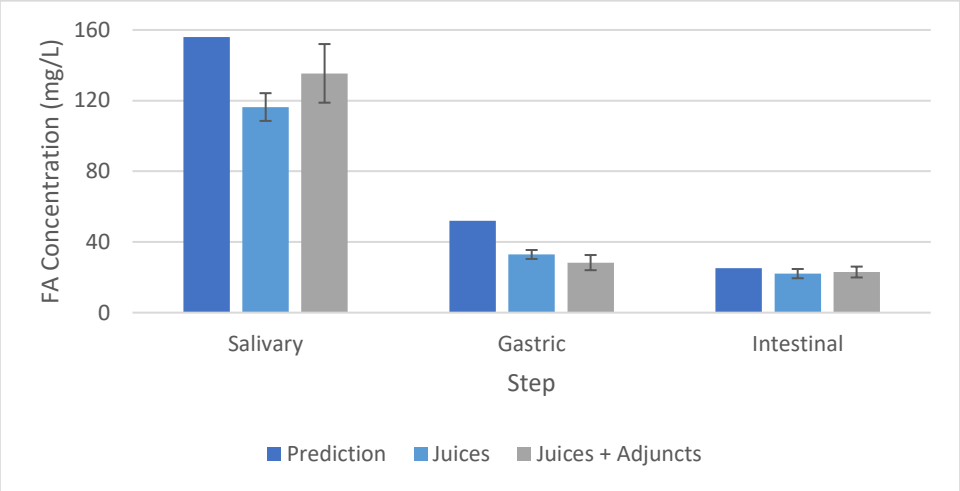


Figure 30 - FA's concentration in the digestion media. The presented values are the average of three assays ± SD. In the case of the Prediction the values refer to theoretical calculations.

As Figure 30 depicts, the concentrations obtained after digestion were, in general, similar to the prediction and were always lower, as expected. After the two digestion methods, the concentrations obtained for both cases can be considered identical, suggesting that the presence of adjuncts has no influence over FA.

If the enzymes in the juices had some effect towards FA, there could be some degradation or chemical changes that would be reflected during the HPLC quantification. However, the concentrations obtained with and without the adjuncts were similar. As such, it can be concluded that the two methods give equivalent results regarding the stability of FA during the *in vitro* digestion, in the conditions of this work.

This outcome is not unexpected as the used enzymes act upon specific types of molecules other than phenols, and so, degradation by enzyme activity in these assays was not expected to occur: mucin is a glycoprotein and the main component of mucus, therefore is important in the emulsification of drugs; α-amylase breaks down starch into glucose; pepsin and trypsin are proteases, they convert polypeptides into smaller units;[60] lipases produce monoacylglycerol and free fatty acids from triacylglycerols;

and pancreatin is a complex mixture of biological components and digestive enzymes, such as amylases, proteases, and lipases.[61]

Even though there may not be any perceived enzymatic activity over these samples, when assaying the behaviour of the loaded resin *in vitro* adjuncts should be used, as the complex mixture of enzymes, added in sequential order, simulates the gastrointestinal conditions and the results obtained provide a better understanding on the expected behaviour *in vivo*.[42]

2.3. Solid samples' digestion

Considering the reasons stated previously, while studying the stability of FA, only the assays using adjuncts will be discussed.

The amount of compound released from the resin samples in all digestion steps (including the assay with the gelatine capsule) can be found in Figure 31 and was calculated using the equation below. This equation takes into account the volume of the digestion media, and consequently the dilutions, so the values are comparable.

$$qr = \frac{C \times V}{m}$$

Where:

qr = amount of FA (mg) released from the Lewatit resin (g);

C = concentration of FA in solution (mg/mL);

V = volume of the digestion medium (mL);

m = amount of weighted resin (g).

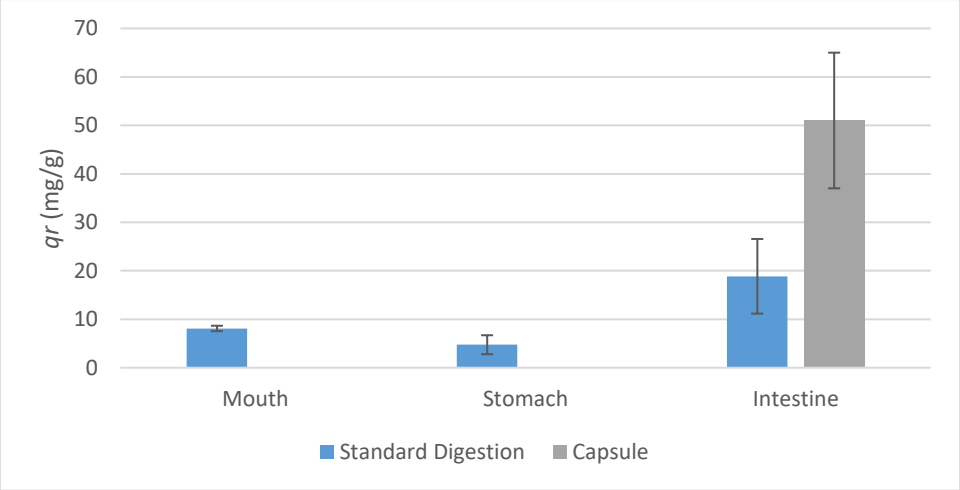


Figure 31 - Amount of resin-bound FA released in gastrointestinal conditions. The variant "Capsule" refers to the full *in vitro* digestion using the gelatine capsule. The presented values are the average ± SD of three assays.

Analysing the first three assays (standard digestion), one can see that the full digestion provided the major release of FA (19 mg/g), followed by the mouth (8 mg/g) and stomach steps (5 mg/g).

From an efficiency point of view, the release of FA was very poor, because at the end of the digestion only 13 % of the loaded compound was detected in the medium. However, the 19 mg/g obtained are comparable to the quantities found in several foods stuffs, as these range from 0.19 x 10^{-3} mg to 33 mg/g (fresh weight)[12].[7]

The release observed in the mouth may be attributed to the ionization of FA at basic pH, which weakens the intensity of the hydrophobic interactions between it and the polystyrene resin. In the stomach, however, there seems to be a re-adsorption of the compound that had already been released in the mouth, due to the low pH, and this would explain why the amount of compound in solution decreases after the first step. Finally, in the intestine it is possible that the ionization of FA is what triggers the release, like in the salivary step, but the longer transit time (2 hours in the intestine vs. 5 min. in the mouth) and the higher pH may cause the increase in the amount released. In the adsorption assays, the low pH favoured the adsorption process, so it would not be unexpected if for the desorption it was the other way around.

During the *in vitro* digestion with the gelatine capsule, a release of 51 mg/g was obtained (36 % release), an improvement to the 19 mg/g without the capsule. The high deviations are probability due to the mixing and to the dissolution rate of the capsule itself, affecting the contact time of the resin with the media in all assays. It was observed (Figure 32) that the release of the resin from the capsule was a gradual process (as already mentioned in the Methods) and was different in all experiments.

[12] Table 1 in the Introduction.

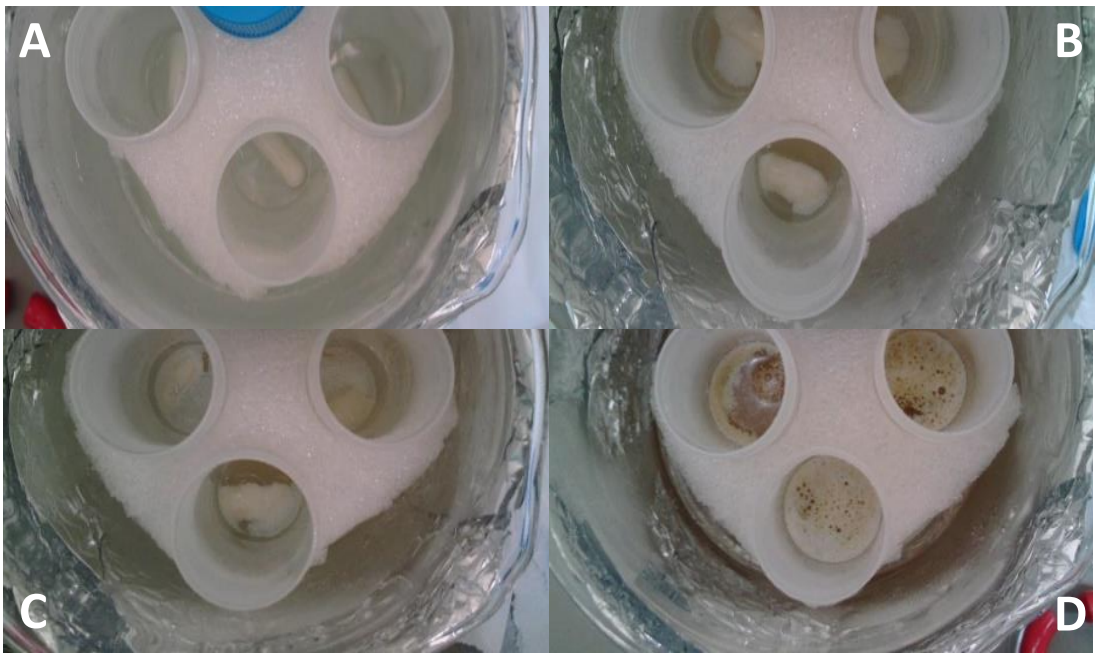


Figure 32 - Digestion of the encapsulated resin-bound FA: A - before the addition of the salivary juice; B – middle of the gastric digestion; C – end of the gastric step; D – intestinal stage.

Despite the greater difficulty with the mixing of the several components, the amount of FA released was increased in comparison to the experiments without encapsulation. The gelatine capsules served mainly as carriers, but the presence of surfactants in their composition may improve the dissolution of FA. This is a common strategy for the enhancement of the dissolution of poorly soluble drugs, and surfactants like Tween-80 are added to the formulations for this purpose.

The encapsulation of the loaded resin samples may be the more realistic approach given the practical issue with the swallowing of the spheres, so the amount of FA released in that experiment should be the value considered, 51 mg/g, further supporting the notion that the delivery of FA in loaded resins is an alternative to the dietary intake as discussed earlier.

In the paper by Kammerer, D. R. *et al.*, the recovery of phenols from apple juice using the food grade polymethylmethacrylate adsorbent resin, Alimentech P-495, was studied. Unfortunately, there was no mention of FA, but other hydroxycinnamates such as chlorogenic acids were analysed, and the desorption of these in water at 40 °C was found to be around 25 %, a value close to that obtained in the present work. It was also determined that adding ethanol to the elution medium favoured the desorption of these compounds from Alimentech P-495. A parallel to this work can be made with this

information and one must wonder if the administration of this formulation with an alcoholic beverage is beneficial.[62]

In terms of *in vivo-in vitro* correlation (IVIVC), if the administered dose is not very high, an IVIVC can be achieved for a BCS Class II drug. Still, there are variations to the digestion method that could be made, resulting in a clearer understanding of the release behaviour of the drug. For instance, the bile secretions present in the small intestine contain phospholipids such as lecithin, both in the fasted and fed states, however this component was not included in the intestinal or bile juices. The presence of phospholipids is important for the emulsification of poorly soluble drugs, by the creation of micelles and wetting of solids, thus increasing their solubility in the digestive medium. Also, the production of digestive enzymes in the fed state influences the dissolution of BCS Class II compounds, compromising their stability, specially lipases and peptidases. This means that the obtained results could still be closer to reality, and should not be taken as absolute.[63]

The improvement of the dissolution of FA is the main focus, because for a BCS Class II drug (poor solubility, high permeability) it must be assumed that if the drug is in solution, it will most likely be absorbed, since the rate-limiting step is the release and dissolution.[64] Although only 13 % and 36 % release was achieved in the assays without and with the capsule, respectively, inappropriate mixing may be the cause for the poor release, meaning these values could be improved. Additionally, LANXESS states that the grinding of the resin prior to its administration *per os* is the usual procedure for the application of these materials. If this is the case, it would be expected that this pre-treatment would ease the release of the adsorbed compound, making it more efficient.[13]

Finally, as the digestion progresses, the deviations increase, particularly in the case of the intestinal digestions. This may be caused by inappropriate mixing of the loaded resin samples with the digestion juices, especially since the resin is hydrophobic, very light and it is dehydrated, resulting in the beads floating in the medium (Figure 33). Following the method implemented in our lab, magnetic stirring was used in this work, however it is reported that head-over-heels rotation, as used in the adsorption studies, provides greater repeatability.[65,66] Still, this type of rotation could be difficult to apply as there would be a limitation with the heating of the mixtures, because to run the experiments at 37 °C the rotator would have to be placed inside a large oven. Nonetheless,

[13] See Figure S2 of the Supplementary Information.

these results possibly represent the worst-case scenario, as it is expected that with proper agitation, the amount of released compound will increase.

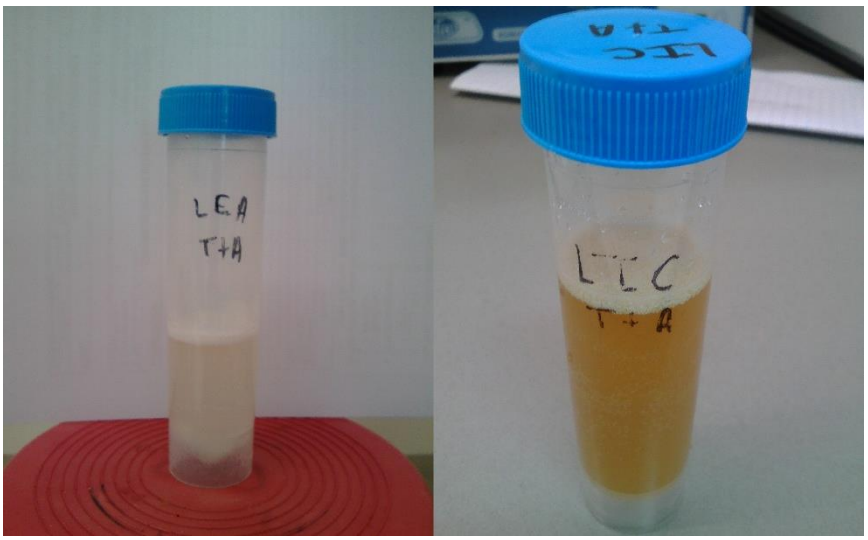


Figure 33 - Loaded resin's simulated digestion in the stomach (left) and the intestine (right).

SEM analysis of the digested Lewatit+FA

After the full digestion, the loaded resin remaining was recovered and, after lyophilization, was analysed in the SEM to observe if there were any changes in the structure of the resin.

In Figure 34 the SEM images of the loaded resin after the digestion with and without the adjuncts are shown. Comparing to the images in Figure 27, the surface of the resin seems rougher than before the digestion, what can possibly be explained by the friction between the spheres during the digestion. Also, there seems to be deposition of crystalized salts from the buffer at the surface of the resin from the digestion without the adjuncts, as can be seen by the bright spots on the spheres, whereas in the digestion with the adjuncts there is no apparent deposition, suggesting that the adjuncts may "clean" the surface of the resin. However, this deposition is probably only visible because the resin was not washed after the samples were collected and was stored in the freezer causing the crystallization and consequent deposition. Still, the physical integrity of the resin seems have been kept during the digestive process.

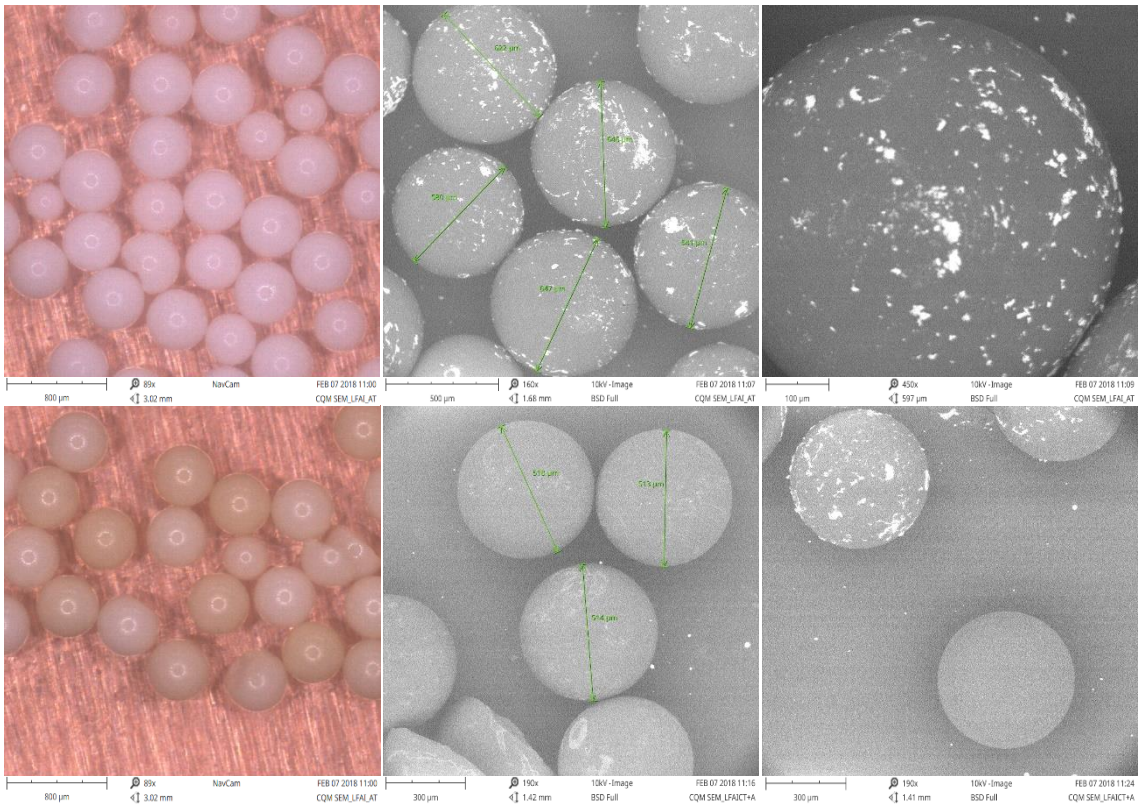


Figure 34 - SEM images of the loaded resin after a full digestion. Legend: A, B, C and top spheres on F – digestion only without the adjuncts; D, E and bottom sphere – digestion with the adjuncts.

Lewatit+FA for lowering cholesterol levels

During the digestion studies it was noticed that the release experienced in the intestinal step may be aided by a competitive and simultaneous adsorption of bile acids, as suggested by the colour change in the resin (Figure 35). As mentioned in the Introduction, adsorbent resins have been used as bile sequestrants, making this a real possibility.

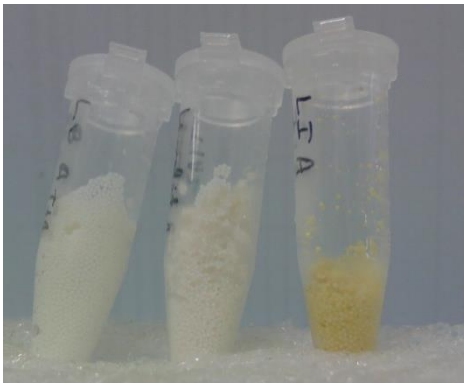


Figure 35 - From left to right: digested resin after the salivary, gastric and intestinal steps.

The usual treatment for high cholesterol levels is the administration of drugs known as *statins*, that inhibit the action of HMG-CoA reductase, an enzyme essential in the biosynthesis of cholesterol. However, there are a few setbacks with potential side effects, so their use on pregnant women, children, and patients with liver failure is unadvisable.

An alternative is the use of polymeric materials for the sequestration of bile acids. These compounds share the same metabolic pathway, with cholesterol being the precursor of bile acids, so when the amount of the latter drops, their metabolism is up-regulated to maintain bile levels and consumes the former. Although this sequestration is commonly achieved with the use of cationic resins, because of the electrostatic interactions with the negative groups of bile acids (Figure 36), the hydrophobic forces keep them from desorbing back into the gastrointestinal medium, and styrene backboned (cationic) resins have been used for this purpose, suggesting that Lewatit could also be a candidate.

Considering this information while knowing that FA has anti-cholesterolemic activity, it is interesting and only natural to speculate if the administration of FA loaded resins would be an effective treatment for this condition, inhibiting the production of cholesterol, while consuming it simultaneously. The combination of sequestrants with statins is not a novelty, and this could help solve the major problem with bile sequestrants which is their low clinical efficacy, where 16-24 g of resin are needed to achieve a 20 % decrease in cholesterol levels.[13,29]

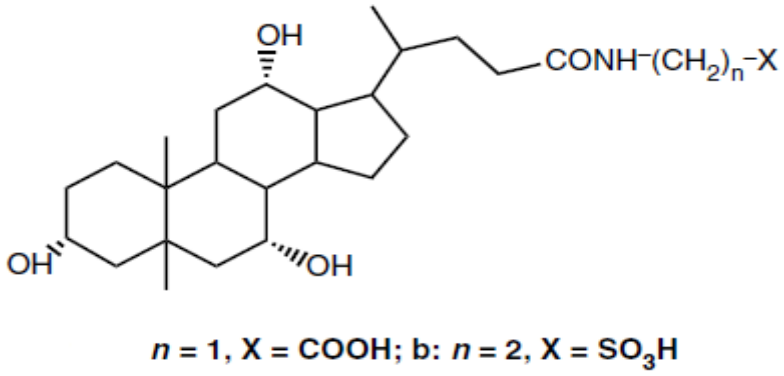


Figure 36 - Structure of bile acids. Adapted from Dhal, PK et al. (2006).[29]

Final remarks

These kind of studies are very complex, with many variables and parameters to take into consideration: the properties of the drugs (BCS Class and others), the encapsulating and delivery forms, the dosage, the rotation and mixing, the pH of the

media (which can be surprisingly different from study to study), the presence/absence of food and the resulting modifications in the juice's composition.

As stated in the literature, there is no universal dissolution methodology. Each analysis is different and new, so adjustments should be made when necessary, optimizing the IVIVC. Unfortunately, there are always limitations that need to be overcome, and, in this work, they can be identified as the issue with the mixing, the need for the gelatine capsules for the delivery of the loaded resin spheres, and the absence of surfactants in the formulation. Nonetheless, these experiments proved it is possible to orally deliver FA adsorbed onto a polymeric resin, with its major site of release in the intestine.[63]

In terms of bioavailability, it is greatly affected by absorption, which, in turn, is influenced by the dissolution/solubility rate and by the chemical stability in the gastrointestinal media. It has been suggested presently that FA is stable throughout the *in vitro* digestion, meaning that the dissolution is the main limitation to be tackled. This corroborates the information obtained previously by the log D and the classification of FA as a BCS class II compound. Additionally, permeation enhancement is more successfully achieved with drug design rather than drug delivery studies, which is the main topic in this work.[67]

Additionally, drawing comparisons with the literature regarding the release/desorption of FA or any HCA analogue was very difficult, and this is felt on the lack of said information, for a number of reasons. In some studies, the compounds do not belong to the same class as FA; also, there are reports where the desorption experiments are not performed; and, in other cases the elution assays are made with solvents other than water, that obviously are not found in the gastrointestinal tract, so comparisons would be futile. However, there are studies where FA is released from encapsulating agents such as electrospun fibers, with 60 % and 100 % of release after the gastric and intestinal digestions, respectively. These results were ascribed to the ultrathin structure of the fibers and also to conformation changes of the fibers in the different pH of the media.[68]

Also, the amount of FA present in food may be not all bioaccessible, since it is mostly covalently linked to other molecules. In this work, it is assumed that the compound released from the resin in these conditions is bioaccessible, since it was the free form. As such, comparing the effective bioaccessible amount of FA in this work with those present is food stuffs, it seems that the delivery of FA through the oral administration of a formulation of Lewatit+FA is more advantageous than through the diet.[65]

3. Antioxidant activity evaluation

3.1. Standard ferulic acid solutions

Starting with a 1 mg/mL standard FA solution, an *in vitro* digestion was performed as described previously. At the end of each stage, aliquots were withdrawn, the FA concentration was determined by HPLC-DAD and the DPPH assay was performed. The DPPH inhibition (%) of said samples are depicted in Figure 37 and were calculated as such:

$$DPPH\ inhibition\ (\%) = 100 - (\frac{A_{sample}}{A_{DPPH}} * 100)$$

Where:

Asample = Absorbance of the sample at 550 nm after the reaction;

A_{DPPH} = Absorbance of the DPPH solution at 550 nm;

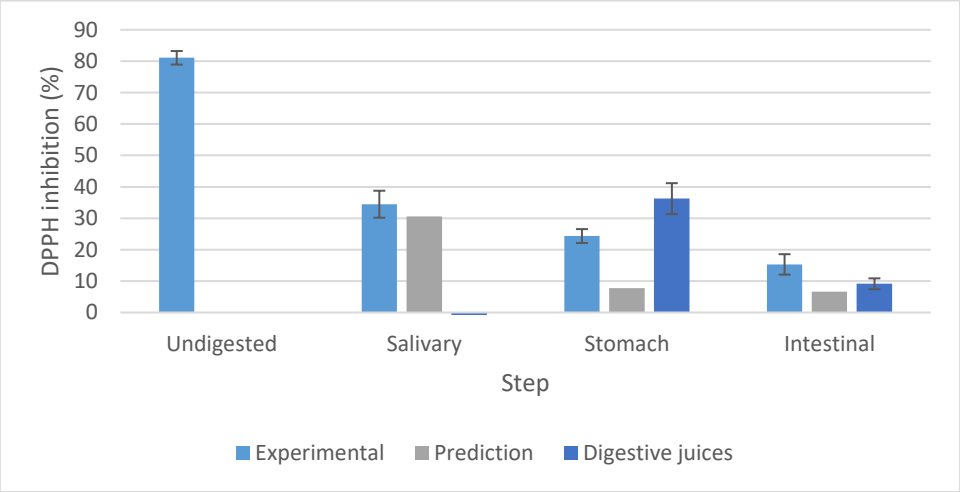


Figure 37 – DPPH inhibitions (%) obtained for the standard FA solution before and after each digestion step, the predicted values for each concentration, and for the simulated digestive juices. The values presented are the mean ± SD of several assays: n = 3 for the undigested solution and simulated digestive juices; and n = 9 for each of the digested samples.

From the concentration of each sample determined by HPLC-DAD after each digestion step (Table 9), the predicted values of the DPPH inhibitions (%) were calculated using the calibration curve prepared using standard FA solutions (Figure S8 in the Supplementary Information).

Table 9 - FA's concentration in each step of the standard FA solutions' digestion.

Step	Salivary	Stomach	Intestinal
FA (mg/L)	135	28	23

The DPPH inhibitions get increasingly lower as the digestion progresses, i.e. as the concentrations decreases. However, after the gastric step there is a big difference

between the predicted inhibitions and the experimental values, what could be explained by the simulated juices exhibiting AA. The salivary juice shows no AA, and the predicted and experimental values obtained for the FA samples are similar.

This is a problem, as the juices mask the results of the samples, especially at low concentrations of antioxidants. Unfortunately, to the best of the author's knowledge, the AA of the digestive juices have never been debated in the literature, and consequently, the reason for their AA is unclear.

3.2. Supernatants of the Lewatit+FA digestions

The DPPH inhibitions calculated for the supernatants of the Lewatit+FA digestion, as calculated previously, are presented in Figure 38. Also, in Table 10 the concentrations of FA in each step are shown.

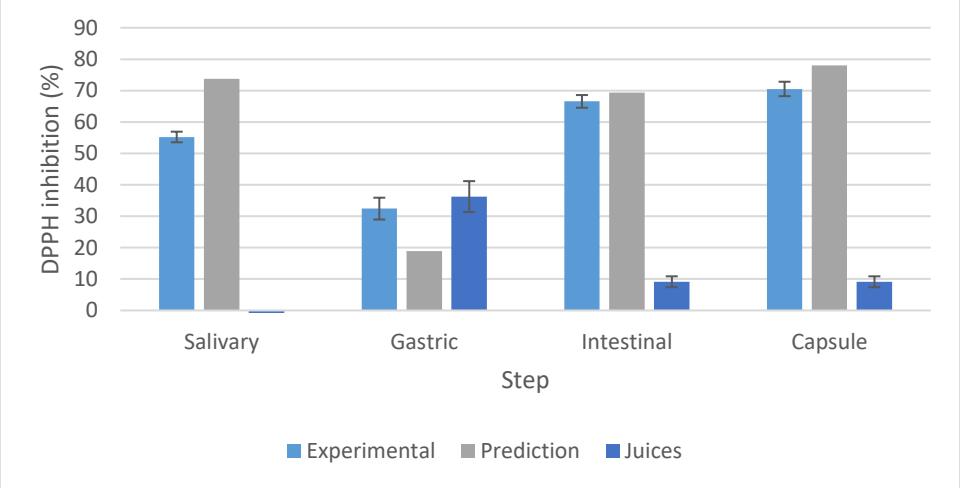


Figure 38 - DPPH inhibitions (%) of the supernatants of each step of the Lewatit+FA digestion, as well as the prediction based on each sample's concentration. The values presented are the mean ± SD of nine assays.

Table 10 - FA concentration in the supernatants of the Lewatit+FA digestion after each step.

Step	Salivary	Gastric	Intestinal	Capsule
FA (mg/L)	338	81	317	358

The intestinal step and the capsule assay provided the highest inhibitions, with similar results, followed by the salivary sample, and finally by the gastric step. Since these samples are the supernatants of the digestion of the loaded resin, there is no initial undigested sample for comparison.

The experimental values were always lower than those predicted, except for the gastric step, as in the previous situation. Similarly, the influence of the gastric juice seems to be responsible for the inhibition at this stage once more, so the real AA of FA after its

release from the resin in the stomach cannot be accurately described. However, in the intestinal step, the major site of release of FA, the digestive juice seems to have little influence in the AA of the sample, maybe due to the high concentration of FA present in solution.

Using the equation below, the Trolox eq. (mM/g/L FA) were calculated, dividing the Trolox eq., obtained from the calibration curve (Figure 18, Chapter II, section 3), by the concentration determined by HPLC-DAD after the digestion. The results are displayed in Figure 39.

$$Trolox\ eq.(mM/g/L\ FA) = \frac{Trolox\ eq.(mM)}{[FA]\ (g/L)}$$

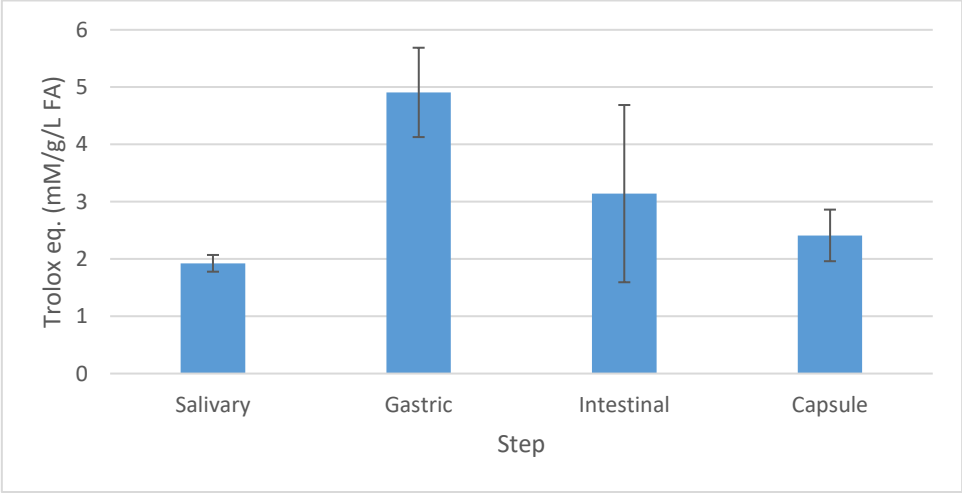


Figure 39 - Trolox eq. (mM/g/L FA) of the supernatants of the Lewatit+FA digestion.

When analysing the information depicted in Figure 39, one must be cautious while considering the gastric value, as it most likely reflects the AA of the gastric juice and not the one from FA. It is natural this value is so high, because of the low concentration of FA and the high inhibition possibly provided by the juice.

Even so, disregarding the gastric step, the intestinal step and the capsule assay provided the highest Trolox equivalents. However, the big error bars seem to suggest that the samples from the intestinal and mouth steps have similar AA. These big error bars are caused by differences in the concentration of FA within the same digested samples.

These experiments suggest that even after the digestion, FA still exhibits a noteworthy AA, especially in the intestinal steps. In terms of IVIVC this would mean that after the release of FA from Lewatit in the intestines, it is available to scavenge locally found endogenous radical species.

3.3. DPPH assay on the Lewatit+FA complex

The Lewatit recovered after each step was analysed, as well as the undigested loaded resin, and the results of the DPPH inhibitions (%) are displayed in Figure 40. The unloaded resin and the empty gelatine capsule's AA were also assessed, and the respective inhibitions are presented alongside those for the other samples.

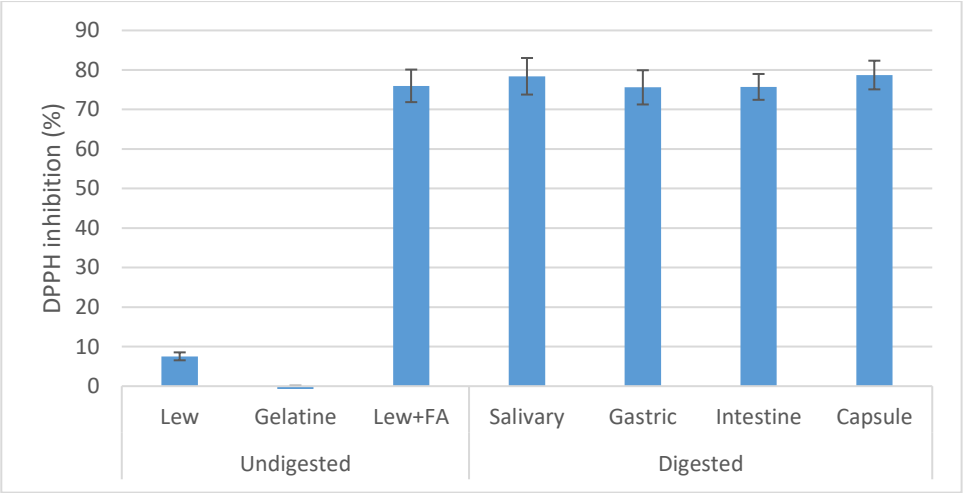


Figure 40 - DPPH inhibitions of the undigested unloaded and loaded Lewatit resin and gelatine capsule; and of the digested Lewatit+FA after each step of the simulated *in vitro* digestion. Values presented are the mean ± SD of three and nine assays for the undigested samples and the digested samples, respectively. The sample "Gelatine" refers to the empty gelatine capsule.

Contrary to the unloaded resin and the gelatine capsule that presented little or no inhibition, all the loaded resin samples were capable of efficiently scavenge the DPPH radical. Unfortunately, they were all capable of completely scavenge the radical, so it is not possible to accurately point out which sample presented the highest AA. The assay needs to be repeated, with the proper adjustments – increasing the radical/resin ratio; so accurate conclusions can be withdrawn.

Since the unloaded resin showed little AA, that of the loaded resins seems to be dependent of too steps, with the first being the desorption of FA to the methanolic medium, followed by FA's radical scavenging mechanism described previously in the Introduction (Section 1.2.2.2). To test this hypothesis, the assay was repeated with the initial Lewatit+FA sample and a parallel experiment was made but using only methanol, instead of the DPPH solution, so the supernatants could be analysed by HPLC-DAD and compared. Both experiments were done in triplicate.

FA was successfully detected in both cases (chromatograms in Figure S9 of the Supplementary Information), proving that in the context of this specific AA assessment test, the solvent causes the release of FA, that becomes available to react with radical

species. It is thus possible to determine which step is rate-limiting in the scavenging of the DPPH by the resin-bound FA, however this study is yet to be made.

The amount of FA in solution was very different, as methanol alone was able to almost completely release all FA adsorbed, whereas the DPPH solution resulted in a 51 % desorption efficiency, as depicted in Table 11. Even so, although only half of the bound compound is released during the DPPH assay, the amount present in solution is capable of completely reduce the radical. It is assumed this behaviour is common to the other loaded resin samples, since their AA was similar. This suggests that the compound remaining adsorbed after each stage of the digestion process is ready to act as an antioxidant upon release.

Table 11 - Release of FA during the DPPH assay and in methanol.

Assay	FA before release (mg/g)	FA after release (mg/g)	Release (%)
Lewatit+FA Methanol	144.0	15.8	89.0
Lewatit+FA DPPH		70.6	51.0

Final remarks

The goal of this experiment was to determine if FA maintained its AA throughout the *in vitro* digestion simulation, after its adsorption on the Lewatit resin. The supernatants of the digestions of the loaded resin proved this to be true, as all samples were capable of scavenging the DPPH radical. The intestinal aliquot was where the highest AA was measured.

The simulated juices somehow influenced the analysis, in the gastric and intestinal steps, however their real influence is unclear. When the supernatants of the Lewatit+FA were analysed, the intestinal juice's influence was unnoticeable, and since the site of major release of FA is in the intestine, seemingly accurate assessments were made, with the FA released at this stage having the AA of 3.1 mM Trolox eq./g/L FA.

IV. Conclusion and Future Perspectives

To the best of our knowledge, this is the first study of the *in vitro* release of ferulic acid from an adsorbent resin in gastrointestinal simulated media. This work proved that free ferulic acid immobilized on a non-charged material can successfully overpass the low pH of the stomach and be released in the intestine, where its action is much needed. The dietary intake of ferulic acid presents some limitations in terms of its bioavailability and this study showed that by using solid matrixes for the delivery of the free compound, this problem can be effectively solved.

The antioxidant activity assay was helpful in the qualitative assessment of the bioactivity of the samples before and after the various steps of the *in vitro* digestion, but the influences of the simulated juices suggest that perhaps the DPPH assay was not the most appropriate. During this work, a biorelevant antioxidant test, the Oxidative Haemolysis Inhibition Assay[14] was studied. This assay measures the inhibition of the oxidative haemolysis of red blood cells, in the presence of the naturally occurring radical APPH, by an antioxidant and preliminary experiments were made, using sheep erythrocytes provided by the Regional Veterinary and Food Safety Laboratory (*Laboratório Regional de Veterinária e Segurança Alimentar*), but unfortunately the assay was not successfully implemented.

Lewatit® VP OC 1064 MD PH being a macroscopic resin, may not be the most appropriate immobilizer since its administration would require a second type of encapsulation. Also, the physical properties of the beads made it difficult to analyse by the usual techniques, like in the case of the ATR-FTIR. Additionally, Lewatit is a synthetic polymeric resin, falling into the category of microplastic, which is something that has raised very serious environmental concerns. Perhaps a micro or nano immobilization would make it possible to disperse the resulting powders in water or another neutral beverage.

In past works in our lab, the production of electrospun fibers of the polymer Eudragit® 100-55 loaded with ferulic acid was attempted, but unfortunately the immobilization was unsuccessful. This could, however, be solved by incorporating ferulic acid in Eudragit by different methods.[15] This material is interesting because it can be

[14] See: Takebayashi J, Iwahashi N, Ishimi Y, Tai A. Development of a Simple 96-well Plate Method for Evaluation of Antioxidant Activity Based on the Oxidative Haemolysis Inhibition Assay (OxHLIA). Food Chem.; 2012;134(1):606–10.
[15] See: Joshi GV, Kevadiya BD, Bajaj HC. Design and Evaluation of Controlled Drug Delivery System of Buspirone Using Inorganic Layered Clay Mineral. Microporous Mesoporous Mater. 2010;132(3):526–30.

orally administered as a powder and the formulations are resistant to the gastric pH, ensuring that ferulic acid is delivered to the intestine. Additionally, studies have showed that Eudragit® 100-55 can lower the pH of the intestinal medium[16], possibly stabilizing ferulic acid in the undissociated form, increasing its absorption through passive diffusion.

As for future work and perspectives, it would be interesting to:

- Optimize the experimental parameters with influence in the adsorption studies that have been discussed previously such as the pH, the resin dose, and time;
- Use a different type of rotation during the *in vitro* digestion simulation in order to obtain more accurate results;
- Simulate the post-prandial conditions of the gastrointestinal tract by adding, for instance, milk and perform the *in vitro* digestion;
- Study the possible adsorption of bile acids by the resin;
- Assess the inhibition of HMG-CoA reductase by ferulic acid after the digestion; and
- Perform biorelevant antioxidant activity assays such as the Oxidative Haemolysis Inhibition Assay.

[16] See: Terao T, Matsuda K, Shouji H. Improvement in Site-specific Intestinal Absorption of Furosemide by Eudragit L100-55. J. Pharm. Pharmacol. 2001;53(4):433–40.

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VI. Supplementary Information

Table S1 - Properties of ferulic acid. Legend: a = Graf, E. (1992) [8]; b = Mota et al. (2008) [69]; c = Sohn, Y. T. and Oh, J. H. (2003) [70]; d = Serafim, T. L. et al. (2011) [41]; e = Ozkurucuklu et al. (2009) [71] (e1 = SPRAC prediction; e2= Capillary Electrophoresis [CE]; e3 = CE-Diode Array Detector [CE-DAD]; e4 = Potenciometric); f = Dávila-Guzman et al. (2012) [32])

Common name	*trans*-ferulic acid
IUPAC name	(E)-3-(4-hydroxy-3-methoxy-phenyl)-prop-2-enoic acid)
Cas number	1135-24-6
Chemical formula	$C_{10}H_{10}O_4$
Linear formula	$HOC_6H_3(OCH_3)CH=CHCO_2H$
Molecular weight	194.18 g/mol
Form	Colourless orthorhombic needles[a]
Melting point	174 °C[a]
Solubility	Ethanol, ethyl acetate, and hot water[a] S = 0.78 g/L ± 0.01 g/L in pure water (T = 298 K, pH = 3.46)[b]
***n*-octanol to water apparent partition coefficient**	0.3753 at pH = 3[c] 0.489 at pH = 10[c]
log D	0.42[d]
Dissociation constants	$pK_{a,1}$ = 4.27[e1]; 4.30[e2]; 4.38[e3]; 4.46[f]; 4.56[e4] $pK_{a,2}$ = 8.65[e4]; 8.75[e3]; 8.77[f]; 8.81[e2]; 8.83[e1]

Table S2 - Equipment and materials used throughout the work.

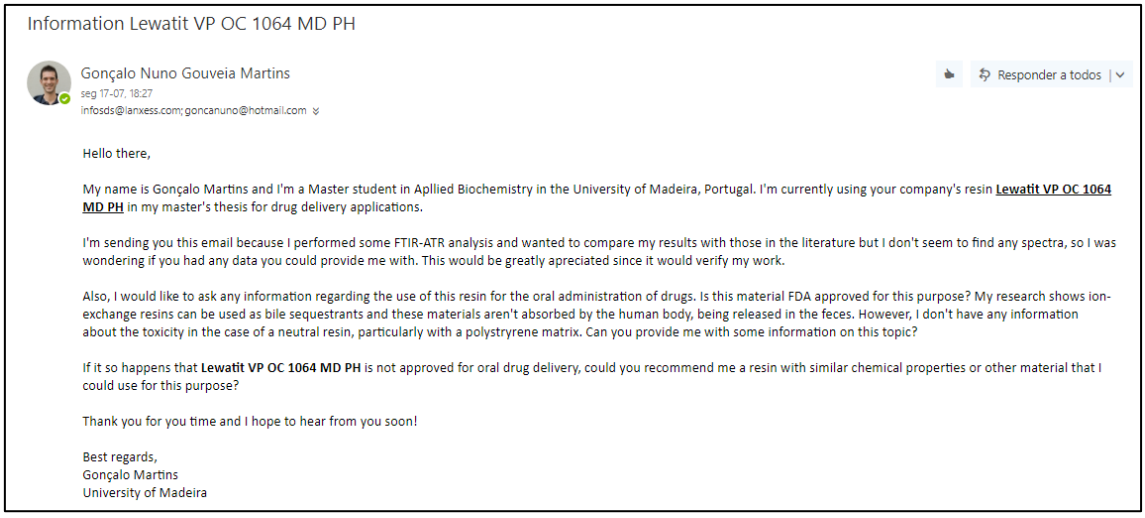
Equipment/material	**Model, *Brand***
Heating and stirring plate	Isotemp, *Fisher Scientific*
Head-over-heels rotation device	Deax 2, *Heindolph*
Ultrasonic bath	Sonorex Super RK 102 H, *Bandelin*
Centrifuge	Sigma 3K30, *Bioblock Scientific*
Centrifuge	Rotofix 32 A, *Hettich Zentrifugen*
Freeze drier	Alpha 1-2 LD Plus, *Martin Christ*
Analytical scale	AE200, *Metler Toledo*
Analytical scale	AB240, *Metler Toledo*
Moisture analyser	DBS 60-3, *Kern*
Refrigerator/circulator	Alpha RA8, *Lauda*
Dewar (circulator/refrigerator)	*Cole Parmer*
Thermostatic bath	W28, *Grant*
HPLC-DAD	Dionex UltiMate 3000, *Thermo Fisher Scientific*
Column (HPLC)	Gemini C18 Column, *Phenomenex*
UV-Vis spectrometer	Lambda 2, *Perkin Elmer*
FT-IR spectrometer	Spectrum Two, *Perkin Elmer*
Attenuated Total Reflectance (ATR) apparatus	Dura Sample*IR* II, *Smiths Detection*
Scanning Electron Microscope	Phenom ProX, *Phenom World*
Microplate reader	Victor^3 1420 Multilabel Counter, *Perkin Elmer*
0.45 µm cellulose acetate sterile filters	N. A., *Frilabo*
pH meter	744 pH Meter, *Metrohm*
Adsorbent resin	Lewatit® VP OC 1064 MD PH, *LANXESS Chemicals*
Gelatine capsules	Capsulas 00 incoloras, *Fagron*
3D-printer[17]	Prusa Mendel Iteration 2, *N. A.*

[17] For additional information contact: jorge.lopes@guest.uma.pt.

Table S3 - Reagents used in the experimental procedure. N.A. = Not applicable/available.

Compound – Formula	**Manufacturer**	**Purity**
Ferulic acid – $C_{10}H_{10}O_4$	Sigma-Aldrich	≥99 % (HPLC)
Hydrochloric acid – HCl	Vaz Pereira	37 %
Sodium hydroxide – NaOH	Panreac	>98.0 %
p-coumaric acid – $C_9H_8O_3$	Fluka	purum. ≥98 %
Caffeic acid – $C_9H_8O_4$	Sigma-Aldrich	≥98.0 %
Sinapic acid – $C_{11}H_{12}O_5$	Fluka	puriss. p.a. ≥99 %
Chlorogenic acid (3-*O*-caffeoylquinic acid) – $C_{16}H_{18}O_9$	Sigma-Aldrich	≥95 %
Ethanol – CH_3CH_2OH	Chem-lab	p.a. Abs. 100 %
Methanol – CH_3OH	Fisher Scientific	Analytical reagent grade 99.99 %
Formic acid – CHOOH	Sigma-Aldrich	puriss. p.a. ≥98 %
Acetonitrile – CH_3CN	Carlo Erba	HPLC Plus Gradient grade 99.9 %
Sodium chloride – NaCl	Sigma-Aldrich	puriss. p.a. ≥99.8 %
Potassium chloride – KCl	Panreac	p.a. 99.5 % to 100.5 %
Sodium bicarbonate – $NaHCO_3$	Fluka Analytical	purum. p.a. ≥99.0 %
Sodium dihydrogenphosphate – NaH_2PO_4	Merck	p.a. 99.0-102.0 %
Calcium chloride – $CaCl_2{\cdot}2H_2O$	Panreac	p.a. 99.0 % to 102.0 %
Ammonium chloride – NH_4Cl	Merck	p.a. >99.8 %
Potassium dihydrogenphosphate – KH_2PO_4	Riedel-deHaën	p.a. >99.5 %
Magnesium chloride – $MgCl_2$	Merck	p.a. 99.0 % to 102.0 %
Urea - CH_4N_2O	Merck-Schuchardt	p.a. >99 %

α-amylase (from porcine pancreas) – N. A.	Sigma-Aldrich	N. A.
Mucin (from porcine stomach) – N. A.	Sigma-Aldrich	N. A.
Pepsin (from porcine gastric mucosa) – N. A.	Sigma-Aldrich	N. A.
Lipase (from porcine pancreas) – N. A.	Sigma-Aldrich	N. A.
Bile salts (Bile extract from porcine) – N. A.	Sigma-Aldrich	N. A.
DPPH – $C_{18}H_{12}N_5O_6$	Fluka	pract. ≥85 %
Trolox – $C_{14}H_{18}O_4$	Fluka	purum. ≥98 %


Information Lewatit VP OC 1064 MD PH

Gonçalo Nuno Gouveia Martins
seg 17-07, 18:27
infosds@lanxess.com; goncanuno@hotmail.com

Responder a todos

Hello there,

My name is Gonçalo Martins and I'm a Master student in Apllied Biochemistry in the University of Madeira, Portugal. I'm currently using your company's resin **Lewatit VP OC 1064 MD PH** in my master's thesis for drug delivery applications.

I'm sending you this email because I performed some FTIR-ATR analysis and wanted to compare my results with those in the literature but I don't seem to find any spectra, so I was wondering if you had any data you could provide me with. This would be greatly apreciated since it would verify my work.

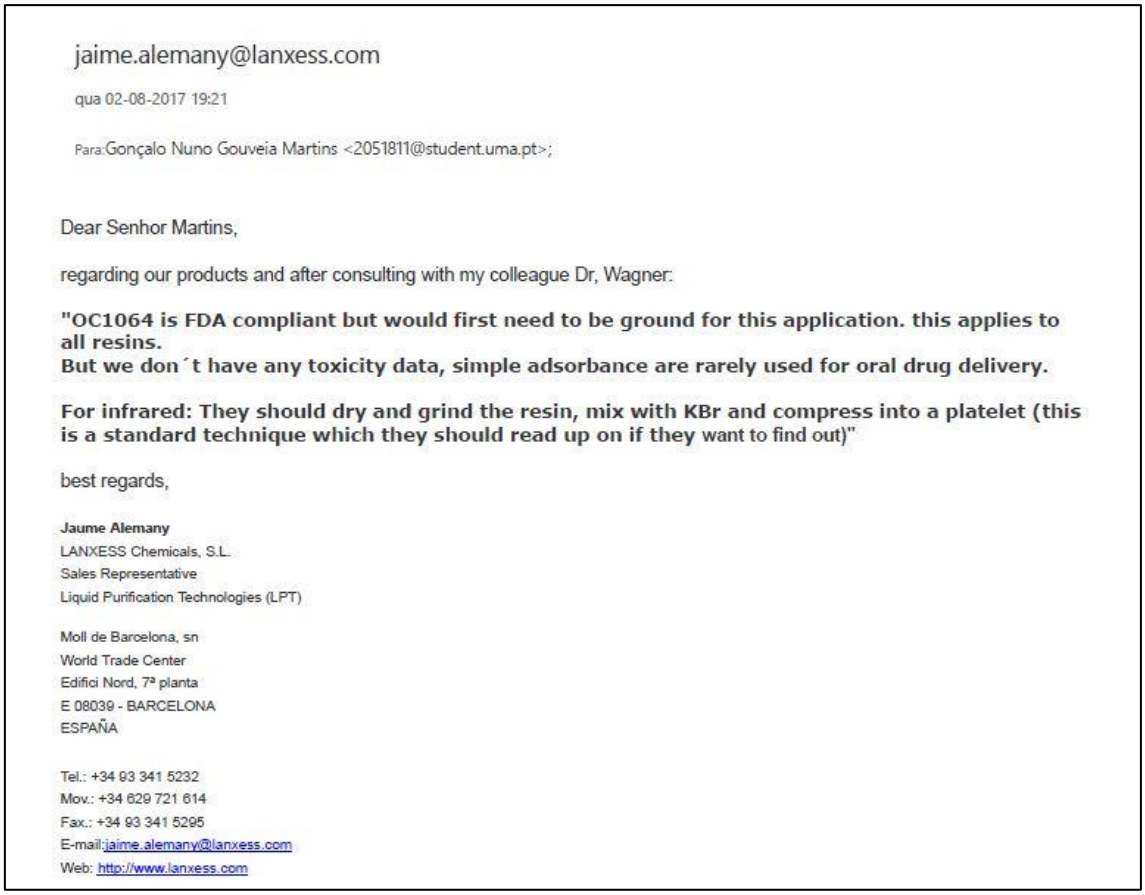
Also, I would like to ask any information regarding the use of this resin for the oral administration of drugs. Is this material FDA approved for this purpose? My research shows ion-exchange resins can be used as bile sequestrants and these materials aren't absorbed by the human body, being released in the feces. However, I don't have any information about the toxicity in the case of a neutral resin, particularly with a polystryrene matrix. Can you provide me with some information on this topic?

If it so happens that **Lewatit VP OC 1064 MD PH** is not approved for oral drug delivery, could you recommend me a resin with similar chemical properties or other material that I could use for this purpose?

Thank you for you time and I hope to hear from you soon!

Best regards,
Gonçalo Martins
University of Madeira

Figure S1 - Email sent to the customer support at LANXESS on the 17th of July of 2017.



jaime.alemany@lanxess.com

qua 02-08-2017 19:21

Para:Gonçalo Nuno Gouveia Martins <2051811@student.uma.pt>;

Dear Senhor Martins,

regarding our products and after consulting with my colleague Dr, Wagner:

"OC1064 is FDA compliant but would first need to be ground for this application. this applies to all resins.
But we don´t have any toxicity data, simple adsorbance are rarely used for oral drug delivery.

For infrared: They should dry and grind the resin, mix with KBr and compress into a platelet (this is a standard technique which they should read up on if they want to find out)"

best regards,

Jaume Alemany
LANXESS Chemicals, S.L.
Sales Representative
Liquid Purification Technologies (LPT)

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Figure S2 - Screenshot of the email contact with Jaime Alemany, LANXESS, in the 2nd of August of 2017.

Table S4 - Calibration curves obtained for each hydroxycinnamic acid by HPLC-DAD at 320 nm, using the chromatographic run designed for the detection of the five compounds.

Acid	Concentration range (mg/L)	Equation	R^2
Chlorogenic	1-100	y = 0.5004x + 0.2791	0.9994
Caffeic	1-100	y = 0.7212x + 0.3732	0.9995
p-coumaric	1-100	y = 0.8851x + 0.6752	0.9991
Sinapic	1-100	y = 0.7001x + 0.1599	0.9992
Ferulic	1-100	y = 0.8125x – 0.8351	0.9987

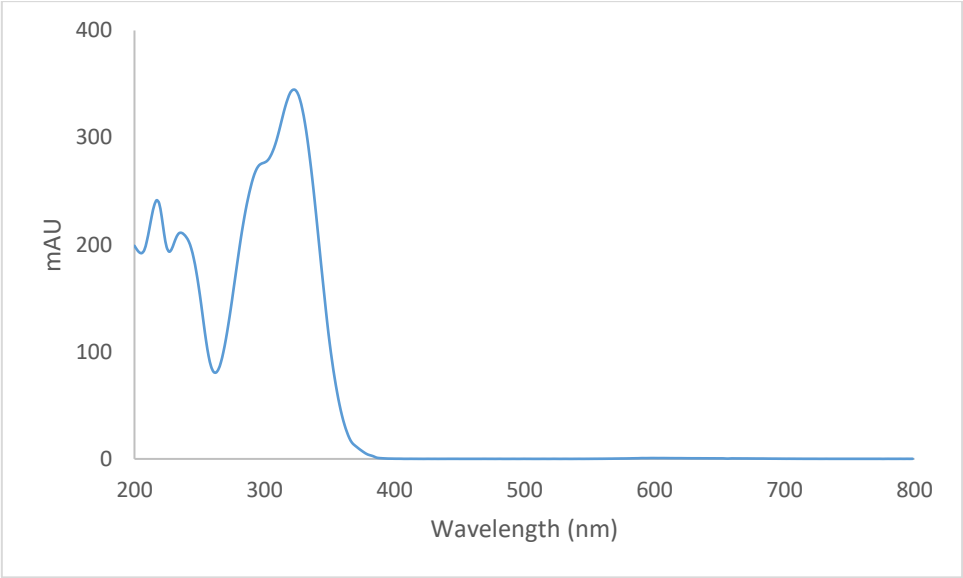

Figure S3 - UV spectrum of ferulic acid obtained by HPLC-DAD.

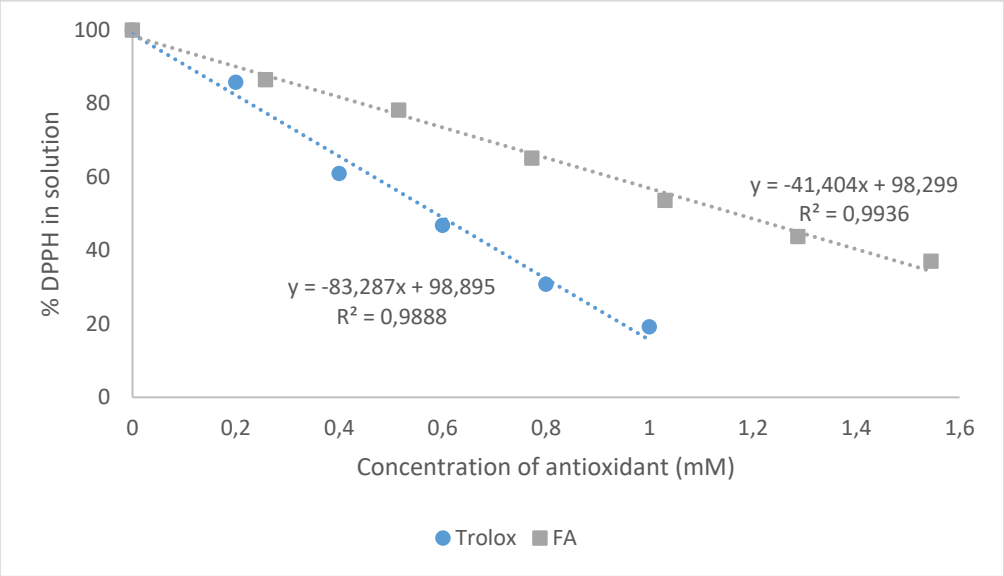

Figure S4 - Percentage of DPPH in solution versus the concentration of Trolox and ferulic acid.

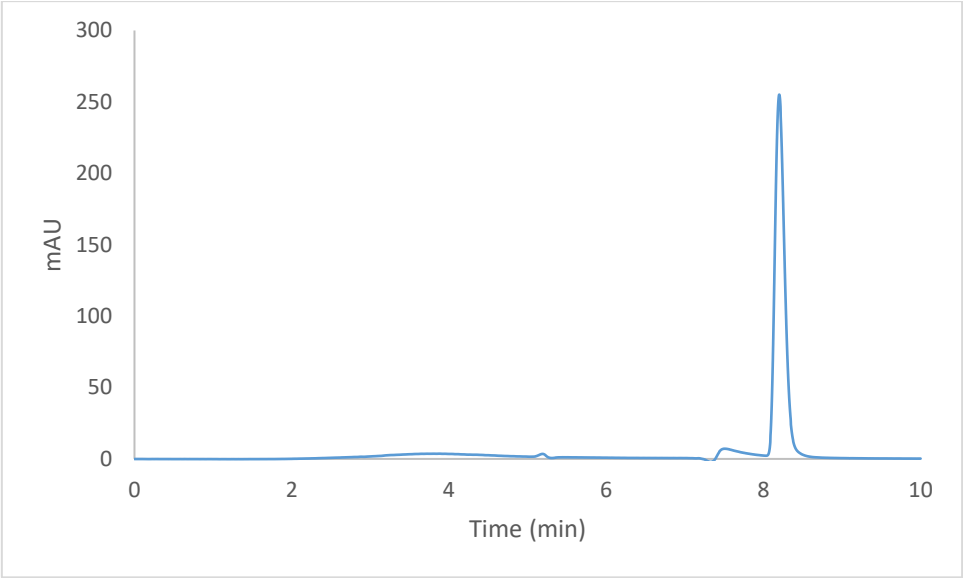

Figure S5 – Chromatogram of ferulic acid obtained at 320 nm by HPLC-DAD after a simulated digestion with adjuncts in the mouth.

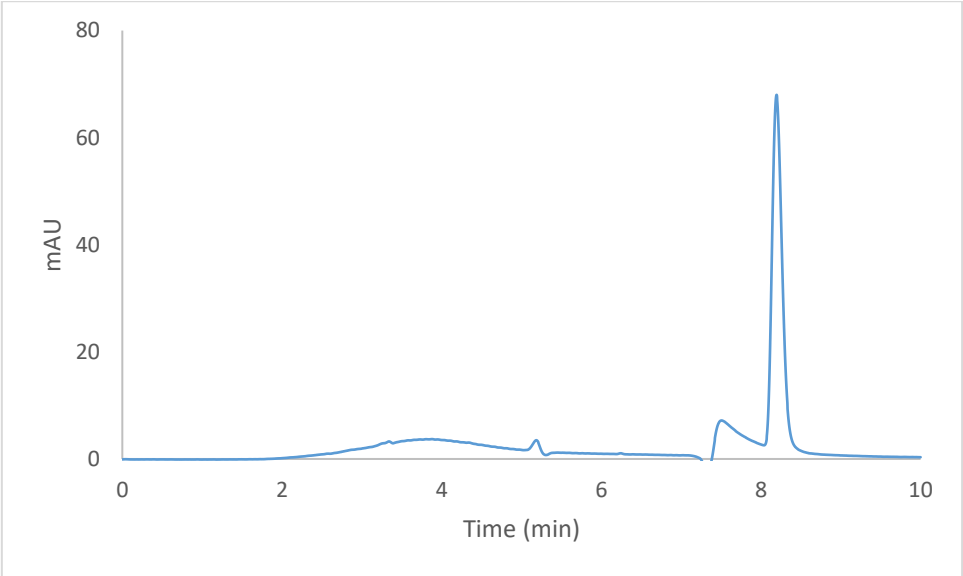

Figure S6 – Chromatogram of ferulic acid obtained at 320 nm by HPLC-DAD after a simulated digestion with adjuncts in the stomach.

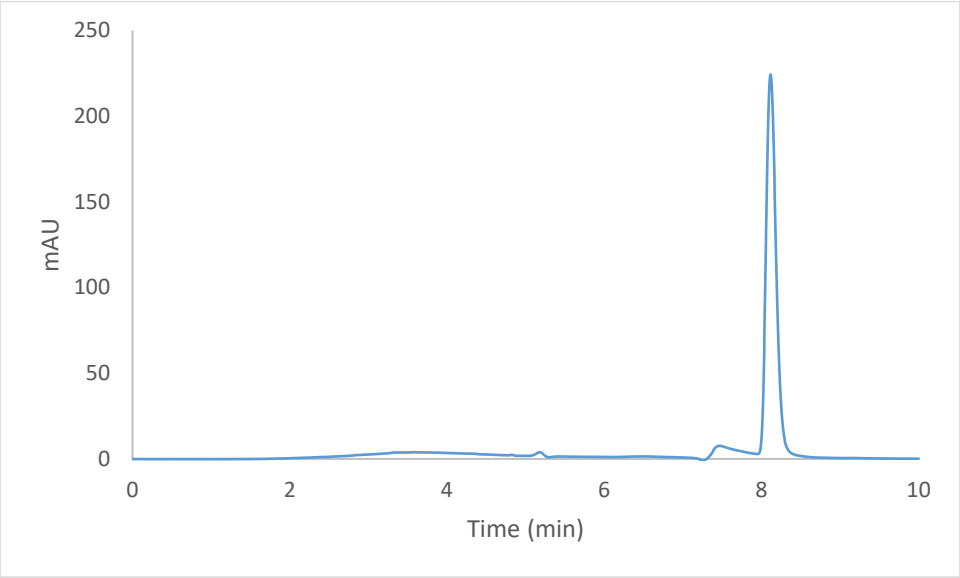

Figure S7 – Chromatogram of ferulic acid obtained at 320 nm by HPLC-DAD after a full digestion with adjuncts, using the gelatine capsule.

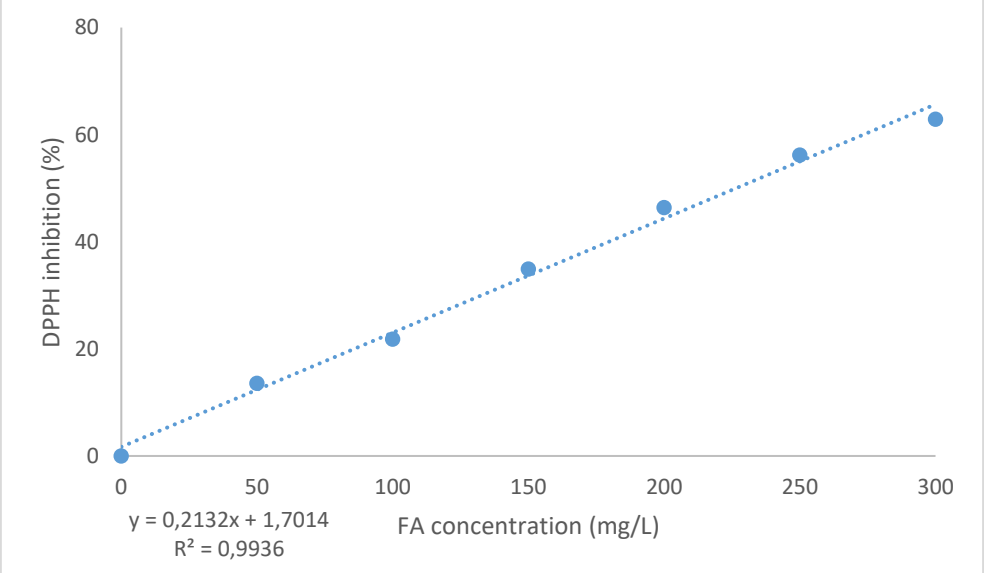

Figure S8 - Calibration curve of the DPPH inhibition vs FA concentration (mg/L).

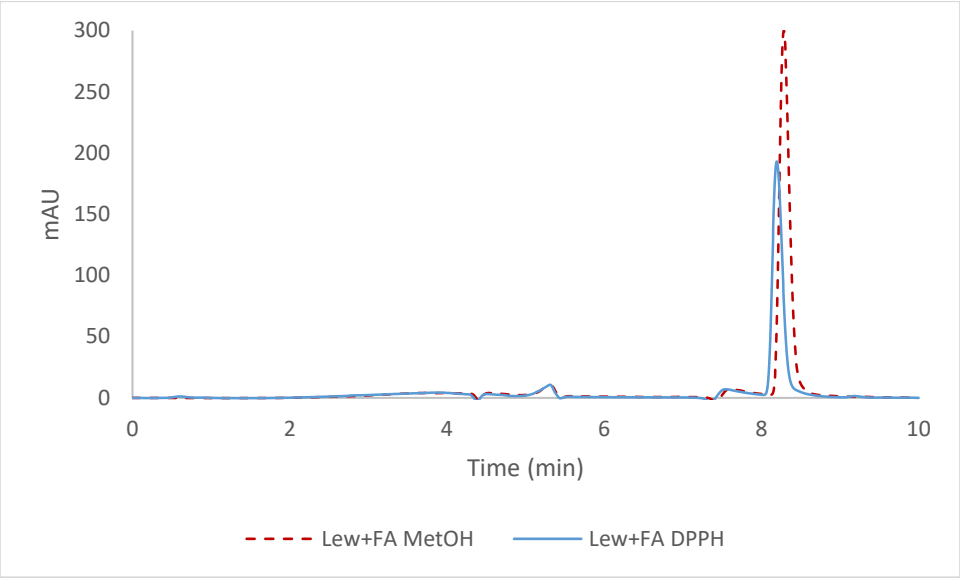

Figure S9 - Chromatograms of ferulic acid after desorption in methanol and after the DPPH assay.