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TRANSPORT AND METABOLISM OF FERULIC ACID THROUGH THE COLONIC EPITHELIUM

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Running title: Ferulic acid transport and metabolism

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Abbreviations: DTT, dithiothreitol; LDL, low-density lipoprotein; LC-MS, liquid chromatography-mass spectrometry; MCT, monocarboxylic acid transporter; MRP, multidrug resistance protein; m/z, mass to charge ratio; TEER, transepithelial electrical resistance; TIC, total ion count.

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ABSTRACT

Ferulic acid is an important antioxidant found in food, beverages, supplements and herbal medicines. However, its mechanism of absorption in the colon has never been examined, even though this is its main site of *in vivo* absorption. Ferulic acid was efficiently transported as the free form through an *in vitro* model for the colonic epithelium consisting of co-cultured Caco-2 and mucus-producing HT29-MTX cells, with only a small amount transported as feruloyl-glucuronide or sulfate, together with some free dihydroferulic acid. This pattern of metabolism and permeation was also seen using rat everted ascending and descending colon sacs. In the cell model, free ferulic acid permeated by passive diffusion, as judged by the linearity of the uptake over time and non-saturable concentration dependence. The permeation was independent of tight junctions but strongly linked to the hydrophobicity of the different phenolic acids tested, suggesting a transcellular rather than a paracellular transport. Using inhibitors, we showed that only a small proportion (< 20 %) of the free ferulic acid transport was carrier-mediated. The production of metabolites in the basal chamber was lowered by MK571 and increased by cyclosporine A, implying an involvement of MRP and P-gp transporters in the efflux of metabolites respectively to the serosal and luminal sides. These results show that the form of ferulic acid available to the blood after passage across the colonic barrier would be mainly the free form, together with only a small percentage of conjugated and reduced ferulic acid.

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Phenolic acids represent a subgroup of secondary metabolites found in plants, referred to as "phenolics" and involved in the defense of the plant against ultraviolet radiation and pathogens. There are two classes of phenolic acids, the hydroxybenzoic and the hydroxycinnamic acids, which respectively derive from benzoic and cinnamic acids (Clifford, 2000). While the content of hydroxybenzoic acids in edible plants is low, hydroxycinnamic acids are more abundant. The most commonly found hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic and sinapic acids (Shahidi and Wanasundara, 1992). They are rarely present as free forms, except in processed food, but occur more frequently as simple esters with quinic acid or glucose, with polysaccharides but also with other carboxylic acids such as tartaric or shikimic acids (Herrmann, 1989). The foods containing phenolic acids are fruits, vegetables, beverages and cereals. Cereals and coffee are abundant sources of ferulic acid where it is respectively found esterified to arabinose residues in primary cell wall arabinoxylans and as feruloylquinic acid (Clifford, 2000). However, phenolic acids are not only found in food but also in traditional herbal Chinese medicines and supplements (Cai, et al., 2006).

Ferulic acid has been shown to potentially exert several beneficial effects on health. For example, it significantly protected against UVB-induced erythema in humans (Saija, et al., 2000), acted as a peroxyl radical scavenger and increased the resistance of LDL to oxidation (Castelluccio, et al., 1995) and protected against some chronic diseases such as diabetes (Balasubashini, et al., 2004), Alzheimer's (Yan, et al., 2001) and colon and breast cancers (Hudson, et al., 2000).

However, the *in vivo* activity of ferulic acid strongly depends on its absorption, further metabolism and tissue distribution. Ferulic acid absorption has been studied *in*

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vivo after ingestion by animals or humans of the pure compound or contained in food (Rondini, et al., 2004), *in situ* perfusion of small intestinal segments (Silberberg, et al., 2006) and perfusion of rat stomach (Konishi, et al., 2006). Cultures of Caco-2 cells, as an *in vitro* model for the small intestinal epithelium, have also been used for studying bioavailability of ferulic acid (Konishi and Shimizu, 2003). However, the major and most potentially important site of ferulic acid absorption is the colon since ferulic acid is released from parent compounds or from the food matrix by microbial cinnamoyl esterases (Couteau, et al., 2001). Approximately 95 % of the total release of feruloyl groups takes place during fermentation of wheat bran in the human colon after release of feruloylated oligosaccharides by xylanases (Kroon, et al., 1997). These cinnamoyl esterases are located mainly in the lumen of the large intestine (of microbial origin), with only small amounts in the small intestine mucosa, and possibly in pancreatic secretions (Andreasen, et al., 2001). In addition, ferulic acid is a metabolite of caffeic acid and is secreted back into the lumen by intestinal cells after methylation of caffeic acid by the catechol-*O*-methyl transferase as shown by caffeic acid perfusion of rat small intestine (Lafay, et al., 2006). As caffeic acid can also be released by microbial esterase activity on 5-*O*-caffeoylquinic acid (chlorogenic acid) by cleavage of the quinic acid moiety (Plumb, et al., 1999), ferulic acid could also be available in the colon after ingestion of any source of chlorogenic acid. Moreover, ferulic acid found in food esterified with carboxylic acids such as quinic acid (Herrmann, 1989) could also be released and absorbed in the colon, providing these esters reached this segment intact. It cannot be excluded that they could be unstable during their passage through the upper part of the gastro-intestinal tract or could be absorbed intact, or as the free forms in the stomach and/or the small

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intestine after lysis of the ester bond by esterases present in the small intestine or the stomach.

However, the mechanisms of phenolic acid intake and their mammalian metabolism in the colon, where the mucus layer is the thickest, have not yet been investigated. The purpose of this study was to better understand the mechanisms of ferulic acid absorption by the colonic epithelium, in the presence of mucus, and to determine the metabolites released by intestinal cells. The *in vitro* model used was a co-culture of enterocyte (Caco-2) and goblet-like (HT29-MTX) cells and was compared with rat everted ascending and descending colonic sacs; the sample analysis was performed by LC-MS, which allowed direct identification and quantification of both the free form and metabolites.

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Materials and methods

Materials- Dulbelcco's Modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin-streptomycin, amphotericin B, L-glutamine, Hanks' Balanced Salt solution (HBSS), Krebs-Ringer bicarbonate buffer, sodium bicarbonate, calcium chloride, periodic acid, paraformaldehyde, phosphate buffered saline 1 X (PBS), xylol, Schiff's reagent, Alcian Blue, DL-dithiothreitol, cyclosporine A, α-cyano-4-hydroxycinnamic acid (CHC), ibuprofen and fluorescein isothiocyanate-dextran 4000 (FD4) were purchased from Sigma (Buchs, Switzerland). Minimum essential medium (MEM) was purchased from Gibco (Basel, Switzerland). Plastic dishes and Transwell® Polycarbonate semi-permeable membranes of 0.4 µm pore size and 4.7 cm^2 surface area were obtained from Corning (Wohlen, Switzerland). Lab-Tek® glass Chamber Slides™ were obtained from Nalge Nunc (Hereford, UK). Ethyl acetate, sodium acetate anhydrous, HPLC grade water, acetonitrile and glacial acetic acid were purchased from Merck (Dietikon, Switzerland). Aqueous mounting medium was obtained from DakoCytomation AG (Baar, Switzerland). Ferulic, caffeic and dihydrocaffeic acids were obtained from Extrasynthese (Genay, France). Dihydroferulic acid was provided by Alfa Aesar (Karlsruhe, Germany). Feruloyl-4-*O*-β-D-glucuronide, as a HPLC standard, was from NRC collection of standard compounds. MK571 was purchased from Biomol Int. (Zurich, Switzerland).

Cell culture- The human colon carcinoma cell line Caco-2 cells (HTB-37) was obtained from the American Type Culture Collection (ATCC, LGC Promochem, Molsheim, France) at passage 21. The HT29-MTX cells were elaborated and kindly provided by Dr T. Lesuffleur (Villejuif, France) at passage 7. Caco-2 and HT29-MTX cells were used between p 30 and 70. The cells were maintained in 75 cm^2 culture

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flasks at 37 °C under a humidified 5 % CO_2 / 95 % O_2 atmosphere in DMEM supplemented with 584 mg/L of L-glutamine, 1 % (v/v) MEM, 100 U/ml of penicillin-streptomycin, 0.25 µg/ml amphotericin B and 15 or 10 % heat inactivated FCS, for Caco-2 or HT29-MTX respectively.

Mucus detection- 6 x 10^4 cells (76 % Caco-2 and 24 % HT29-MTX) were seeded per cm^2 in 4-wells Lab-Tek® glass Chamber Slide™ and grown for 21 days in 10 % FCS supplemented DMEM, medium being changed every other day. For mucus staining, slides were washed with HBSS and fixed overnight with 4 % paraformaldehyde in PBS 1 X at 4 °C. Cells were re-hydrated by successively bathing in water containing decreasing amounts of EtOH (from 100 to 50 %), rinsed in PBS 1 X and finally in distilled water. Slides were then immersed 2 min in 3 % acetic acid, incubated 10 min in a solution of 1 % Alcian Blue prepared in 3 % acetic acid, washed twice in water, treated with 1 % aqueous periodic acid for 5 min, rinsed in distilled water, treated with Schiff's reagent for 5 min, washed in running tap water, dehydrated by ascending amounts of EtOH (from 50 to 100 %), cleared by incubation in xylol and finally mounted with aqueous mounting medium.

Preparation of transport and metabolism solutions- Ferulic acid in powder form was weighed and dissolved in HBSS for 10 min under stirring at room temperature. The solutions were then adjusted to pH 6 or 7.4 and centrifuged for 5 min at 5 000g. The amount of ferulic acid present in solution was determined by measuring the optical density at 310 nm using a UV/Vis Spectrophotometer (Uvikon, Kontron, Basel, Switzerland) after dilution if necessary. Ferulic acid stock solutions were prepared in EtOH or DMSO depending on the intended concentration.

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In vitro transepithelial transport studies- Caco-2 and HT29-MTX were seeded together in Transwells at 6 x 10^4 cells/cm^2 (76 % Caco-2 and 24 % HT29-MTX). Co-cultures were allowed to grow and differentiate over a period of 21 days, medium (DMEM containing 10 % FCS) being changed every other day. The integrity of the monolayers was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell®-ERS device (Millipore, Zug, Switzerland) before and after the treatments. To evaluate transepithelial permeability, medium was removed from the apical and basal sides of the cultures and replaced by 2 ml of the transport solution consisting of HBSS containing 1.8 mM calcium (calcium concentration required for maintaining integrity of the tight junctions) and phenolic acids and/or inhibitors, and pH was adjusted to 6 or 7.4. After an incubation of a desired period of time at 37 °C, apical and basal solutions were collected, acetic acid added to obtain a final concentration of 10 mM and samples stored at -20 °C until further analysis. To investigate the effect of mucus, permeation was evaluated after removal of the mucus layer by a pre-incubation of 10 min at 37 °C with 1 mM DL-dithiothreitol (DTT) solubilized into HBSS followed by two successive washes in HBSS. To study the effect of sodium azide on transport, cells were pre-incubated with 10 mM NaN_3 for 30 min and co-incubated with 10 mM NaN_3 and 30 µM ferulic acid for 1 h.

Metabolism experiments- Cells were seeded at 6 x 10^4 cells/cm^2 in 3.8 cm^2 wells and grown over a period of 6 days in 10 % FCS supplemented DMEM, the medium being changed every second day. Metabolism of ferulic acid was studied by incubating each well with 100 µM ferulic acid. After 3 h at 37 °C, supernatant was collected, acidified with acetic acid to obtain a final concentration of 10 mM and stored at -20 °C until analysis.

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Transport study using everted sacs- Six week old male Sprague Dawley rats were adapted to the laboratory conditions (normal 12 h dark / 12 h light cycles) with free access to tap water and to a conventional diet for one week and then to a polyphenol-free semi-synthetic diet of AIN 93M type for another week. Rats were randomly sacrificed by decapitation. The ascending and descending parts of the colon were quickly excised, everted and rinsed in room temperature Krebs-Ringer bicarbonate buffer, prepared according to the manufacturer's recommendations and gassed with 95 % O_2 / 5 % CO_2 for 60 min prior to use. The segments were then mounted at one extremity on a 1 ml plastic syringe which contained 0.7 ml of Krebs-Ringer bicarbonate buffer, pre-warmed to 37 °C and supplemented with 1 mg / ml FD4, while the other extremity was sealed with chirurgical linen. The content of the syringes was emptied into the sacs, which were then incubated in chambers filled with 30 ml of Krebs-Ringer bicarbonate buffer, containing ferulic acid at a concentration of 500 µM and pre-warmed to 37°C. The everted sacs were incubated for 30 min in the chambers maintained at 37 °C and continuously gassed with 95 % O_2 / 5 % CO_2. At the end of the incubation, the content of the sacs was recovered by filling back the syringe, further acidified with acetic acid to a final concentration of 20 mM and stored at -20 °C until analysis. The content of the chambers was recovered in order to control the sacs integrity by measuring the FD4 concentration by fluorescence spectrophotometry (excitation at 490 nm and emission at 520 nm).

Sample preparation and LC-MS analysis- Room temperature defrosted samples (150 µl) were mixed with an equal volume of 0.1 mM sodium acetate buffer pH 5. HPLC grade water (150 µl) and 300 µl 200 mM HCl/methanol were added. After 30 sec of sonication, the mixture was vortexed and extracted 3 times with ethyl acetate. After each centrifugation at 5000 g for 5 min, organic phases were collected, pooled and

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dried under nitrogen. The dried extracts were re-suspended into 5 % acetonitrile in water containing 0.1 % formic acid and injected onto an Acquity UPLC™ BEH Shield RP_{18} column (2.1 x 100 mm, 1.7 µm; Waters, Rupperswil, Switzerland). Elution was performed with a flow rate of 0.3 ml/min and a gradient of solvent A (water) and B (acetonitrile), both acidified with 0.1 % formic acid. HPLC analysis was started with 3 % of solvent B. This condition was maintained for 1 min and then the percentage of solvent B was linearly increased to 5 within 1 min, to 10 within 18 min, to 15 within 10 min and finally to 100 within 1 min. 100 % solvent B was maintained for 3 min, then initial conditions were reached within 1 min and the column equilibrated in 3 % solvent B for 5 min.

The HPLC system (Acquity UPLC system, Waters, Rupperswil, Switzerland) was connected to a triple Quadrupole Micromass Quattro micro API mass spectrometer (Waters, Rupperswil, Switzerland), with an electrospray ionization (ESI) interface. The LC eluate was introduced directly from the absorbance monitor (Photo Diode Array, PDA) into the ESI probe without flow splitting. A nebulizing gas flow of 150 L/h and a drying gas flow of 550 L/h were applied for ionization using nitrogen in both cases. Samples were analyzed using the negative ion mode. ESI-MS parameters were as follows: voltage, 3 kV; capillary temperature, 90 °C.

The results obtained were normalized using an internal standard, syringic acid, added at a known concentration to the samples to be extracted.

Data analysis- Data are shown as mean ± SD. Differences between test and control conditions were assessed by Student's *t*-test and differences with value of $P < 0.05$ were considered as significant.

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Results

Mucus production by Caco-2/HT29-MTX co-cultures- Since most ferulic acid available from the diet is likely to be released in the colon after microbial metabolism of bound ferulic acid, an *in vitro* model consisting of co-cultures of enterocytes and goblet-like cells was set up in order to study ferulic acid transport and metabolism in the presence of mucus, which is abundant in the colon. The co-cultures, prepared with 76 % Caco-2 and 24 % HT29-MTX seeded on inserts and grown for 21 days, exhibited TEER values of 950 ± 70 Ohm.cm^2 (n=10) and were almost entirely covered by mucus (Fig. 1).

Dissolution of ferulic acid in transport solutions- The procedure to prepare transport solutions and the concentrations of ferulic acid to use were investigated, since several papers have reported the use of millimolar concentrations to study permeation of different compounds. Transport solutions were firstly prepared by dissolving ferulic acid in powder form directly into HBSS. In some experiments, when the pH was adjusted to 6, the concentration of ferulic acid measured corresponded to the intended concentration, up to 50 µM. Over 50 µM, the measured concentration corresponded only to a certain percentage of the intended concentration (from 100 % for 50 µM to 76 % for 3 mM). The measured concentrations decreased to 70 % of the intended concentrations in the range of 3 to 10 mM. In other experiments, when the pH was adjusted to 7.4, the measured concentration was between 100 and 80 % of the intended concentration in the range of 50 µM to 10 mM. As a consequence, ferulic acid was pre-dissolved as stock solutions in ethanol (up to 100 mM) which were further dissolved into HBSS to obtain concentrations from 10 to 100 µM. However, stock solutions above 100 mM were prepared in DMSO and further dissolved into

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HBSS for concentrations from 100 µM to 2 mM. The maximum solvent concentration in the cultures during treatments never exceeded 0.1 %.

Time-dependent transport of ferulic acid- Quantification of ferulic acid was performed using LC-MS by detection of the corresponding negative ion (*m/z* = 193), without any pre-treatment of the samples with de-conjugating enzymes. The transport of ferulic acid by Caco-2/HT29-MTX co-cultures was rapid and linear up to 3 h at 30 µM (Fig. 2), and was linear up to at least 2 h for the other concentrations (data not shown). For further experiments investigating the mechanisms of ferulic acid transport, an incubation time of 60 min was chosen, as this time was well within the linear phase.

Concentration and direction-dependent permeation of ferulic acid- The relationship between the amount of ferulic acid transported and its initial concentration in the donor compartment was determined. Permeation from the apical to the basal side (A to B) was compared with the transport in the opposite direction (B to A). Fig. 3a shows that ferulic acid transport in the absence of a proton gradient (A and B at pH 7.4) was linear in both directions over the range of concentrations tested (from 2 µM to 2 mM), without any saturation for the highest concentration, and being a little faster from B to A. The transport of ferulic acid was also linear (Fig. 3b) in the presence of a proton gradient (A, pH 6 and B, pH 7.4), over the range of the concentrations tested, and almost twice as high compared with transport in the absence of a proton gradient, pH 6 being close to the immediate surface of the rat and human colon measured at around 6.4 (McNeil, et al., 1987). For all the conditions tested, the transport displayed

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on a normal or log-log scale was linear over the range of concentrations tested ($R^2 = 1$; data not shown).

Effect of pH and micro-environment created by the mucus layer on ferulic acid transport- Permeation of 30 µM ferulic acid through the co-cultures was higher in the presence ($3.8 \pm 0.2 \times 10^{-2}$ nmol/cm^2.min) than in the absence ($1.6 \pm 0.4 \times 10^{-2}$ nmol/cm^2.min) of a proton gradient. This permeation in the presence of a proton gradient was slightly but significantly higher after removal of the mucus layer by DTT treatment (123 ± 11 % of the ferulic acid transported in control condition; $P < 0.05$).

Role of tight junctions on ferulic acid permeation- Tightness of the intercellular junctions was monitored by measuring the transepithelial electrical resistance (TEER) of the cultures. When transport studies were performed at low calcium concentrations (18 µM), the TEER measured was 495 ± 55 Ohm.cm^2 (n=3) lower than when studies were preformed at 1.8 mM, as extracellular calcium plays a role in the maintenance of tight junction integrity (Ma, et al., 2000). However, TEER was 520 ± 100 Ohm.cm^2 (n=3) higher when the HT29-MTX cell population was decreased from 24 to 10 % in the co-culture, as shown previously (Hilgendorf, et al., 2000). None of these treatments affecting TEER had a significant effect on ferulic acid permeation. Moreover, cultures of Caco-2 cells only transported ferulic acid similarly to mixed cultures, even with a TEER value 1.8 times higher than that of mixed cultures (data not shown).

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Inhibition of ferulic acid transport- Sodium azide, a metabolic inhibitor, was used to test for the involvement of an active transport mechanism in ferulic acid permeation. 10 mM sodium azide reduced the permeation of ferulic acid to 80 ± 4 % ($P < 0.05$) of the control only in the presence of a proton gradient. In addition, isoferulic acid did not compete for transport with the closely related compound ferulic acid, even if used at a concentration ten times higher. Ferulic acid transport in the presence of isoferulic acid was 106 ± 8 and 94 ± 10 % of the transport of ferulic acid alone in the presence and absence of a proton gradient respectively ($P > 0.05$). Since possibly up to ~ 15-20 % of transport of ferulic acid is carrier-mediated , based on the results obtained with sodium azide and albeit only in the presence of a proton gradient, transport was studied in the presence of inhibitors (150 μM): α-cyano-4-hydroxycinnamic acid (CHC), an inhibitor of the monocarboxylic acid transporter 1 (MCT1) which binds to MCT1 without being translocated, or ibuprofen, a strong ligand of the sodium-dependent monocarboxylic acid transporters (S-MCTs). Both compounds showed a small inhibitory effect on free ferulic acid permeation, which was 90.1 ± 3.1 and 89.5 ± 2.6 % of the control in the presence of ibuprofen and α-cyano-4-hydroxycinamic acid respectively ($P < 0.05$).

Relationship between HPLC retention time of phenolic acids and their permeation rate- Since about 80 % of ferulic acid transport is by a passive mechanism, we explored this further. Paracellular transport was ruled out (see above), so permeation of ferulic acid was compared with transport of other phenolic acids with a similar chemical structure (Fig. 4) and used at the same concentration (30 μM). The results, summarized in Table 1, show that permeation of phenolic acids was dependent on pH, and strongly proportional to their retention time on a reverse phase HPLC column. As

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seen for ferulic acid, the permeation rate of the other phenolic acids tested was higher in the presence of a proton gradient. Table 1 also shows that there is no direct relationship between the permeation rate of the phenolic acids and their calculated radius. These data support the theory that the transport mechanism is transcellular passive diffusion rather than paracellular diffusion.

Capacity of Caco-2/HT29-MTX co-cultures to metabolize ferulic acid- All the experiments cited above measured the transport of the free form of ferulic acid by the cultures under different conditions, samples being analyzed after liquid-liquid extraction, without any enzymatic de-conjugation. However, it was of importance to determine to what extent the co-culture model used was also competent for conjugation and metabolism. In order to focus the experiment on metabolism rather than on transport, Caco-2 and HT29-MTX cells were seeded on dishes and incubated with ferulic acid for 3 h. Supernatants of cultures analyzed by LC-MS in the negative ion mode contained three ferulic acid metabolites: 2 conjugates, feruloyl-sulfate (m/z = 273) and feruloyl-glucuronide (m/z = 369), and a reduced form of ferulic acid, dihydroferulic acid (m/z = 195; Fig. 4). Identity of the compounds was confirmed by fragmentation of the parent ions, which gave the expected daughter ions, with mass to charge ratio 193 for the glucuronide and sulfate and 136 for dihydroferulic acid (Fig. 5). The conjugation reactions of ferulic acid are hypothesized to be on the free hydroxyl group, on the 4 position of the phenyl ring. The presence of dihydroferulic acid and feruloyl-4-*O*-β-D-glucuronide was also confirmed by comparing their retention time and fragmentation pattern with the characteristics of the standard compounds. There was a difference in the capacity of Caco-2 and HT29-MTX cells to metabolize ferulic acid when grown separately, especially for glucuronidation which

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was not detected in the Caco-2 supernatant. Since the amount of metabolites was much lower than the free form of ferulic acid, it was not possible to detect any increase in the amount of ferulic acid free form after enzymatic de-conjugation. Therefore only feruloyl-4-*O*-β-D-glucuronide and dihydroferulic acid, for which standards were available, were quantified, while the relative amount of the sulfate was evaluated by measuring the area under the peak. Protons (pH 6) favored the production of dihydroferulic acid compared with feruloyl-4-*O*-β-D-glucuronide which was more abundant at pH 7.4 (Table 2). Moreover, the area under the peak obtained for feruloyl-sulfate was also slightly lower at pH 6 for both types of cells, reflecting a lower production of metabolites in the presence of a higher concentration of protons (data not shown). While HT29-MTX seemed to be more competent than Caco-2 cells to conjugate ferulic acid with sulfate and glucuronide groups, there was no major difference in the production of dihydroferulic acid between the two types of cell lines.

Effect of different culture conditions on metabolite production- Although most of the ferulic acid transported was as the parent compound, a small proportion was metabolized into dihydroferulic acid, found in equivalent amounts on apical and basal sides of the cultures, and conjugated to feruloyl-4-*O*-β-D-glucuronide, released more abundantly on the basal side (Table 3). Feruloyl-sulfate was also detected on both sides but more abundantly on the basal one. As the results suggested the involvement of MCT1 and S-MCT in the transport of up to 20 % of free ferulic acid, we tested if inhibitors of MCT1 and S-MCT also had an effect on production of ferulic acid metabolites. Inhibitors of potential efflux by P-gp/MDR1 (cyclosporine A) and by MRP1/2 (MK571) were tested. Results show that ibuprofen and MK571 significantly reduced the release of feruloyl-4-*O*-β-D-glucuronide while only MK571 inhibited the

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release of dihydroferulic acid (Table 4). However, cyclosporine A significantly stimulated the basal production of both metabolites.

Ex vivo ferulic acid uptake and metabolism by everted sacs of rat colon- In order to compare the *in vitro* model of Caco-2/HT29-MTX co-cultures with colonic tissue, ferulic acid uptake and metabolism were studied using an *ex vivo* model of colonic epithelium consisting of everted sacs of ascending and descending colon. All the ferulic acid metabolites identified using the *in vitro* model were detected in the content of the everted sacs. Their identity was confirmed by multiple reactions monitoring (MRM), fragmenting the parent compounds and looking for pairs of daughter and parent ions (Fig. 6). Interestingly, a second peak was observed with mass spectral and fragmentation behavior identical to that obtained for the *trans*-feruloyl-4-*O*-β-D-glucuronide standard. An equivalent compound was detected in culture supernatants but in very low quantities. This second peak might be the *cis* isomer of feruloyl-4-*O*-β-D-glucuronide. The free form and putative *trans*-feruloyl-4-*O*-β-D-glucuronide (RT = 11.12), for which standards were available, were quantified, but dihydroferulic acid was below the limit of quantification. Results are summarized in Table 5. The amount of ferulic acid glucuronide as a percentage of total amounts of free form and feruloyl-glucuronide appearing in the rat colon sacs was 16.9 ± 2.8 for the ascending and 20.6 ± 2.1 for the descending part of the colon.

DISCUSSION

The aim of the present study was to better understand mechanisms of ferulic acid absorption and metabolism by the colonic epithelium since this is its major site of

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absorption after consumption of foods containing conjugated ferulic acid, which is by far the major form in the diet. Use of co-cultured human enterocytes and goblet-like cells, as an *in vitro* model for colonic epithelium, showed that ferulic acid was mainly transported as the free form. This uptake was linear over time and not saturable at the highest concentrations we could obtain in physiological buffers (2 mM), both in the presence and absence of a proton gradient. These results show, as already suggested, that ferulic acid is mostly transported by passive diffusion (Silberberg, et al., 2006), which is mainly transcellular rather than paracellular, as ferulic acid permeation does not seem to be restricted by tight junctions nor dependent on their tightness. Phenolic acid permeation through the intestinal epithelium by transcellular diffusion could also occur in other tissues, such as the gastric mucosa, shown to be a site of absorption of ferulic, caffeic, 5-caffeoylquinic, *p*-coumaric and gallic acids (Konishi, et al., 2006). Transcellular diffusion is governed by the law of non-ionic diffusion suggesting that a dissociable substance permeates through a membrane in the non-ionized form, the ratio between the ionized and non-ionized forms being dependent on the pH of the solution and on the pKa of the compound. This would explain why ferulic acid, whose pKa is 4.44 ± 0.03 (Maegawa et al., 2007), is more transported at pH 6 than at 7.4. Moreover, permeation rate would also be expected to be proportional to the hydrophobicity of the phenolic acid, and this hypothesis was tested by comparing the permeation rates of caffeic, ferulic, dihydrocaffeic and dihydroferulic acids. As clearly indicated by their retention time on reversed phase chromatography, caffeic and dihydrocaffeic acids (with two hydroxyl groups on the phenyl ring) are respectively less hydrophobic and absorbed than ferulic and dihydroferulic acids (a methoxyl group replacing one of the hydroxyls); similarly, dihydrocaffeic and dihydroferulic acids (both with a saturated side chain) are also respectively less

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hydrophobic and absorbed than caffeic and ferulic acids (both with an unsaturated side chain). pKa values of these phenolic acids, being very similar (Maegawa et al., 2007) and their estimated radius (~ 2.6 Å), being lower than the size of the smallest pore (3 Å) formed by tight junctions in the intercellular space (Sawada, et al., 1989), cannot explain the permeation differences obtained.

The pH dependency of ferulic acid permeation due to ionization does not exclude an involvement of a facilitated mechanism of transport coupled to a proton pump. Indeed, phenolic acids have been earlier proposed to be transported by a monocarboxylic acid transporter (MCT) (Konishi and Shimizu, 2003;Konishi and Kobayashi, 2004), recently suggested to be an isoform different from MCT1 (Watanabe, et al., 2006). Protons and energy have also been shown to be necessary for the transport of some drugs possessing a carboxylic acid moiety such as ibuprofen. First suggested to be MCT related (Tamai, et al., 1995), ibuprofen permeation was later shown to be MCT1-independent (Legen and Kristl, 2003), and more recently clarified as being driven by a sodium monocarboxylate co-transporter (S-MCT) (Coady, et al., 2004). In the present report, we propose that only a small percentage of the free ferulic acid permeating would be transported via an energy-dependent system, which could be MCT1 and/or S-MCT.

It has long been proposed that adjacent to the mucosa of the gastrointestinal tract is a layer whose pH is maintained around neutrality (McNeil, et al., 1987). As the non-ionic diffusion rule suggests that the pH of a solution has a strong effect on the permeation of ionizable molecules, it was also hypothesized that the microclimate present at the surface of the epithelium could play a role in ferulic acid permeation.

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The mucus layer present on the intestinal epithelium has been demonstrated to play a key role in the maintenance of the pH microclimate, as DTT treatment significantly reduced the proton concentration close to the cell surface of excised rat proximal jejunum (Shimada, 1987). DTT treatment of the co-cultures slightly, but significantly, increased the permeability of ferulic acid in the presence of a proton gradient. This suggests that the mucus layer may create a micro-environment of neutral pH, even though the pH of the chamber is lower, thereby increasing the number of ionized molecules and lowering the effective concentration of molecules that can cross the membrane. However, because it has only a slight effect, the mucus layer is not a rate-limiting step in ferulic acid transport across the intestine.

In the supernatant from co-cultures, three different ferulic acid metabolites were identified: dihydroferulic acid, feruloyl-sulfate and *trans*-feruloyl-4-*O*-β-D-glucuronide, the latter being only released by HT29-MTX cells. Caco-2 cells have already been used for studying ferulic acid transport and metabolism. Grown as tri-dimensional cultures, they were unable to conjugate ferulic acid (Konishi and Shimizu, 2003) whereas grown as monolayers, they exported ferulic acid as sulfate but not glucuronide conjugates (Kern, et al., 2003). The results obtained, showing free ferulic acid as the major form transported followed by its glucuronide, and also the presence of sulfate and dihydroferulic acid in lower amounts, were also seen with everted sacs of rat colon. Our results are consistent with earlier *in vivo* studies showing considerable excretion of feruloyl-glucuronide in human urine after intake of tomato (Bourne and Rice-Evans, 1998) and its appearance in significant amounts, but lower than un-conjugated form, on the serosal side of rat small intestinal segments perfused with ferulic acid (Spencer, et al., 1999). However, two other studies reported no free

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form detected in unhydrolyzed plasma after ferulic acid *in situ* perfusion of rat small intestine and after ferulic acid intake from a semi-purified diet (Adam, et al., 2002) and free ferulic acid and glucuronide detected in plasma but with sulfate and sulfoglucuronide as the most abundant forms after ingestion of a diet enriched with ferulic acid (Rondini, et al., 2002). In the former study, the blood was collected at the end of the perfusion period, allowing absorbed free ferulic acid to reach the liver for further metabolism. In the work reported by Rondini *et al.*, ferulic acid was present as the free form in the diets and therefore part of it could have been absorbed directly from the stomach and later conjugated in the liver. Feruloyl-sulfoglucuronide, proposed earlier as one of the metabolites (Rondini, et al., 2004), was neither detected *in vitro* nor with everted sacs but could *in vivo* result from the hepatic metabolism of the feruloyl-glucuronide secreted by the intestinal epithelium. Dihydroferulic acid has been detected in urine following coffee consumption (Rechner, et al., 2001) and proposed to derive from ferulic acid, itself a metabolite of caffeic acid. Firstly suggested to be restricted to microflora only (Scheline, 1968), the capacity to convert ferulic acid into dihydroferulic acid was also detected in hepatocytes (Moridani, et al., 2002). However, this is the first time that intestinal cells are shown to display such activity.

In the tridimensional co-cultures, the concentration of conjugates was higher in the basal chamber whereas dihydroferulic acid was equally present on both sides of the epithelium. The results obtained in the presence of MK571 suggest that an MRP transporter could contribute to a major part of the efflux of feruloyl-glucuronide and dihydroferulic acid to the serosal side. The strongest candidate for this function is MRP3 (ABCC3), as it has been shown to be expressed on the basolateral side of Caco-2 cells (Prime-Chapman, et al., 2004). MRP6 (ABCC6), localized to the

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basolateral side of cells in kidney and liver (Kool, et al., 1999), should also be considered since Caco-2 cells express the gene corresponding to ABCC6 (Taipalensuu, et al., 2001). Cyclosporine A increased the release of glucuronide and dihydroferulic acid in the basal compartment, suggesting that these two metabolites could also be effluxed to some extent to the apical side through the P-gp protein, localized on the apical side of enterocytes. In addition to its active efflux, dihydroferulic acid could diffuse transcellularly, as shown for ferulic acid, until equilibrium is reached. Ibuprofen strongly inhibited the release of feruloyl-glucuronide either by competition with ferulic acid for the transport via S-MCT and/or by competition for glucuronidation by UDP-glucuronosyl-transferase. In both cases, it is of interest to mention that non-steroidal anti-inflammatory drugs could compete for bioavailability, meaning absorption and/or metabolism, with food-derived phenolic acids.

The data reported in this paper suggest that ferulic acid is absorbed by the colonic epithelium by a combination of mechanisms, passive transcellular diffusion and facilitated transport (MCT1 and S-MCT). Both these mechanisms give free ferulic acid on the serosal side with only a small amount of conjugated forms; efflux of the conjugates is also transporter(s) dependent. The form available to the body after passage across the intestinal barrier would be mainly the free form but also a small percentage of conjugates, feruloyl-glucuronide being the major metabolite, followed by the sulfate and dihydroferulic acid. Both free and conjugated forms could then be further metabolized by the liver. However, source, form and amount of ferulic acid ingested will influence the ratio of the different forms available in blood for further uptake by the tissues.

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FIGURE LEGENDS

Fig. 1: Distribution of mucus on Caco-2/HT29-MTX co-cultures.

Mucus was detected by using the periodic acid-Schiff-Alcian Blue procedure (mucus is stained in red).

Fig. 2: Time-dependent permeability of ferulic acid.

Caco-2/HT29-MTX co-cultures grown on inserts were incubated in HBSS (pH 7.4) and the apical side supplemented with ferulic acid (30 μM). Permeation of ferulic acid was evaluated by measuring the amount transported to the basal compartment after different times of incubation at 37 °C. Values are mean ± SD of 3 independent cultures.

Fig. 3: Concentration and direction dependence of ferulic acid permeation.

Permeation of ferulic acid (2 mM, 200, 20 and 2 μM) was measured after 60 min incubation at 37 °C; (a) the basal and apical chambers were at pH 7.4 and permeation from apical to basal (♦) and from basal to apical side (■) was determined; (b) the apical chamber was at pH 6 and the basal one at 7.4, recording permeation from apical to basal side. Values are mean ± SD of 3 different cultures.

Fig. 4: Chemical structure of the phenolic acids tested.

R1, R2 = H: dihydrocaffeic acid; R1 = H; R2 = CH_3: dihydroferulic acid; R3, R4 = H: caffeic acid; R3 = H; R4 = CH_3: ferulic acid; R3 = CH_3; R4 = H: isoferulic acid

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Fig. 5: Identification of the ferulic acid metabolites by MRM (multiple reactions monitoring). Supernatants of Caco-2 or HT29-MTX cultures, grown on dishes for 6 days, were collected after 3 h incubation with 100µM ferulic acid in HBSS, pH adjusted to 6. Supernatants were analyzed by MRM in the negative ion mode, for the following pairs of parent and daughter ion masses: 273 > 193, 195 > 136 and 369 > 193. TIC, total ion count.

Fig. 6: Confirmation by MRM of the identity of the metabolites produced in everted sacs and of the transport of ferulic acid. Contents of everted sacs of ascending and descending colon incubated with ferulic acid were analyzed by MRM in the negative ion mode, with static parent and daughter ion masses: 273 > 193, 195 > 136, 193 > 134 and 369 > 193. TIC, total ion count.

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Table 1. Permeation of phenolic acids compared with their HPLC retention time[a].

Phenolic acid	RT (min)	Permeation rate (nmol/cm^2.min)		Radius (Å)
		pH 6	**pH 7.4**	
dihydrocaffeic	7.5	$< 3 \times 10^{-4}$	$< 3 \times 10^{-4}$	2.64
caffeic	12.2	$4 \times 10^{-4} \pm 1 \times 10^{-5}$	$3 \times 10^{-4} \pm 1 \times 10^{-5}$	2.56
dihydroferulic	14.5	$3 \times 10^{-2} \pm 1 \times 10^{-3}$	$1 \times 10^{-2} \pm 8 \times 10^{-3}$	2.75
ferulic	22.6	$4 \times 10^{-2} \pm 2 \times 10^{-3}$	$2 \times 10^{-2} \pm 4 \times 10^{-3}$	2.67

[a] The cells were exposed to each phenolic acid (30 µM) for 1 h (n = 3).

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Table 2. Metabolism of ferulic acid by Caco-2 and HT29-MTX cells[a].

		Metabolite production (pmol/min.mg protein)	
	pH	**Caco-2**	**HT29-MTX**
feruloyl-glucuronide	**6**	ND[b]	61.3 ± 5.8
	7.4	ND[b]	109.4 ± 8.9
dihydroferulic acid	**6**	6.8 ± 0.8	7.1 ± 0.1
	7.4	3.6 ± 0.8	2.3 ± 0.4

[a] The cultures were incubated with ferulic acid (100 μM) for 3 h before analysis of the supernatant by LC-MS for quantification of metabolites.

[b] ND, not detected in the samples.

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Table 3. Release of ferulic acid metabolites from differentiated Caco-2/HT29-MTX co-cultures[a].

		Metabolite production ($pmol/cm^2.min$)	
	pH gradient	**Apical**	**Basal**
feruloyl-glucuronide	**6 / 7.4**	1.4 ± 0.1	24.9 ± 4.7
	7.4 / 7.4	1.6 ± 0.3	15.4 ± 1.1
dihydroferulic acid	**6 / 7.4**	3.0 ± 0.1	3.2 ± 0.4
	7.4 / 7.4	2.0 ± 0.3	1.7 ± 0.2

[a] The cultures were incubated with ferulic acid (1 mM) for 1 h before analysis of the samples by LC-MS for quantification of metabolites.

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Table 4. Inhibition of metabolite production on the basal side by different treatments.

	feruloyl- glucuronide[a]	**dihydroferulic acid**[a]
ibuprofen[b]	37.7 ± 6.3*	95.1 ± 17.3
CHC[b]	114.7 ± 20.7	111.3 ± 10.0
MK571[c]	40.9 ± 3.6*	64.6 ± 4.8*
cylcosporin A[c]	117.5 ± 5.3*	128.5 ± 6.3*

[a] Metabolites (in percentage of control), released on basal side after 1 h of incubation of the apical compartment with ferulic acid at 1mM
[b] 150 µM of inhibitor on the apical side
[c] 50 µM of inhibitor on the basal side
* Significantly different from the control value ($P < 0.05$, *t-test*)

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Table 5. Transport of ferulic acid and its glucuronide by ascending and descending rat colon everted sacs[a].

Segment of the colon		Concentration (µM)	Rate (pmol/cm^2.min)
Ascending	**free form**	23.3 ± 7.4	83.0 ± 31.7
	glucuronide	4.7 ± 1.4	15.4 ± 5.2
Descending	**free form**	23.8 ± 8.9	196.2 ± 89.3
	glucuronide	6.1 ± 1.9	50.7 ± 23.8

[a] Everted sacs of colon were incubated for 30 min with ferulic acid (500 µM) and content of the sacs analysed by LC-MS.

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Fig 1

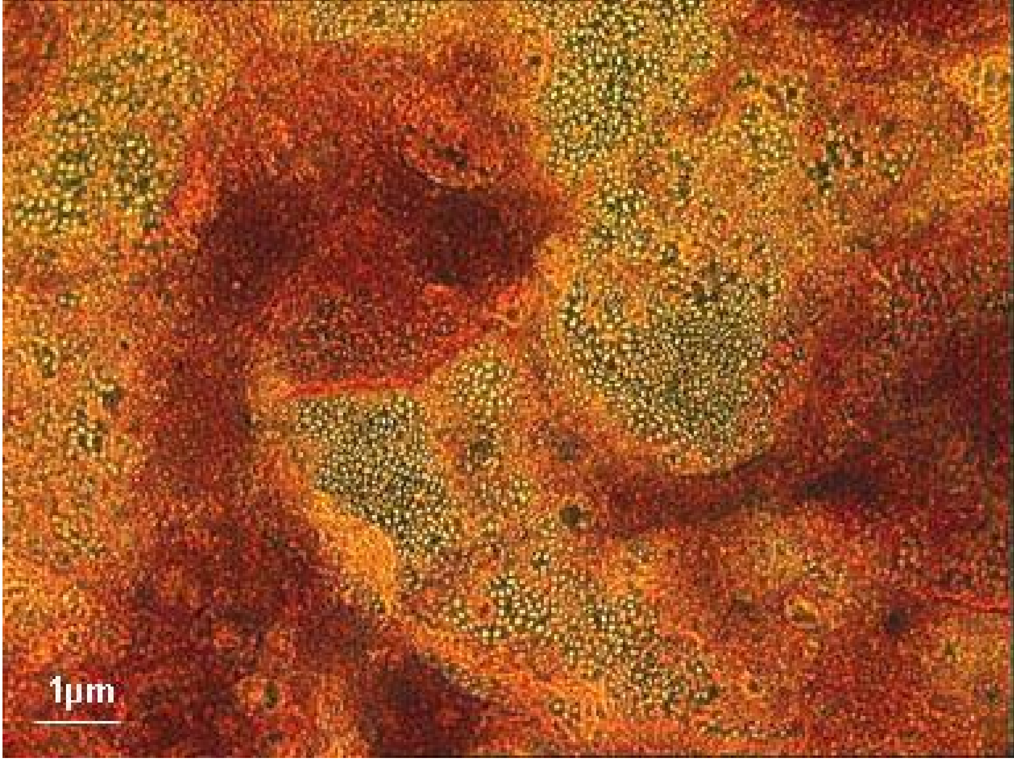

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Fig 2

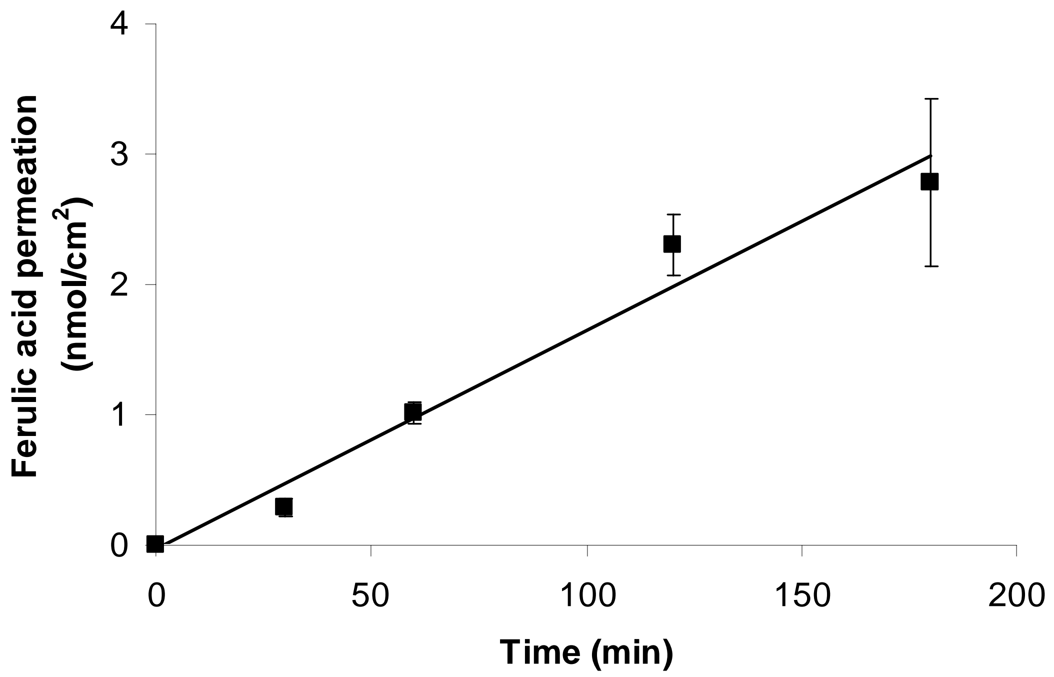

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Fig 3a

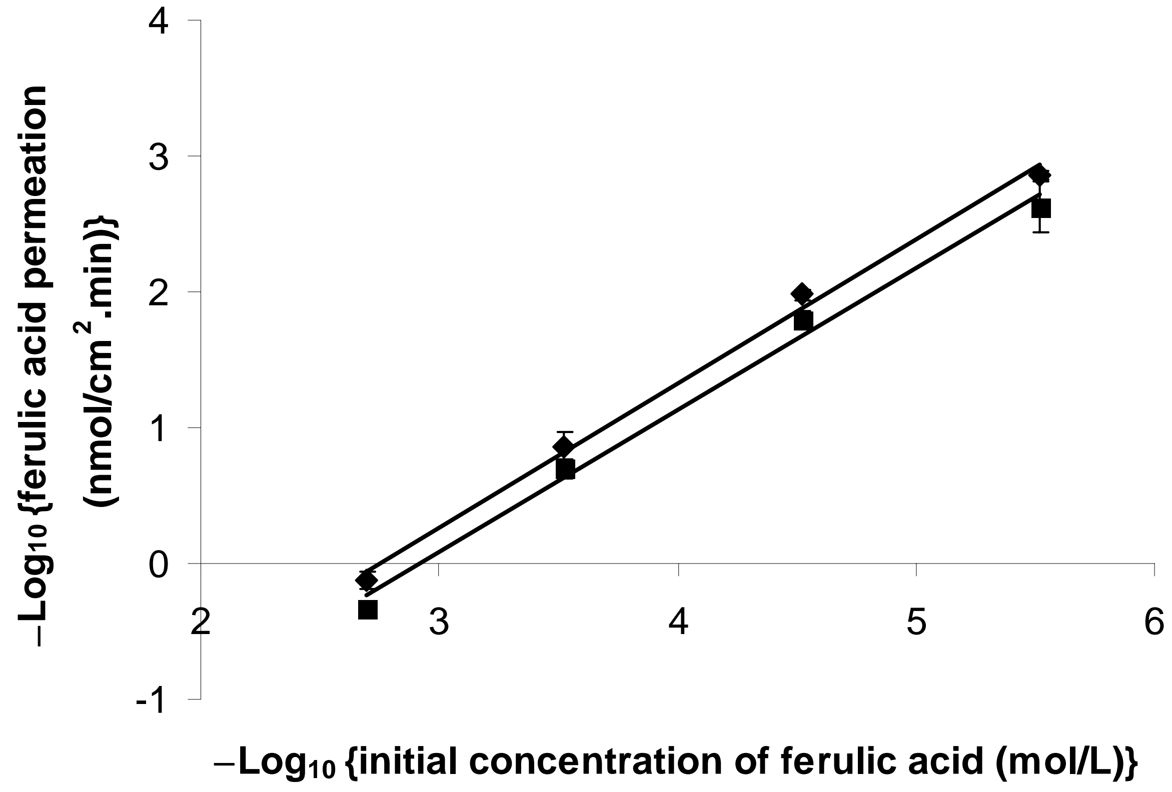

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Fig 3b

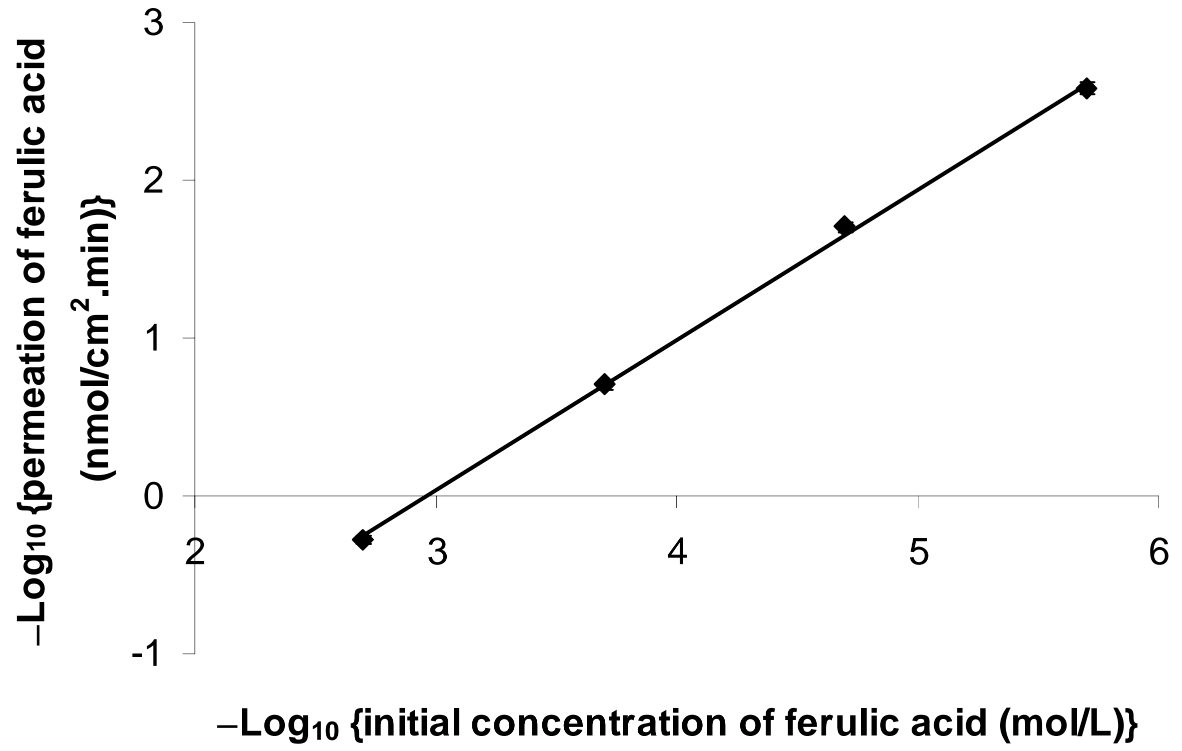

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Fig 4

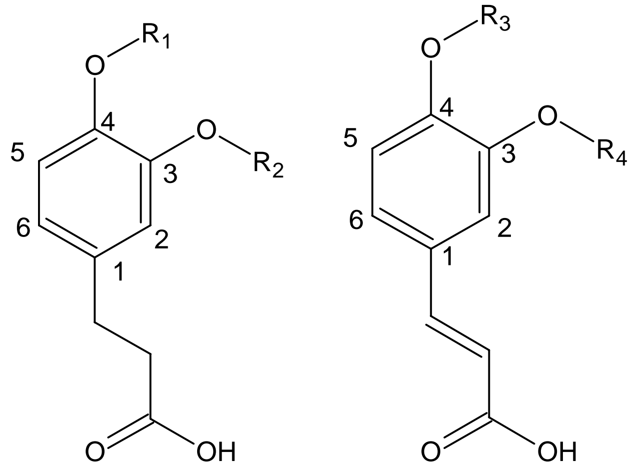

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Fig 5

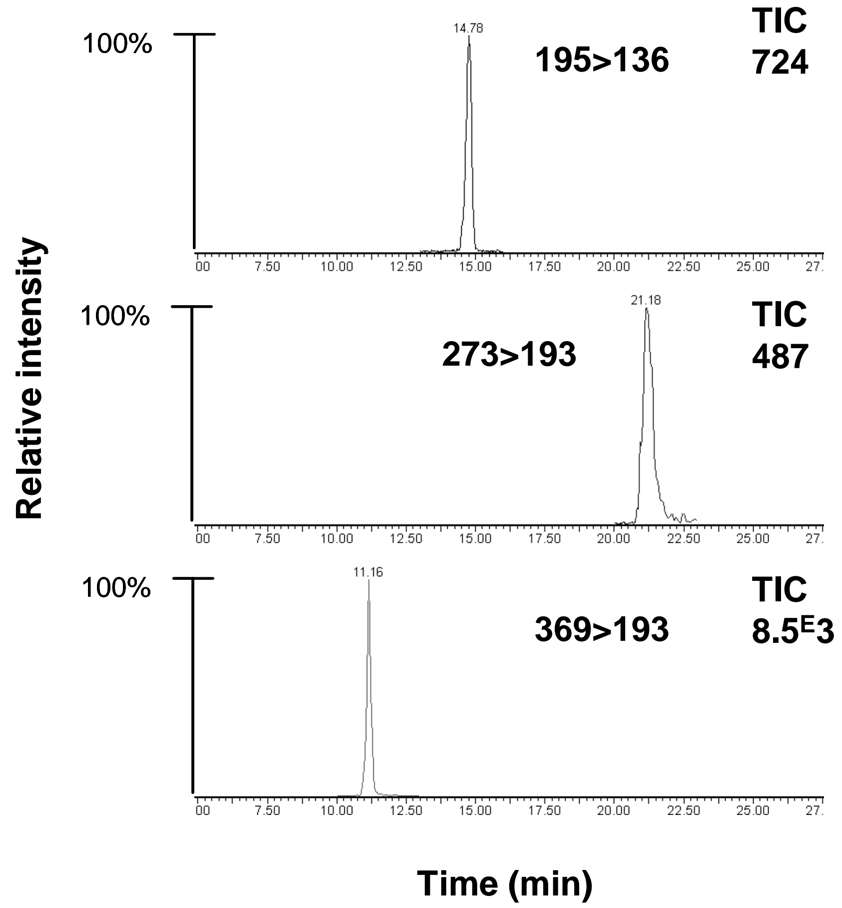

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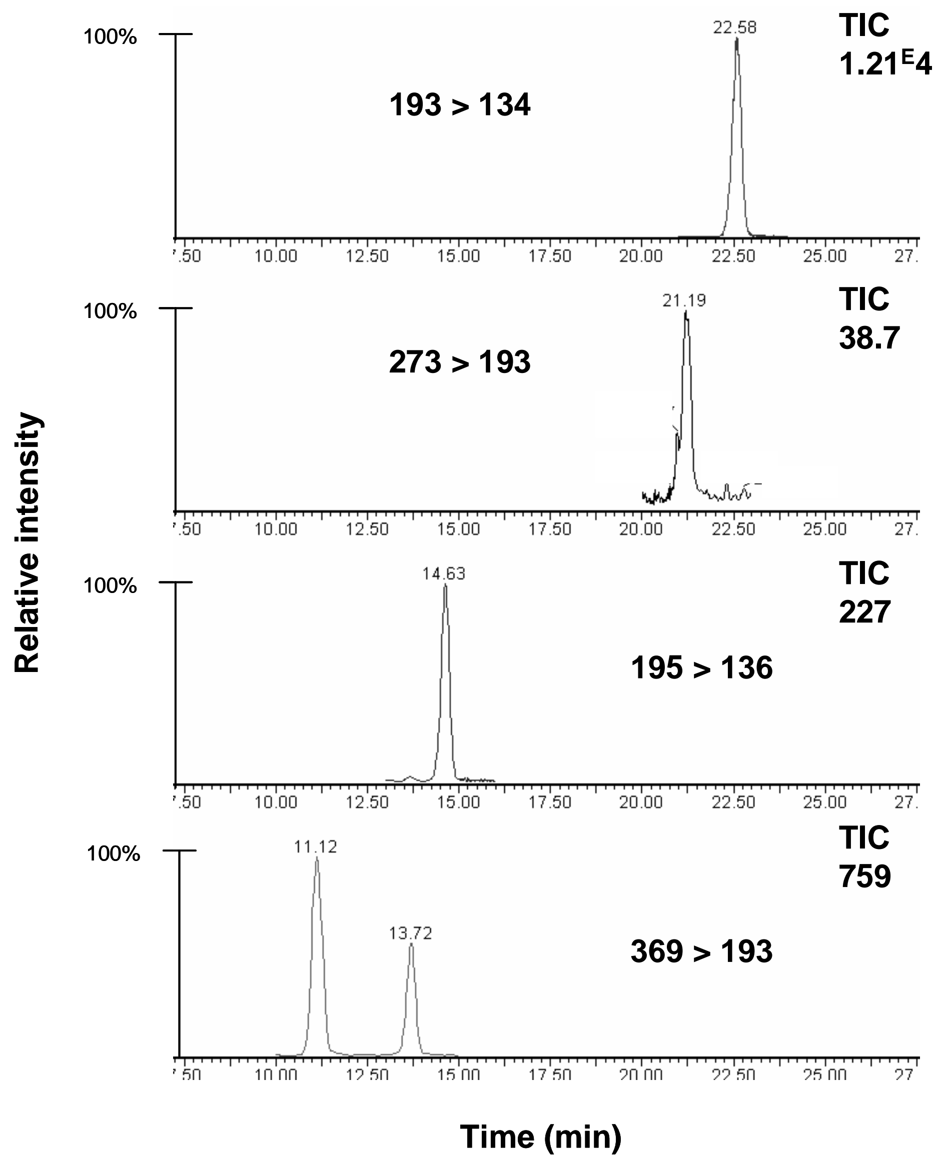
Fig 6



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