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Characterization of Metabolites of Hydroxycinnamates in the in Vitro Model of Human Small Intestinal Epithelium Caco-2 Cells

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Hydroxycinnamic acids are antioxidant phenolic compounds which are widespread in plant foods, contribute significantly to total polyphenol intakes, and are absorbed by humans. The extent of their putative health benefit in vivo depends largely on their bioavailability. However, the mechanisms of absorption and metabolism of these phenolic compounds have not been described. In this study, we used the in vitro Caco-2 model of human small intestinal epithelium to investigate the metabolism of the major dietary hydroxycinnamates (ferulate, sinapate, *p*-coumarate, and caffeate) and of diferulates. The appearance of metabolites in the medium versus time was monitored, and the various conjugates and derivatives produced were identified by HPLC-DAD, LC/MS, and enzyme treatment with β-glucuronidase or sulfatase. Enterocyte-like differentiated Caco-2 cells have extra- and intracellular esterases able to de-esterify hydroxycinnamate and diferulate esters. In addition, intracellular UDP-glucuronosyltransferases and sulfotransferases existing in Caco-2 cells are able to form the sulfate and the glucuronide conjugates of methyl ferulate, methyl sinapate, methyl caffeate, and methyl *p*-coumarate. However, only the sulfate conjugates of the free acids, ferulic acid, sinapic acid, and *p*-coumaric acid, were detected after 24 h. The O-methylated derivatives, ferulic and isoferulic acid, were the only metabolites detected following incubation of Caco-2 cells with caffeic acid. These results show that the in vitro model system differentiated Caco-2 cells have the capacity to metabolize dietary hydroxycinnamates, including various phase I (de-esterification) and phase II (glucuronidation, sulfation, and O-methylation) reactions, and suggests that the human small intestinal epithelium plays a role in the metabolism and bioavailability of these phenolic compounds.

KEYWORDS: Hydroxycinnamates; diferulic acids; human metabolism; small intestine; glucuronides; sulfates; esterases; O-methylation; ferulic acid; sinapic acid; caffeic acid; *p*-coumaric acid

INTRODUCTION

Hydroxycinnamic acids and diferulic acids are a group of polyphenols that are widely distributed in the diet, mostly in whole grains, fruits, vegetables, and beverages (*1*−*3*). These phenolic compounds exhibit good in vitro antioxidant (*4*−*7*) and chemoprotective properties (*8*−*10*) which may have some beneficial effects in vivo. The extent of their protective effect in vivo depends on their bioavailability. Hydroxycinnamates and diferulates are present in plant-derived food predominantly in esterified forms, linked to polymers (*11*, *12*) or small molecules, e.g., caffeoyl−quinic derivatives or 'chlorogenic acids' (*13*). Various studies have now shown that a proportion of these hydroxycinnamates and diferulates can be cleaved by esterases and released into the gut as free acids (*14*, *15*). In humans, some hydroxycinnamic acids, mostly ferulic acid and caffeic acid, can be absorbed from a range of dietary sources into the circulatory system, conjugated by phase II reactions glucuronidation and/or sulfation and excreted in the urine (*16*−*24*). Diferulic acids are absorbed in the rat (*15*), but the absorption of diferulic acids in humans and the existence and nature of their putative conjugates has not yet been established.

As for any other dietary compound, the systemic bioavailability of dietary hydroxycinnamates and diferulates depends on absorption in the intestine and first-past metabolism by the intestine and/or liver. The extent and role of hepatic and intestinal metabolism on these dietary phenolic compounds has not been established. Although the liver is the major organ for metabolism of many drugs and xenobiotics, the small intestine

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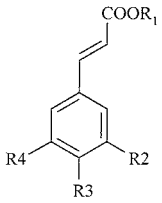

Compound	R_1	R_2	R_3	R_4
1 Ferulic acid-sulfate	H	OCH_3	OSO_3	H
2 Sinapic acid-sulfate	H	OCH_3	OSO_3	OCH_3
3 *p*-Coumaric acid-sulfate	H	H	OSO_3	H
4 Methyl ferulate-sulfate	CH_3	OCH_3	OSO_3	H
5 Methyl sinapate-sulfate	CH_3	OCH_3	OSO_3	OCH_3
6 Methyl *p*-coumarate-sulfate	CH_3	H	OSO_3	H
7 Methyl caffeate-sulfate	CH_3	OH	OSO_3	H
	CH_3	OSO_3	OH	H
8 Methyl ferulate-glucuronide	CH_3	OCH_3	GlcUA	H
9 Methyl sinapate-glucuronide	CH_3	OCH_3	GlcUA	OCH_3
10 Methyl caffeate-glucuronide	CH_3	OH	GlcUA	H
	CH_3	GlcUA	OH	H
11 Methyl *p*-coumarate-glucuronide	CH_3	H	GlcUA	H
12 Ferulic acid	H	OCH_3	OH	H
13 iso-Ferulic acid	H	OH	OCH_3	H
14 Sinapic acid	H	OCH_3	OH	OCH_3
15 *p*-Coumaric acid	H	H	OH	H
16 Caffeic acid	H	OH	OH	H
17 Methyl ferulate	CH_3	OCH_3	OH	H
18 Methyl sinapate	CH_3	OCH_3	OH	OCH_3
19 Methyl *p*-coumarate	CH_3	H	OH	H
20 Methyl caffeate	CH_3	OH	OH	H

Figure 1. Structures of the main dietary hydroxycinnamates and their metabolites formed by differentiated Caco-2 cells.

also contributes substantially to it by several pathways involving phase I and phase II reactions. Glucuronidation and sulfation are major pathways of phase II xenobiotic metabolism in the human intestine (*25*). Two main multigenic families of enzymes are responsible, namely, UDP-glucuronosyltransferases (UGTs) (*26*) and sulfotransferases (STs) (*27*).

Enterocyte-like differentiated Caco-2 cells are one of the most utilized in vitro models for investigating the molecular events associated with the small intestinal epithelium metabolism and uptake of numerous xenobiotics including pharmaceuticals (*28*). These cells exhibit many morphological and biochemical features of adult human enterocytes including the expression of phase II enzymes such as phenol STs (*29, 30*) and UGTs (*31, 32*). Caco-2 cells have been used in many studies looking at absorption and metabolism of flavonoids (*33*), isoflavones (*34*), and other dietary phenolic antioxidants (*35*). In the present study, we investigated the possible role of the small intestinal epithelium in the metabolism of hydroxycinnamates and diferulates, prevalent dietary antioxidants, using a well-established in vitro model differentiated Caco-2 cells. For this purpose, we monitored the formation versus time of some of the conjugates and metabolites formed from hydroxycinnamates and diferulates by these cells and characterized these derivatives using HPLC-DAD, LC/MS, and enzyme treatment with glucuronidase or sulfatase.

MATERIALS AND METHODS

Chemicals. For structures of all the hydroxycinnamates used in this study as well as their conjugates and derivatives, see **Figure 1**. Methyl ferulate (**17**), methyl sinapate (**18**), methyl *p*-coumarate (**19**), and methyl caffeate (**20**) were obtained from Apin Chemicals Ltd. (Abingdon, Oxon, U.K.). Ferulic acid (**12**), sinapic acid (**14**), *p*-coumaric acid (**15**), caffeic acid (**16**), β-glucuronidase from *E. coli* (3.2.1.31, type IX-A), and sulfatase from *Helix pomatia* (EC 3.1.6.1, type H-1) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). *trans*-Isoferulic acid (**13**) was purchased from Extrasynthese (Genay, France). Diethyl 5,5-diferulate, diethyl 8-*O*-4-diferulate, diethyl 8,5-benzofuran diferulate, and their monoester and free acid products were prepared as previously described (*36*). Ferulic and sinapic acid sulfates (**1, 2**) were synthesized as described elsewhere (*37*). All other chemicals were analytical or HPLC grade where applicable. Ultrapure water (18Ω) was used throughout the study.

Cell Culture. Human colon adenocarcinoma cells (Caco-2 cells) were obtained from the European Collection of Cell Culture and were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 1% (v/v) nonessential amino acids, 10% fetal calf serum, and 2 mM l-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were grown in a humidified incubator of 5% CO_2 at 37 °C and were subcultured at 80% confluence every week.

Metabolic Studies. Cells (passages 30−45) were seeded at a density of approximately 2×10^4 cells/cm^2 in 55 cm^2 dishes and allowed to adhere overnight. The medium was changed every second day, and cells were allowed to grow and differentiate up to 10 days after reaching confluence. At this stage, the cells are heterogeneously polarized and differentiated and they have not started detaching from the edges (*38, 39*). On the day of the experiment, fresh medium was added and cells were incubated with either methyl ferulate, methyl sinapate, methyl caffeate, methyl *p*-coumarate, or each of the corresponding hydroxycinnamic acids (50 μM; 0.1% methanol) for 0, 0.5, 1, 2, 4, 6, and 24 h. Caco-2 cells were also incubated with either 5,5-diethyl diferulate (60 μM), 8-*O*-4-diethyl diferulate (35 μM), 8,5-benzofuran diethyl diferulate (60 μM) in DMSO (0.1%), or 5,5-diferulic acid (15 μM) in methanol (0.1%) for 16 h. All metabolic experiments were carried out in duplicate. The stability of the test compounds throughout the treatment was checked by incubating fresh medium (+ fetal calf serum) with each individual compound for 24 h. A set of cultures was exposed to the equivalent concentration of vehicle solvent to search for background peaks which could interfere with the analysis. Also, each individual parent compound was incubated with medium that had been preincubated with cells for 22 h and subsequently removed to determine the extra- or intracellular origin of the detected metabolic activities. At the end of the incubation period, the medium was processed for HPLC-DAD and LC/MS analysis as follows: methanol was added to the collected medium (1:1) and the mixture was kept at 4 °C for 16 h to allow protein precipitation. Samples were then centrifuged at 13 000 rpm for 15 min at 4 °C and filtered (0.22 μm) prior to analysis.

HPLC Analysis. Samples were analyzed by reverse-phase HPLC-DAD using a slightly modified version of a previously published method (*15*). Hydroxycinnamates, their metabolites, and diferulates were separated using a Luna C18 (2) column (25 cm × 4.60 mm i.d., 5 μm; Phenomenex). Solvents A (10% acetonitrile, 1 mM trifluoroacetic acid (TFA)) and B (40% acetonitrile, 40% methanol, 1 mM TFA) were run at a flow rate of 1 mL/min using the following gradient: solvent B increases from 10% to 38% over a 30 min period followed by an increase up to 100% over 5 min. These conditions were held isocratically for 20 min to decrease to 10% B over 5 min and held isocratically for 10 min (reequilibration). The eluent was monitored at 325 and 280 nm with a diode array detector. Methyl hydroxycinnamates and hydroxycinnamic acids were quantified by integration of peak areas at 280 or 325 nm, with reference to calibration made using known amounts of pure compounds. The concentration of identified conjugates was estimated using the response factor of the corresponding parent compound.

Liquid Chromatography−Mass Spectrometry. Metabolites were identified by positive (ES^+) or negative (ES^-) ion electrospray LC/MS measurements using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) coupled to a Jasco PU-1585 triple-pump HPLC with an AS-1559 cooled autoinjector, CO-1560 column oven, and UV detector (Jasco Ltd., Great Dunmow, U.K.). The HPLC column temperature was maintained at 25 °C and the autoinjector at 4 °C. The eluent flow of 1 mL/min was split between the mass spectrometer and the UV detector in a ratio of 1:5 using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, U.K.). Approximately 800 μL of the flow split, monitored using a Humonics Optiflow 1000 flow meter (Sigma-Aldrich, Dorset, U.K.), was diverted to the UV detector for detection at 280 nm. HPLC conditions were as described above. All mass spectra were performed using a Micromass Z-spray ion source with the following tuning parameters: electrospray capillary voltage 3.5 kV, cone voltage 18 V, source block temperature 120 °C, and desolvation temperature 350 °C. The flow rates of nitrogen

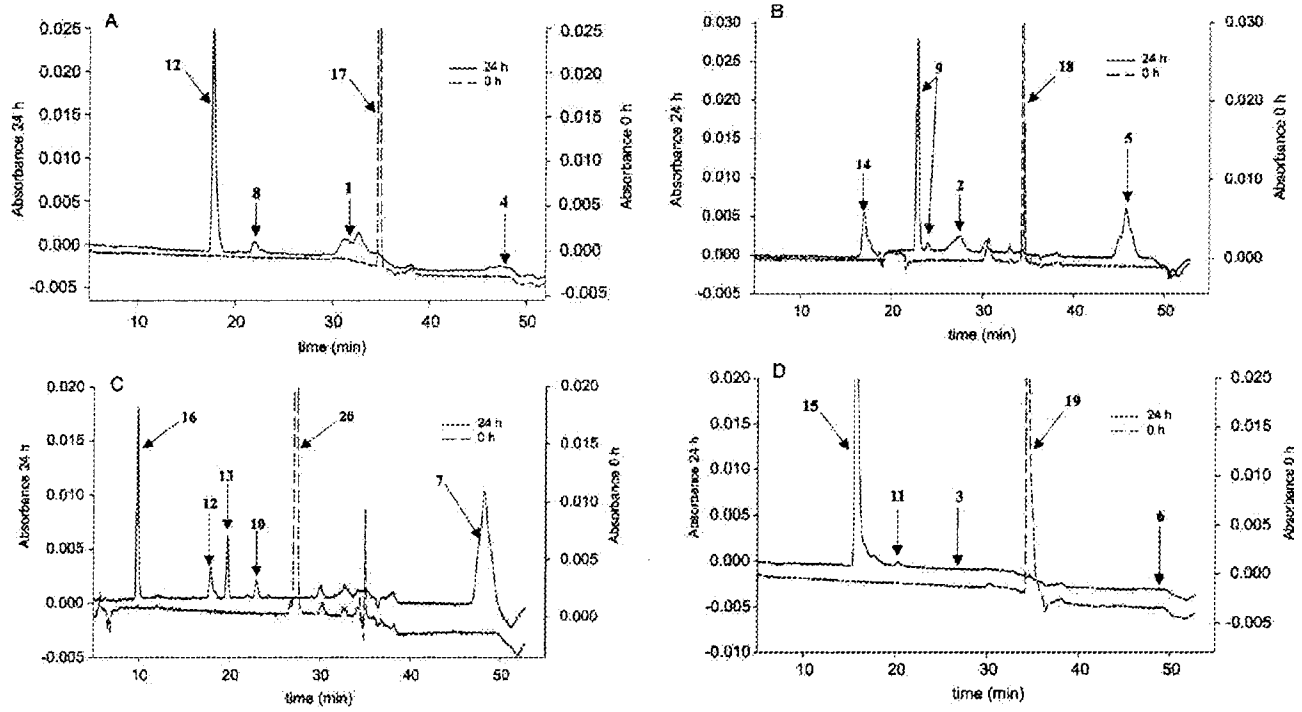

Figure 2. HPLC chromatograms of cell culture medium from Caco-2 cells at 0 and 24 h: (A) methyl ferulate metabolism, (B) methyl sinapate metabolism, (C) methyl caffeate metabolism, and (D) methyl *p*-coumarate metabolism. Differentiated cells (10 days postconfluency) were incubated with 50 μM substrate over a 24 h period (UV absorption at 325 nm). Numbers in bold correspond to the numbering identification system given to these compounds in **Table 1** and **Figure 1**.

as drying and nebulizing gas were 500 and 15 L/h, respectively. Full scan spectra were obtained in positive- or negative-ion mode between *m*/*z* 50 and 1000 with a scan duration of 1.5 s and an interchannel delay of 0.1 s, respectively (detection limit ~50 pmol as injected). Instrument control, data acquisition, and processing were performed using Micromass MassLynx version 3.4 data system and software.

Indirect Determination of Hydroxycinnamates Metabolites. Samples of medium (1 mL) containing hydroxycinnamate conjugates/metabolites (24 h incubation) were treated with β-glucuronidase or sulfatase as follows: after acidification (50% acetic acid; pH ~6.0), 300 μL aliquots were incubated with (a) glucuronidase (5 μL = 25 U) in phosphate-buffered saline (PBS), (b) sulfatase (5 μL = 5 U) in PBS, and (c) PBS (5 μL) at 37 °C for 1 h (control). Reactions were stopped by addition of methanol (300 μL), and the mixtures were stored at 4 °C for 16 h. Samples were then centrifuged (13 000 rpm for 15 min at 4 °C) and filtered (0.22 μm) prior to HPLC-DAD analysis. The synthesized sulfate conjugates from ferulic acid and sinapic acid were also treated with either enzyme as follows: samples containing the synthetic sulfate were dried under a light stream of N_2, redissolved in PBS, and acidified with 50% acetic acid to pH ~6.0. Aliquots (300 μL) of the acidified solution were treated as described above.

RESULTS

Detection and Identification of Metabolites of Hydroxycinnamates and Diferulates Formed by Differentiated Caco-2 Cells. The formation of metabolites from hydroxycinnamates and diferulates was investigated using cultured differentiated Caco-2 cells. Cells were grown on dishes, and the metabolites were determined only in the medium, i.e., either produced intracellularly and excreted to the apical side of the monolayer or produced directly in the medium by secreted enzymes. Methyl esters of ferulic (**17**), sinapic acid (**18**), caffeic (**20**), and *p*-coumaric (**19**) and ethyl esters of various diferulic acids were used as model compounds to reflect the prevalence of these compounds as esters in the diet.

Hydroxycinnamates. Analysis by HPLC-DAD of initial samples (0 h) and samples over the 24 h incubation period with each of the parent compounds demonstrated the capability of these cells to metabolize the hydroxycinnamates generating several products. **Figure 2A**−**D** shows the comparison between the HPLC traces of initial samples and samples after 24 h incubation. The analysis of control cells incubated only with vehicle solvent showed the absence of any background signal, which could interfere with the analysis of the samples. Products were identified by retention time (RT) and spectroscopic properties (as compared to synthetic standards where available), LC/MS analysis, and/or sensitivity to treatment with β-glucuronidase or sulfatase. Results are summarized in **Table 1** and structures presented in **Figure 1**. HPLC-DAD analysis of the metabolites formed from methyl ferulate, methyl sinapate, methyl caffeate, or methyl *p*-coumarate showed the formation of the corresponding free hydroxycinnamic acids (products **12**, **14**, **16,** and **15,** respectively). The hydroxycinnamate esters were not hydrolyzed by culture medium alone, indicating that the esterase activity originated from the cells. In contrast, medium that had been preincubated with differentiated cells and subsequently removed showed esterase activity against methyl ferulate, methyl caffeate, and methyl *p*-coumarate. However, methyl sinapate was only hydrolyzed in the presence of Caco-2 cells and not by culture medium subsequently isolated from cells.

Incubation of each of the methyl substrates with Caco-2 cells resulted also in the formation of the corresponding methyl hydroxycinnamate-glucuronides (**8**−**11**) and methyl hydroxycinnamate-sulfates (**4**−**7**). Caco-2 cells were also capable of forming a sulfate conjugate from ferulic acid (**1**), sinapic acid (**2**), and *p*-coumaric acid (**3**), respectively. The molecular mass of all the sulfate derivatives detected exhibited a characteristic single sulfur isotope pattern. Ferulic acid (**12**) and isoferulic acid (**13**) were the only metabolites identified from caffeic acid. No glucuronides of any of the hydroxycinnamic acids were detected. Following incubation of the parent compounds with

Table 1. Summary of Identification Parameters for Main Dietary Hydroxycinnamates Metabolites Formed by Caco-2 Cells

conjugates	RT[a] (min)	λ_{max1}[a] (nm)	λ_{max2}[a] (nm)	λ_{min}[a] (nm)	comparison to standard	LC/MS (ES^-) $[M - H]^-$, *m/z*	reaction to (G) or (S)[b]	identification
1	32.0	283	311	252	available	273 (100%)[d]	+(S)	ferulic acid–sulfate
2	26.4	300		254	available	303 (100%)	–(S)[c]	sinapic acids–sulfate
3	28.0				not available	243 (100%)		*p*-coumaric acid–sulfate
4	47.4				not available	287 (100%)	+(S)	methyl ferulate–sulfate
5	45.6	303		262	not available	317 (100%)	–(S)	methyl sinapate–sulfate
6	47.4	-			not available	257 (100%)		methyl *p*-coumarate–sulfate
7	48.0	312		262	not available	273 (100%)	+(S)	methyl caffeate–sulfate
8	22.5	-			not available	383 (100%)	+(G)	methyl ferulate–glucuronide
9	23.2	300		255	not available	413 (100%)	+(G)	methyl sinapate–glucuronide
10	22.8	-			not available	369 (100%)	+(G)	methyl caffeate–glucuronide

free acids	RT[a] (min)	λ_{max1}[a] (nm)	λ_{max2}[a] (nm)	λ_{min}[a] (nm)	comparison to standard	LC/MS (ES^+) $[M + H]^-$, *m/z*	$[M - H_2O + H]^+$, *m/z*	identification
12	18.0	323	299	262	available	195 (46%)	177 (100%)	ferulic acid
13	19.5	323	296	262	available	195 (23%)	177 (100%)	isoferulic acid
14	17.4	324		264	available	225 (23%)	207 (100%)	sinapic acid
15	15.8	310	299	266	available	165 (55%)	147 (100%)	*p*-coumaric acid
16	9.8	323	296	248	available	181 (34%)	163 (100%)	caffeic acid

[a] Retention time (RT) and spectroscopic properties by HPLC-DAD. [b] (G): β-Glucuronidase, *E. coli* (3.2.1.31, type IX-A). (S): Sulfatase, *Helix pomatia* (EC 3.1.6.1, type H-1). (+): Peak decreased after incubation with enzyme. (–) Peak unchanged after incubation with enzyme. [c] Absence of activity on sinapic acid–sulfate was confirmed using the synthetic standard. [d] Relative abundance of the molecular ions given in brackets.

culture medium that had been preincubated with cells and subsequently isolated from the cells, we were not able to detect any of the glucuronide or sulfate conjugates or other metabolites. Additional confirmation of the conjugates was obtained by treatment of the medium samples with glucuronidase or sulfatase. Incubation with buffer alone did not modify the peak area corresponding to any of the identified conjugates. Treatment with pure β-glucuronidase (*40*) resulted in the disappearance of the glucuronide peak only and an increase of the corresponding methyl hydroxycinnamate peak. The sulfate conjugates of methyl ferulate, methyl caffeate, and ferulic acid were verified by treatment with sulfatase type H-1 from *Helix pomatia*. This enzyme has been reported to contain some glucuronidase activity (*40*), and thus, we detected some hydrolysis of the glucuronides present in our samples. The sulfate conjugates of methyl sinapate and sinapic acid were not cleaved by this sulfatase, and this was confirmed by incubating the enzyme with the synthetic conjugate. The enzyme was not capable of hydrolyzing the synthesized sinapic acid−sulfate, although it did hydrolyze the synthetic ferulic acid−sulfate. It has been shown that some phenyl sulfates can be very resistant to hydrolysis by sulfatases and that the presence of substituents groups ortho to the position of the sulfate ester group may result in a very low rate of hydrolysis by some sulfatases (*41*). This may explain why the sulfate derivatives of sinapic acid methyl sinapate are not hydrolyzed by the sulfatase from *Helix pomatia*.

Diferulates. Incubation of Caco-2 cells with each of the diferulate diesters (5-5-, 8-*O*-4-, or 8-5-benzofuran) also demonstrated the ability of these cells to de-esterify the dimeric phenolics. The diester 8-*O*-4-diferulate was almost completely hydrolyzed to form the free acid after 16 h of incubation. Over the same period, the 8-5-benzofuran diester diferulate was hydrolyzed at a much lower rate, forming one 8-5-benzofuran monoester product and small quantities of the free acid. The 5-5-diester diferulate was also hydrolyzed to form small quantities of the monoester product, but no free acid was detected after 16 h. All products were identified from their retention time and spectroscopic properties, and the corresponding molecular masses were confirmed by LC/MS (monoester, $[M + H]^+$ at *m/z* 415; free acid, $[M + H]^+$ at *m/z* 387) (*36*). No other metabolite from any of the three diferulates or from 5-5-diferulic acid was found.

Formation of Metabolites versus Time. Differentiated Caco-2 cells were incubated with each of the methyl hydroxycinnamates or corresponding hydroxycinnamic acid over a period of 24 h. **Figure 3** illustrates the formation of metabolites versus time. Concentrations of the glucuronide or sulfate derivatives are estimates only, since pure conjugates were not available, and calculations were carried out using the response factors for the corresponding parent compounds. Methyl ferulate disappeared rapidly from the medium (**Figure 3A**). Only about 10% of this substrate was remaining in the media after the first 2 h of incubation, and the substrate had disappeared completely by 4 h. The concentration of ferulic acid in the medium increased rapidly and reached a maximum (30−35 μM) between 2 and 4 h to then decrease slowly until 24 h ($\sim$17 μM at the end of the incubation period). In addition, small quantities of a glucuronide and a sulfate conjugate of methyl ferulate were also formed over the 24 h period. Incubation of Caco-2 cells with ferulic acid (**Figure 3B**) showed a slow loss of this hydroxycinnamic acid from the medium; about one-half of the initial concentration was still left in the media after 24 h. The ferulic acid−sulfate was only detected after 6 h. The formation of metabolites from methyl sinapate and sinapic acid by Caco-2 cells versus time is shown in **Figure 3C** and **D**. Methyl sinapate disappeared from the media at a lower rate than methyl ferulate since approximately 50% of the starting methyl ester substrate was present in the media after 4 h of incubation and traces of methyl sinapate were still detected after 24 h. The product of its de-esterification, sinapic acid, was first detected in the media after 2 h, but only small amounts were quantified over the incubation period (<5 μM). Two major conjugates were detected after 2 h, a methyl sinapate−sulfate and a methyl sinapate−glucuronide, the levels of which increased rapidly during the first 6 h and then more slowly up to 24 h. A second compound with the same molecular mass as the methyl sinapate−glucuronide appeared in minor quantities approximately 2 h later, and traces of sinapic acid−sulfate were detected after 6 h. Incubation of Caco-2 cells with sinapic acid (**Figure 3D**) showed a slow disappearance of this hydroxycinnamic acid from

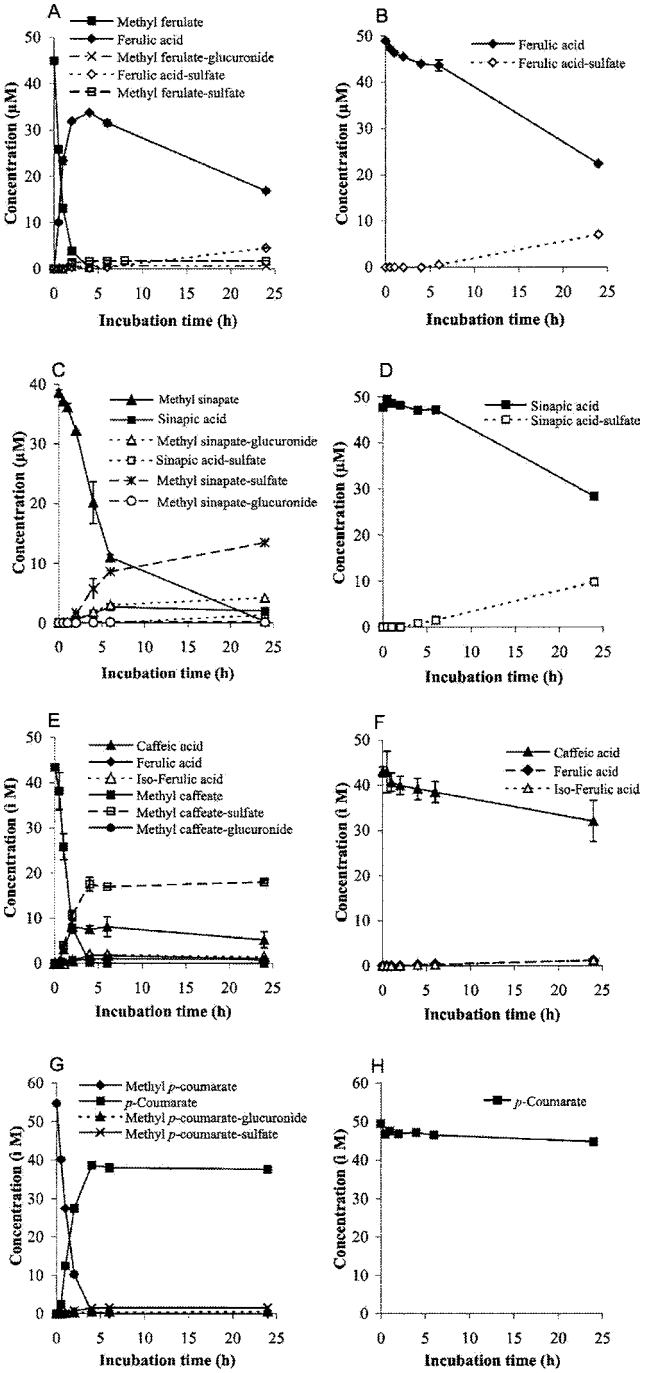

Figure 3. Time course of metabolism by Caco-2 cells of (A) methyl ferulate, (B) ferulic acid, (C) methyl sinapate, and (D) sinapic acid. Differentiated cells were incubated with each substrate (50 μM) over a 24 h period. Error bars represent mean ± SD of duplicates. Error bars, where not visible, were smaller than the size of the symbol. Time course of metabolism by Caco-2 cells of (E) methyl caffeate, (F) caffeic acid, (G) methyl *p*-coumarate, and (H) *p*-coumaric acid. Differentiated cells were incubated with each substrate (50 μM) over a 24 h period. Error bars represent mean ± SD of duplicates. Error bars, where not visible, were smaller than the size of the symbol.

the media; approximately 58% of the initial concentration was present in the media after 24 h. The sinapic acid−sulfate first appeared after 4 h.

Methyl caffeate was also rapidly metabolized by Caco-2 cells, and no methyl ester compound was left in the media after 4 h (**Figure 3E**). Caffeic acid was detected only after 30 min but increased only up to a maximum concentration of ~8 μM after 6 h incubation to then decrease slowly by 24 h. A major metabolite, the sulfate derivative of methyl caffeate, was however detected only after 1 h incubation. Methyl caffeate−glucuronide, ferulic acid, and isoferulic acid were also detected in small quantities. Incubation of Caco-2 cells with caffeic acid led to the slow formation of ferulic acid and isoferulic acid (<2 μM after 24 h incubation; **Figure 3F**). It should be noted that a proportion of caffeic acid or methyl caffeate disappeared from the media, in the absence of cells, after incubation for 24 h, probably due to oxidation (*42*) or adsorption to proteins present in the serum (*1*). The rest of the test compounds used in this study were stable under the incubation conditions. Metabolism of methyl *p*-coumarate by Caco-2 cells was rapid (concentration of methyl *p*-coumarate in the media decreased from 55 μM to less than 1.0 μM within 4 h; **Figure 3G**) and resulted mainly in the formation of the free acid (~38 μM by 4 h), the concentration of which remained stable in the media up to 24 h. Incubation of Caco-2 cells with *p*-coumaric acid (**Figure 3H**) showed a minor decrease in the concentration of this phenolic over the 24 h incubation period. Traces of methyl *p*-coumarate-sulfate and *p*-coumaric acid-sulfate were detected only by LC/MS. A small peak was tentatively identified as methyl *p*-coumarate−glucuronide; this peak was sensitive to treatment with β-glucuronidase, producing *p*-coumaric acid as product.

DISCUSSION

Differentiated Caco-2 cells express phase I and phase II enzymes and provide a useful model for investigating first-pass metabolism of many xenobiotic, drugs, and dietary compounds by the human small intestinal epithelium (*26*−*35*). The aim of this study was to investigate whether the small intestinal epithelium plays a role in the metabolism of dietary hydroxycinnamates and diferulates using this in vitro model system. Our data provide first evidence that enterocyte-like Caco-2 cells are capable of metabolizing hydroxycinnamates by means of phase I and phase II enzymes. The results show that differentiated Caco-2 cells exhibit hydrolase activity toward esters of the major dietary hydroxycinnamates and diferulates. This esterase activity was also detected in medium that had been preincubated with cells and subsequently separated and incubated with methyl ferulate, methyl caffeate, and methyl *p*-coumarate, indicating that some of the esterase(s) were secreted to the medium, but we could not eliminate the possibility that some of the detected activity originates from floating (dead) cells. Notably, medium preincubated with cells and subsequently separated was not active on methyl sinapate, suggesting the presence also in Caco-2 cells of intracellular esterase(s) active on cinnamoyl substrates. The data obtained here indicated that methyl ferulate, methyl caffeate, and methyl *p*-coumarate were more rapidly metabolized than methyl sinapate. These differences can be due to a combination of factors, i.e., different rates of hydrolysis by the esterases and different rates of diffusion across the membrane of the methyl ester hydroxycinnamates and of the hydroxycinnamic acids. The presence of esterase(s) in the human small intestine mucosa with activity against ferulate and diferulates has been previously reported (*14*, *15*), and our results confirm that differentiated Caco-2 cells do exhibit similar enzymatic activity. Various ester-type prodrugs are known to be metabolized in enterocytes to form the active drugs (carboxylates) by carboxylesterases (*43*). These enzymes have been identified in both the human small intestine and in Caco-2 cells (*44*) and may be responsible for the activity described here on methyl hydroxycinnamates.

Our data also provide evidence for sulfation, glucuronidation, and methylation of various hydroxycinnamates and hydroxy-

cinnamic acids by Caco-2 cells. Previous studies in humans have shown that conjugated metabolites of hydroxycinnamic acids such as ferulic acid−glucuronide and the glucuronide/sulfate conjugates of caffeic acid are present in the urine or plasma of volunteers fed hydroxycinnamate-rich foods, but the only evidence supporting the identification was the susceptibility to β-glucuronidases and/or sulfatases (*16*−*24*). For sinapic acid and *p*-coumaric acid, there is a marked lack of information concerning their absorption and metabolism in the available literature; there is some indication that trace quantities of sinapic acid and *p*-coumaric acid can be absorbed, but the presence of conjugates was not determined (*19*, *20*). We identified several conjugates of hydroxycinnamates and hydroxycinnamic acids formed by differentiated Caco-2 cells using a combination of methods including HPLC-DAD, LC/MS, as well as enzyme treatments. Medium that had been preincubated with cells and subsequently removed had no detectable phase II metabolic activity on any substrate, indicating that the responsible enzymes were not secreted to the medium or that if present in the medium (e.g., derived from dead cells) the levels were negligible. These results suggest that all the conjugation reactions must have occurred intracellularly and that once formed some of the metabolites and conjugates were excreted to the media. It has been suggested that excretion of these molecules may occur via transporters specific for organic anions (*45*), but we cannot discard that a proportion of the detected metabolites was originated from dead cells.

Our data show that ferulic acid, sinapic acid, and *p*-coumaric acid can enter the cells, be conjugated by sulfation, and then be excreted to the media. If glucuronidation of these hydroxycinnamic acids has occurred and the glucuronidated derivatives have been excreted to the medium, then their concentration must have been below the detection limits of our method. In contrast, when Caco-2 cells were incubated in the presence of the corresponding methyl esters, both sulfate and glucuronide conjugates of the methyl hydroxycinnamates were formed and detected in the medium. The observed differences in the conjugation pattern between hydroxycinnamic acids and their methyl derivatives may be due to different affinities of the STs and UGTs for these substrates. In general, phenols are substrates for sulfation and for glucuronidation, but the affinity of the STs for these substrates is much higher than the affinity of the UGTs for the same substrates. This implies that at low concentrations sulfation is the preferred route for conjugation, but as the concentration increases, there is a shift toward glucuronidation (*41*). In our experiments the intracellular concentration of hydroxycinnamic acids may have been quite low over the incubation period as shown by a slow decrease of the parent compound in the media (**Figure 3B, D, F**, and **H**). This may be due to transport of the hydroxycinnamates across the membrane being limited. Passive absorption is unlikely to occur since at the pH of the media (~6.5) the hydroxycinnamic acids will be in a predominantly ionized form (*5*). Although the existence of Na^+-dependent active transport processes involved in the intestinal uptake of cinnamic acids has been claimed (*46*), this has yet to be confirmed. The presence or otherwise of such a transporter in Caco-2 cells has not been established. Nevertheless, the more lipophilic methyl hydroxycinnamates can enter the cells by passive diffusion, and so the intracellular concentration may have been higher than that of the free acids. Further, glucuronidation may have been required for detoxification. It is also plausible that the more lipophilic methyl esters are better able to access the UGTs that are found bound to the membrane of the endoplasmic reticulum, whereas the more polar hydroxycinnamic acids remain in the cytosol where the STs are located (*41*).

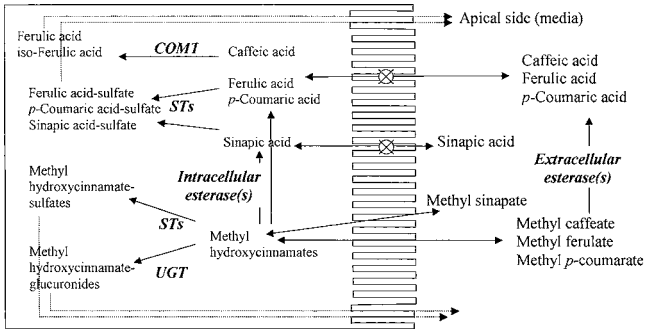

Figure 4. Summary of the mechanism of hydroxycinnamates metabolism by enterocyte-like differentiated Caco-2 cells. Methyl hydroxycinnamates (esters) may go across the apical membrane of the cells by passive diffusion (↔) and hydroxycinnamic acids by unidentified specific transporters (⊗). Methyl ferulate, methyl caffeate, and methyl *p*-coumarate can be de-esterified extra- and/or intracellularly, whereas methyl sinapate is de-esterified intracellularly. Once in the cytosol, the methyl hydroxycinnamates can be further metabolized by phase II enzymes, glucuronosyltransferases (UGTs) or sulfotransferases (STs). Free hydroxycinnamic acids are mostly sulfated by STs except for caffeic acid, which is metabolized to ferulic acid and isoferulic acid by catechol-*O*-methyltransferases (COMT). All these conjugates and metabolites are excreted out to the apical side of the cells by unknown mechanisms (dotted arrows).

Early reports on the metabolism of caffeic acid in humans identified the formation of several hydroxymethoxy derivatives (*47*), and it was indicated that gut microflora and liver (*47*, *48*) were responsible for O-methylation of caffeic acid. More recently, ferulic acid and isoferulic acid glucuronides were detected in the urine of volunteers after consumption of coffee (*21*). In this study, we show that both methoxylated derivatives of caffeic acid, namely, ferulic acid and isoferulic acid, are formed by differentiated Caco-2 cells and excreted to the media. The ability of Caco-2 cells to O-methylate other dietary phenolic substrates has been reported previously (*35*), and our results indicate that the catechol-*O*-methyltransferase (COMT) present in differentiated Caco-2 cells is also active on caffeic acid.

In this work, we identified for the first time some of the metabolites and conjugates derived from some abundant dietary phenolic compounds, hydroxycinnamates, using the in vitro model system differentiated Caco-2 cells. **Figure 4** summarizes the metabolic reactions detected in enterocyte-like Caco-2 cells, showing routes for phase I de-esterification and phase II glucuronidation, sulfation, and O-methylation of the various hydroxycinnamates substrates used in this study. Hydrolysis by esterases has occurred extra- and intracellularly, whereas the phase II conjugates and metabolites were formed intracellularly and excreted to the apical side of the monolayer of cells. With all the cautions and limitations for the extrapolation of results from an in vitro model to in vivo human conditions, our results suggest that these metabolic reactions may occur in the human enterocyte, and thus, the human small intestinal epithelium may contribute to the metabolism and bioavailability of hydroxycinnamates. Our results also suggest that sulfation may be the preferred metabolic pathway for hydroxycinnamic acids in the small intestinal epithelium and that the sulfated derivatives can be excreted to the lumen. The glucuronide derivatives of hydroxycinnamic acids detected in the plasma or urine of humans (*16*−*24*) may be products of liver metabolism.

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LITERATURE CITED

(1) Clifford, M. N. Chlorogenic acids and other cinnamates, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362−372.

(2) Garcia-Conesa, M. T.; Plumb, G. W.; Waldron, K. W.; Ralph, J.; Williamson, G. Ferulic acid dehydrodimers from wheat bran: Isolation, purification and antioxidant properties of 8-O-4-diferulic acid. *Redox Rep.* **1997**, *3*, 319−323.

(3) Andreasen, M. F.; Christensen, L. P.; Meyer, A. S.; Hansen, Å. Ferulic acid dehydrodimers in rye (*Secale cereale* L.). *J. Cereal Sci.* **2001**, *31*, 303−307.

(4) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.***1996**, *20*, 933−956.

(5) Garcia-Conesa, M. T.; Wilson, P. D.; Plumb, G. W.; Ralph, J.; Williamson, G. Antioxidant properties of 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid (8-8′-diferulic acid, noncyclic form). *J. Sci. Food Agric.* **1999**, *79*, 379−384.

(6) Meyer, A. S.; Donovan, J. L.; Pearson, D. A.; Waterhouse, A. L.; Frankel, E. N. Fruit hydroxycinnamic acids inhibit human low-density lipoprotein oxidation in vitro. *J. Agric. Food Chem.* **1998**, *46*, 1783−1787.

(7) Andreasen, M. F.; Landbo, A.-K.; Christensen, L. P.; Hansen, Å.; Meyer, A. S. Antioxidant effects of phenolic rye (*Secale cereale* L.) extracts, monomeric hydroxycinnamates and ferulic acid dehydrodimers on human low-density lipoproteins. *J. Agric. Food Chem.* **2001**, *49*, 4090−4096.

(8) Huang, M.-T.; Smart, R. C.; Wong, C.-Q.; Conney, A. H.. Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.* **1988**, *48*, 5941−5946.

(9) Stich, H. F.; Dunn, B. P.; Pignatelli, B.; Oshima, H.; Bartsh, H. Dietary phenolics and betel nut extracts as modifiers of N-nitrosation in rat and man. *IARC Sci. Publ.* **1984**, *57*, 213−222.

(10) Pieters, L.; Van Dyck, S.; Gao, M.; Bai, R.; Hamel, E.; Vlietinck, A.; Lemière, G. Synthesis and biological evaluation of dihydrobenzofuran lignans and related compounds as potential antitumor agents that inhibit tubulin polymerization. *J. Med. Chem.* **1999**, *42*, 5475−5481.

(11) Ishii, T. Structure and functions of feruloylated polysaccharides, review. *Plant Sci.* **1997**, *127*, 111−127.

(12) Saulnier, L.; Crépeau, M.-J.; Lahaye, M.; Thibault, J.-F.; Garcia-Conesa, M. T.; Kroon, P. A.; Williamson, G. Isolation and structural determination of two 5−5-diferulate oligosaccharides indicates that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. *Carbohydr. Res.* **1999**, *320*, 82−92.

(13) Clifford M. N. Chlorogenic acids and other cinnamates−nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033−1043.

(14) Andreasen, M. F.; Kroon, P. A.; Williamson, G.; Garcia-Conesa, M. T. Esterase activity able to hydrolyse dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. *J. Agric. Food Chem.* **2001**, *49*, 5679−84.

(15) Andreasen, M. F.; Kroon, P. A.; Williamson, G.; Garcia-Conesa, M. T. Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biol. Med.* **2001**, *31*, 304−314.

(16) Bourne, L. C.; Rice-Evans, C. Bioavailability of ferulic acid. *Biochem. Biophys. Res. Commun.* **1998**, *253*, 222−227.

(17) Bourne, L. C.; Rice-Evans, C. Detecting and measuring bioavailability of phenolics and flavonoids in humans: pharmacokinetics of urinary excretion of dietary ferulic acid. *Methods Enzymol.* **1999**, *299*, 91−106.

(18) Graefe, E. U.; Veit, M. Urinary metabolites of flavonoids and hydroxycinnamic acids in humans after application of a crude extract from *Equisetum arvense*. *Phytomedicine* **1999**, *6*, 239−246.

(19) Bourne, L.; Paganga, G.; Baxter, D.; Hughes, P.; Rice-Evans, C. Absorption of ferulic acid from low-alcohol beer. *Free Radical Res.* **2000**, *32*, 273−280.

(20) Virgili, F.; Pagana, G.; Bourne, L.; Rimbach, G.; Natella, F.; Rice-Evans, C.; Packer, L. Ferulic acid excretion as a marker of consumption of a french maritime pine (*pinus maritima*) bark extract. *Free Radical Biol. Med.* **2000**, *28*, 1249−1256.

(21) Rechner, A. R.; Spencer, J. P. E.; Kuhnle, G.; Hahn, U.; Rice-Evans, C. A. Novel biomarkers of the metabolism of caffeic acid derivatives in vivo. *Free Radical Biol. Med.* **2001**, *30*, 1213−1222.

(22) Nardini, M.; Cirillo, E.; Natella, F.; Scaccini, C. Absorption of phenolic acids in humans after coffee consumption. *J. Agric. Food Chem.* **2002**, *50*, 5735−5741.

(23) Cremin, P.; Kasim-Karakas, S.; Waterhouse, A. L. LC/ES-MS detection of hydroxycinnamates in human plasma and urine. *J. Agric. Food Chem.* **2001**, *49*, 1747−1750.

(24) Rechner, A. R.; Kuhnle, G.; Bremner, P.; Hubbard, G. P.; Moore, K. P.; Rice-Evans, C. The metabolic fate of dietary polyphenols in humans. *Free Radical Biol. Med.* **2002**, *33*, 220−235.

(25) Lin, J. H.; Chiba, M.; Baillie, T. A. Is the role of the small intestine in first-pass metabolism overemphasised? *Pharmacol. Rev.* **1999**, *51*, 135−157.

(26) Paine, M. F.; Fisher, M. B. Immunochemical identification of UGT isoforms in human small bowel and in Caco-2 cell monolayers. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 1053−1057.

(27) Chen, G.; Zhang, D.; Jing, N.; Yin, S.; Falany, C. N.; Radominska-Pandya, A. Human gastrointestinal sulfotransferases: Identification and distribution. *Toxicol. Appl. Pharmacol.* **2003**, *187*, 186−197.

(28) Rousset, M. The human colon carcinoma cell lines HT-29 and Caco-2: Two *in vitro* models for the study of intestinal differentiation. *Biochimie* **1986**, *68*, 1035−1040.

(29) Satoh, T.; Matsui, M.; Tamura, H. Sulfotransferases in a human colon carcinoma cell line, Caco-2. *Biol. Pharm. Bull.* **2000**, *23*, 810−814.

(30) Tamura, H.; Taniguchi, K.; Hayashi, E.; Hiyoshi, Y.; Nagai, F. Expression profiling of sulfotransferases in human cell lines derived from extra-hepatic tissues. *Biol. Pharm. Bull.* **2001**, *24*, 1258−1262.

(31) Abid, A.; Bouchon, I.; Siest, G.; Sabolovic, N. Glucuronidation in the Caco-2 human intestinal cell line: induction of UDP-glucuronosyltransferase 1*6. *Biochem. Pharmacol.* **1995**, *5*, 557−561.

(32) Sabolovic, N.; Magdalou, J.; Netter, P.; Abid, A. Nonsteroideal anti-inflammatory drugs and phenols glucuronidation in Caco-2 cells. Identification of the UDP-glucuronosyltransferases UGT1A6, 1A3 and 2B7. *Life Sci.* **2000**, *67*, 185−196.

(33) Walle, U. K.; Galijatovic, A.; Walle, T. Transport of the flavonoids chrysin and its conjugated metabolites by the human intestinal cell line Caco-2. *Biochem. Pharmacol.* **1999**, *58*, 431−438.

(34) Murota, K.; Shimizu, S.; Miyamoto, S.; Izumi, T.; Obata, A.; Kikuchi, M.; Terao, J. Unique uptake and transport of isoflavone aglycones by human intestinal Caco-2 cells: comparison of isoflavonoids and flavonoids. *J. Nutr.* **2002**, *132*, 1956−1961.

(35) Manna, C.; Galletti, P.; Maisto, G.; Cucciolla, V.; D′Angelo, S.; Zappia, V. Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. *FEBS Lett.* **2000**, *470*, 341−344.

(36) Garcia-Conesa, M. T.; Kroon, P. A.; Ralph, J.; Mellon, F. A.; Colquhoun, I. J.; Saulnier, L.; Thibault, J.-F.; Williamson G. A cinnamoyl esterase from *Aspergillus niger* can break plant cell wall cross-links without release of free diferulic acids. *Eur. J. Biochem.* **1999**, *266*, 644−652.

(37) Todd, J. S.; Zimmerman, R. C.; Crews, P.; Alberte, R. S. The antifouling activity of natural and synthetic phenolic acid sulphate esters. *Phytochemistry* **1993**, *34*, 401−404.
(38) Vachon, P. H.; Beaulieu, J. F. Transient mosaic patterns of morphological and functional differentiation in the Caco-2 cell line. *Gastroenterology* **1992**, 103, 414.
(39) Delie, F.; Werner, R. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Crit. Rev. Therap. Drug Carrier Syst.* **1997**, *14*, 221−286.
(40) Rondini, L.; Peyrat-Maillard, M.-N.; Marsset-Baglieri, A.; Berset, C. Sulfated ferulic acid the main in vivo metabolite found after short-term ingestion of free ferulic acid in rats. *J. Agric. Food Chem*. **2002**, *50*, 3037−3041.
(41) Mulder, G. J. Sulfation−metabolic aspects. In *Progress in drug metabolism;* Bridges, J. W., Chasseaud, L. F., Eds.; Taylor & Francis Ltd.: New York, **1984**; Vol. 8, pp 35−100.
(42) Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985−1012.
(43) Zhang, W.; Xu, G.; McLeod, H. L. Comprehensive evaluation of carboxylesterase-2 expression in normal human tissues using array analysis. *Appl. Immunohistochem. Mol. Morphol.* **2002**, *10*, 374−380.
(44) Prueksaritanont, T.; Gorham, L. M.; Hochman, J. H.; Tran, L. O.; Vyas, K. P. Comparative studies of drug-metabolizing enzymes in dog, monkey, and human small intestine, and in Caco-2 cells. *Drug Metabol. Dispos.* **1996**, *24*, 634−642.
(45) Suzuki, H.; Sigiyama, Y. Role of metabolic enzymes and efflux of transporters in the absorption of drugs from the small intestine. *Eur. J. Pharm. Sci.* **2000**, *12*, 3−12.
(46) Wolffram, S.; Weber, T.; Grenacher, B.; Scharrer E. A Na^+-dependent mechanism is involved in mucosal uptake of cinnamic acid across the jejunal brush border in rats. *J. Nutr*. **1995**, *125*, 1300−8.
(47) Scheline, R. R. Metabolism of acids, lactones and esters. In *Handbook of mammalian metabolism of plant compounds*; Scheline, R. R., Ed.; CRC Press, Boca Raton, FL, **1991**; pp 139−196.
(48) Moridani, M. Y.; Scobie, H.; O'Brien, P. J. Metabolism of caffeic acid by isolated rat hepatocytes and subcellular fractions. *Toxicol. Lett.* **2002**, *133*, 141−151.

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