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Hydrolysis of phenolic acid esters by esterase enzymes from Caco-2 cells and rat intestinal tissue.

Name Surname

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Abstract

Regular consumption of coffee has been associated with a number of health benefits including a reduced risk of type 2 diabetes, and cholorgenic acids and their hydroxycinnamic acid metabolites are thought to be important mediators. However their effectiveness is dependent on their hydrolysis, metabolism and absorption from the gastrointestinal tract. Previous research has shown that hydrolysis occurs in the stomach and large intestine, however little is known about the fate of chlorogenic acids in the small intestine. Chlorogenic acid isomers and phenolic acid methyl esters were incubated with Caco-2 cells and rat intestinal tissue extract to determine if esterase enzymes from these models could hydrolyse the ester link in the compounds. Results of the 2 hour incubations showed that methyl ferulate (19.8% hydrolysis) and methyl caffeate (11.4% hydrolysis) were better hydrolysed than 5-O-caffeoylquinic acid (0.02% hydrolysis) and 3-O-caffeoylquinic acid (0.18% hydrolysis). There was also evidence that hydrolysis occurs via a predominantly intracellular mechanism, as during the 24 hour incubation, 57.1% of methyl ferulate incubated directly with Caco-2 cells was hydrolysed to ferulic acid, compared with 0.25% that was hydrolysed when incubated in medium that had been pre-incubated with Caco-2 cells to test for extracellular esterase activity. Incubation with rat intestine extract showed ability to hydrolyse methyl ferulate but not 5-O-caffeoylquinic acid. The differences in hydrolysis could be due to the lipophilicities of the compounds and their structural conformation.

Introduction

Phenolic compounds are widely distributed in fruit, vegetables and beverages such as red wine and coffee. They are secondary plant metabolites which exhibit in vitro antioxidant properties, and a diet containing high amounts of polyphenol- rich foods has been associated with a reduced incidence of chronic diseases caused by free radical and oxidative damage, such as cardiovascular disease and cancer (Bravo 1998).

Coffee, especially, has high levels of a class of phenolics known as chlorogenic acids (CGAs) (Guy *et al.* 2009) which account for 6 – 10% of the dry weight of the bean (Clifford 1999). CGAs are comprised of a quinic acid molecule ester linked to one or more hydroxycinnamic acid (HCAs). Examples of HCAs include ferulic and caffeic acid. Thirteen classes of CGAs have been isolated from coffee beans including caffeoylquinic acids (CQAs) and feruloylquinic acids (FQAs) among others. The abundance of these compounds is partially responsible for the organoleptic properties, as lower CGA content indicates a higher quality of coffee. This helps to explain the difference between the two major varieties of coffee, Coffea arabica (Arabica) and Coffea canephora (Robusta). Arabica is considered to be the superior variety due to its milder and fruiter properties, whereas Robusta is weaker with a more pronounced bitterness (Alonso-Salces *et al.* 2009).

The most abundant CGA found in coffee beans is 5-O-caffeoylquinic acid (Stalmach *et al.* 2009), which is comprised of caffeic acid, ester linked to quinic acid via the carbon 5 position of the quinic acid. Different structural isomers can exist depending on which carbon of the quinic acid the ester link is formed via (Fig. 1). Esterification can occur at positions 3, 4 and 5 (Clifford 2000).

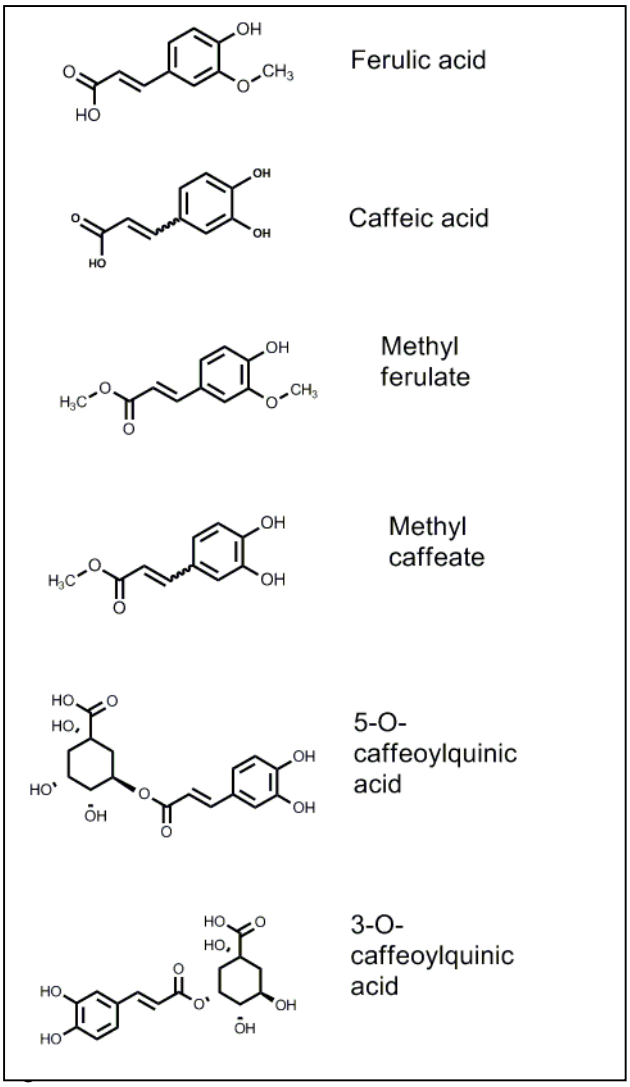
Coffee and CGAs are of interest because coffee is one of the most commonly consumed beverages in the world and regular consumption has been associated with health benefits including regulation of blood glucose (Johnston, Clifford and Morgan 2003), increased insulin sensitivity and reducing the incidence of type 2 diabetes (van Dam *et al.* 2004; Huxley *et al.* 2009). In vitro, CGAs have been shown to have antioxidant properties, and studies have shown that CGAs and HCAs act directly as free radical scavengers (RiceEvans, Miller and Paganga 1996) and can chelate metal ions which catalyse free radical formation (Andreasen *et al.* 2001b). Studies have also shown that in vitro CGAs and HCAs can protect LDL from oxidation (Meyer *et al.* 1998), which is a key step in preventing atherosclerotic plaque formation (Esterbauer *et al.* 1992). However, in vivo their protective action is also thought to occur via indirect biological mechanisms, such as

increasing nitric oxide production for improved endothelial function and inhibiting the formation of inflammatory factors like free radicals (Rocha *et al.* 2009).

However their potential action in vivo, whether via indirect or direct antioxidant mechanisms, is dependent on the bioavailability of the compounds from the diet. Previous research on metabolism has shown that CGA hydrolysis occurs in the colon, where extracellular esterase enzymes secreted by the gut microflora hydrolyse the ester bond between quinic acid and HCA (Plumb *et al.* 1999; Lafay *et al.* 2006). The free HCA can be absorbed and further metabolised, by glucuronidation, methylation or sulphation enzymes for example, into a form that can interact with biological systems (Rechner *et al.* 2002). Also, investigations using rodent gastric epithelial cells have demonstrated that hydrolysis of CGAs occurs in the stomach, as free HCAs were detected following incubation of the cells with 5-O- caffeoylquinic acid and coffee (Farrell *et al.* 2011). With regards to small intestinal hydrolysis, a study showed that medium pre- incubated with small intestinal epithelial cells and subsequently removed had esterase activity towards methyl esters of HCAs such as methyl ferulate and methyl caffeate as free HCAs were detected after incubation (Kern *et al.* 2003). Evidence from a separate study using homogenised cell-free mucosal extracts also supports that methyl esters of HCAs can be hydrolysed by intestinal esterases due to the release of free HCAs (Andreasen *et al.* 2001a).

However there is little in the literature on whether the small intestine is also an important site of hydrolysis of caffeoylquinic acids that are found in coffee, and this study aimed to investigate whether esterase enzymes produced by the small intestine epithelial cells could hydrolyse CGAs.

In order to elucidate whether phenolic acid esters can be hydrolysed by esterase enzymes synthesised by small intestinal enterocytes, the Caco-2 cell model was used in this investigation as an in vitro model of the human small intestine. The cells are derived from a human colon carcinoma cell line (Pinto *et al.* 1983), however when cultured under specific conditions, they differentiate into a confluent monolayer of polarised small intestinal enterocytes (Sun *et al.* 2002). They express microvilli, tight junctions and many of the enzymes, such as esterase enzymes, and transporters that are characteristic of small intestine epithelial cells, therefore they can be used as a suitable in vitro model to predict the metabolism of CGAs (Pinto *et al.* 1983). Rat intestinal tissue extract was also used as an animal model. HCA methyl esters and CGA isomers were incubated with Caco-2 cells and medium pre-incubated with Caco-2 cells to try to determine whether any detected hydrolysis was intracellular or extracellularly localised. Test compounds were also incubated with rat intestinal tissue extract. After incubation any HCA metabolites were detected using HPLC-DAD and the percentage of the test compound hydrolysed, if any, was calculated.



The structures of the compounds that were used during this study: ferulic acid, caffeic acid, methyl ferulate, methyl caffeate, 5-O-caffeoylquinic acid and 3-O-caffeoylquinic acid.

Materials and methods

Chemicals. For structures of all the phenolic acid esters used in this study, see Figure 1. Methyl ferulate (B22657) was obtained from Alfa Aeser (Lancashire, UK). Ferulic acid (46278), caffeic acid (C0625), methyl caffeate, 5-O-caffeoylquinic acid and 3-O-caffeoylquinic acid, protocatechuic acid (156421), p- coumaric acid (C9008), acetonitrile, 98% formic acid, ascorbic acid (A91290-2), ethanol (34870), acetic acid, dimethyl sulfoxide (60153), fetal bovine serum (F7524), Hanks balanced salts (H6648), 100 Units/ ml penicillin and 100µg/ml streptomycin (P0781), sodium phosphate (monobasic – S8282 and dibasic – S9763), intestinal acetone powder from rat (1630) and hydrochloric acid were obtained from Sigma-Aldrich (Dorset, UK). Dulbecco's modified Eagles medium was purchased from LGC Promochem Ltd. All the chemicals were HPLC grade where applicable. Deionised

Millipore water was used throughout the study (Millipore UK Ltd. Hertfordshire, UK).

Cell culture. Human colon adenocarcinoma cells (Caco-2 cells) were obtained from the American Type Culture Collection (LGC Promochem, Middlesex, UK). Transport studies used Caco- 2 cells between passage numbers 44 – 50. The cells were seeded on Transwell inserts at a density of 6 x 10^4 cells/cm^2. They were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and 0.1% amphotericin (1.5ml apical, 2ml basal). Cells were grown in a humidified incubator of 5% CO_2 at 37^{O}C for 21 days, and the medium was replaced every other day.

24 hour transport study of 2mM methyl ferulate. On day 22 under sterile conditions, transport studies were initiated by aspirating the apical and basal culture medium. To the apical chamber, 2ml of a 2mM solution of methyl ferulate mixed in culture medium was added (4.2mg methyl ferulate was dissolved in 20µl DMSO, and was then mixed with 10ml culture medium). To the basal chamber, 2ml of culture medium was added. This was performed in triplicate (n = 3). The stability of the methyl ferulate was assessed by taking a sample of the methyl ferulate and culture medium solution before incubation (0h) at 37^{o}C, and after incubation of the solution without Caco-2 cells for 24 hours. The Transwell plate was incubated for 24 hours. After 24 hours, 1ml of the all the apical and basal solutions were collected individually and 25µl acetic acid was added to each sample, and the samples were frozen at -80^{o}C until needed for HPLC analysis. Methyl ferulate (4.2mg dissolved in 20µl DMSO) was also added to and incubated with cell culture medium (10ml) that had been pre-incubated with Caco-2 cells for 24 hours previously and then removed. This was performed in order to determine whether hydrolysis of methyl ferulate occurred via intracellular or secreted extracellular esterase enzymes.

HPLC preparation of 24 hour incubation cell culture samples. Samples needed to be de-proteinated to remove particulates before HPLC analysis as the culture medium contained proteins. First they were defrosted, and 80µl of the sample was taken and combined with 20µl of 5270µM solution of protocatechuic acid used as an internal standard (5270µM protocatechuic acid, 10mg/ml ascorbic acid, 0.1% formic acid, 5% acetonitrile and 0.1% DMSO), as well as 100µl ethanol. The mixture was vortexed, centrifuged for 10 minutes to remove any proteins, and 95µl was then taken and to it 5µl of 1000µM solution of p-coumaric acid was added (1000µM p-coumaric acid, 10mg/ml ascorbic acid , 0.1% formic acid, 5% acetonitrile and 0.1% DMSO), as a second internal standard to assess variation in HPLC.

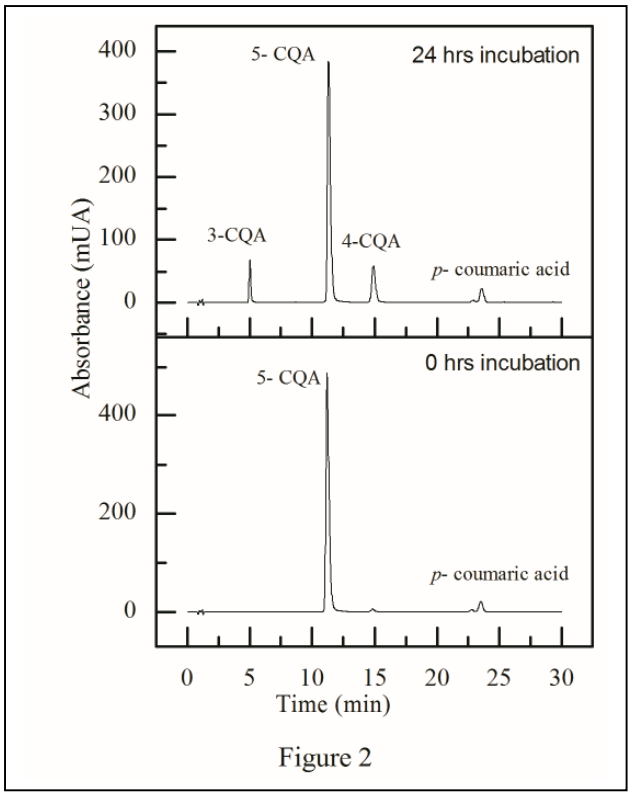


Figure 2
Chromatogram showing the isomerisation of 5-O-caffeoylquinic acid to 4-O-caffeoylquinic acid and 3-O-caffeoylquinic acid, after 24h incubation at 37^{o}C and 5%CO_2.

2 hour transport study of methyl ferulate, methyl caffeate, 3-O-caffeoylquinic acid and 5-O-caffeoylquinic acid. After the 24 hour methyl ferulate transport study was carried out, esterase activity was found to be more intracellularly localised, so incubation of the test compounds with pre-incubated medium was not further investigated. The stability assessment of methyl ferulate had been carried out for the 24 hours study, so stability assessment was carried out on 5-O-caffeoylquinic acid, by taking a sample of a 2mM solution of the compound in cell culture medium before incubation and after incubation for 24 hours without Caco- 2 cells, in order to assess the degradation of the test compound. From the result of the stability assessment and HPLC analysis, the incubation period of the compound with the cells was shortened to 2 hours, due to the isomerisation of 5-O-caffeoylquinic acid to 3-O-caffeoylquinic acid and 4–O-caffeoylquinic acid (Fig 2.). Also the addition of ethanol in the de-proteination step of HPLC preparation was found to cause interference with the recovery of the compound when running the samples through the HPLC, so the sample preparation step was altered. Instead of incubating the test compound with the Caco- 2 cells in cell culture medium, the test compound was incubated with the cells in Hank's balanced salts (HBS) solution modified with 1.8mM calcium chloride (11mg in 45ml HBS) to ensure tight junction integrity between the Caco-2 cells. Also the concentration of the test compound incubated with Caco- 2

cells was reduced to 1mM, which is more realistic of the concentration that could occur in vivo.

On day 22 under sterile conditions, transport studies were initiated by aspirating the apical and basal culture medium. To the apical chamber, 2ml of a 1mM solution of methyl ferulate mixed in HBS was added (2.1mg methyl ferulate was dissolved in 20µl DMSO and then mixed with 10ml of the HBS and calcium chloride solution). To the basal chamber, 2ml of HBS and calcium chloride solution was added. This was performed in triplicate (n = 3). The same procedure was carried out for 1mM solutions of 5-O-caffeoylquinic acid (3.5mg was dissolved in 20µl DMSO and mixed with 10ml HBS and calcium chloride solution), methyl caffeate (1.6mg was dissolved in 15µl DMSO and mixed with 8ml HBS) and 3-O-caffeoylquinic acid (2.8mg was dissolved in 15ml DMSO and mixed with 8ml HBS solution) separately. Reference samples of the phenolic solutions were also incubated without Caco-2 cells to assess any degradation. The cells and test compound solutions were incubated together for 24 hours at 5% CO_2 at 37^{o}C. After 2 hours, 1ml of the apical and basal solutions were collected separately from each of the wells incubated with the test compounds, as well as the control samples incubated without Caco-2 cells, and to these 25µl acetic acid was added, and the samples were frozen at -80^{o}C until needed for HPLC analysis. Sample de-proteination was not needed for this transport study as HBS solution does not contain proteins. In order to prepare the samples for HPLC analysis, they were defrosted, vortexed, centrifuged and 95µl of supernatant was taken and to it was added 5µl of a 1000µM solution of p-coumaric acid as an internal standard, made as described previously.

Trans- Epithelial Electrical Resistance (TEER) values. TEER readings were recorded using a Millicell ERS volt-ohm meter (Millipore Ltd, Watford, UK.) fitted with a chopstick probe in order to determine the integrity of the Caco- 2 cell monolayer. Literature shows that higher TEER readings are due to greater monolayer confluence and tight junction interaction.

Rat intestinal extract hydrolysis study of methyl ferulate and 5-O-caffeoylquinic acid. An enzyme extract was made from intestinal acetone powder from rat, by mixing the powder (30mg) with sodium phosphate buffer pH6.8 (1ml), centrifuging, with the resulting supernatant being the enzyme extract solution. A sample of the enzyme extract (200µl) was then incubated with a 2mM methyl ferulate solution (200µl) for 1 and 2 hours. This was performed in triplicate for each time point (n = 3). The 2mM methyl ferulate solution was made by dissolving 4.2mg methyl ferulate in 40µl DMSO, removing 20µl of this and adding it to a 5ml solution containing 10mg/ml ascorbic acid dissolved in 50mM sodium phosphate buffer pH 6.8. One control sample with buffer (200µl) replacing the enzyme was also incubated with 2mM methyl ferulate solution (200µl) for 1 and 2 hours in order to determine how much HCA formation occurred due to degradation of the methyl ferulate whilst being incubated. After the appropriate time period had elapsed, 1M hydrochloric acid (40µl) was added to both the control and test samples to stop the reaction. All samples were centrifuged and filtered to remove any protein particulates. 1000µM p-coumaric acid (5µl), as prepared previously, was added to 95µl of the resulting supernatant as an internal standard and the test samples and control samples were analysed by Stirling HPLC- DAD. The same procedure was carried out for 5-O-caffeoylquinic acid, using 7mg to prepare the phenolic solution.

HPLC conditions. Samples were analysed by Stirling HPLC- DAD. 5µl of sample was injected into a Rapid Resolution HPLC- DAD. Chromatographic separation was achieved on an Eclipse plus C18 column (30^{o}C, 2.1mm x 100mm, 1.8µm; Aligent Technologies) using a 60 minute gradient of solvent A (5% acetonitrile, 95% water, 0.1% formic acid) and solvent B (95% acetonitrile, 5% water, 0.1% formic acid) at a flow rate of 0.26ml/ min.

Quantification. The compounds were identified from the resulting chromatograms by their retention time in the column, and were quantified using peak areas at 320nm. The analytical wavelength of p- coumaric acid was also 320nm and peak areas were recorded. For each test compound incubated with Caco- 2 cells, pre- incubated medium and rat intestinal tissue extract, a standard curve of concentration against peak area was created for the test compounds and associated HCA in cell culture medium (24h methyl ferulate study) or HBS (2h methyl ferulate, methyl caffeate, 5- O- CQA and 3- O- CQA, and rat intestinal extract studies). The standard curves were created by making a stock solution of the compound (28mM) and carrying out a series of dilutions to result in solutions of 0.1µM, 0.5µM, 1.0µM, 75µM, 599µM and 2394µM. The standard solutions also contained 50µM of protocatechuic acid and 50µM p-coumaric acid which were the internal standards added during the dilution procedure. The peak areas of the test compounds from chromatograms of each concentration were divided by the peak area of p-coumaric acid at that concentration to provide normalised data. The standard curve was used to determine the concentration of phenolic acid ester and HCA in the test samples, and so enabling the calculation of the percentage hydrolysis of each compound that occurred after incubation.

Statistical analysis. Where triplicate samples were run by HPLC, the standard deviation and relative standard deviation was calculated using Microsoft Excel.

Results

Stability assessment. Initial assessment of methyl ferulate revealed stability for 24 hours in cell culture medium under test conditions. An initial assessment of the stability of 5-O-caffeoylquinic acid showed isomerisation to 3-O-caffeoylquinic acid and 4-O-caffeoylquinic acid over 24 hours in cell culture medium (see Fig. 2). A subsequent stability assessment of 5-O-caffeoylquinic acid in HBS, incubated for 2 hours at 37^{o}C showed stability of the compound. In addition, TEER values recorded of the Caco- 2 cells incubated with 2mM solution of 5-O-caffeoylquinic acid for 24 hours indicated that the compound at that concentration caused a loss of integrity of the Caco- 2 cell monolayer therefore the concentration of test compounds used in all the 2 hour incubation studies was decreased to 1mM.

Transport and metabolism of methyl ferulate after 24 hour incubation (Fig 3). A 2mM solution of methyl ferulate was incubated directly with Caco-2 cells, and with pre- incubated medium, for 24 hours in a humidified incubator of 5% CO_2 at 37^{o}C. This study was performed to determine the concentration of methyl ferulate and its primary metabolite, ferulic acid, in the apical and basal chambers of the Transwell insert in order to calculate the percentage hydrolysis that occurred (Table 1). Post incubation, 31.8 ±1.1% (RSD = 3.4%) of methyl ferulate had been hydrolysed to ferulic acid and detected in the apical chamber. In the basal chamber 25.3 ±0.6% (RSD = 2.5%) of methyl ferulate incubated with the cells had been hydrolysed to ferulic acid. Therefore in total, after direct incubation with Caco- 2 cells 57.1% of methyl ferulate had been hydrolysed to ferulic acid and detected by HPLC-DAD. Recovery of the metabolites was 60.4%. To confirm whether hydrolysis occurred via intracellular or extracellular esterase enzymes, the same concentration of methyl ferulate was incubated with medium that had been pre- incubated with Caco- 2 cells and removed, and analysis showed that 0.25 ±0.03% (RSD = 12.3%) of methyl ferulate was hydrolysed to ferulic acid. Recovery of the compounds was 69.3% The results of the 24 hour methyl ferulate study indicate that hydrolysis takes place via a predominantly

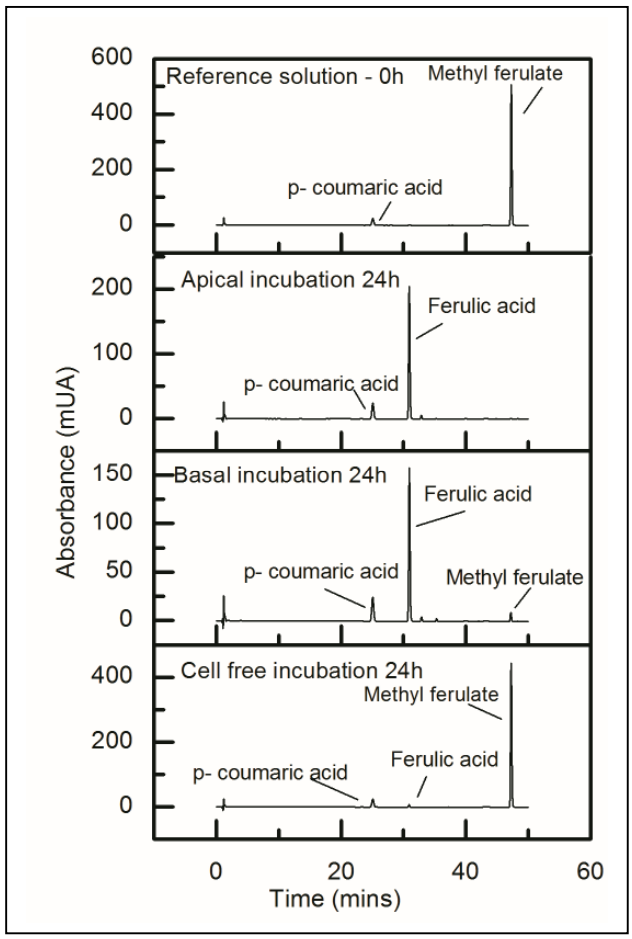


Figure 3
Chromatogram showing the formation of ferulic acid from methyl ferulate after 24h incubation with Caco-2 cells and medium pre-incubated with Caco-2 cells then removed, compared to a reference solution take at 0h. The chromatogram highlights that more metabolite was formed after direct incubation with Caco-2 cells as the ferulic acid peak is larger than for the cell free incubation trace.

Table 1. Summary of the hydrolysis of phenolic acid esters by Caco-2 cell and rat intestine tissue esterases

Phenolic acid ester	24h incubation - 2mM			2 h incuabtion - 1mM		Rat intestinal	
	% Hydrolysis			% Hydrolysis		1h	2 h
	Apical	Basal	Cell free extract	Apical	Basal		
Methyl ferulate	31.8 ±1.1	25.3 ±0.6	0.25 ±0.03	11.1 ±0.4	8.7 ±0.4	3.1 ±0.4	4.7 ±0.3
Methyl caffeate	-	-	-	1.9 ±0.1	9.5 ±0.3	-	-
5-*O*-caffeoylquinic acid	-	-	-	0.0	0.02 ±0.0012	0.0	0.0
3-*O*-caffeoylquinic acid	-	-	-	0.18 ±0.013	0.0	-	-

Test compound (2 mM or 1 mM) was incubated apically in the presence of Caco-2 monolayers (n =3) or cell free extract collected 24 h after incubation with cells. Hydrolysis was determined by detection of metabolite in apical and basal chambers after 24 h or 2 h incubations. Test compound (2mM) was also incubated with rat intestinal enzyme extract. Hydrolysis was determined by detection of metabolite after 1 or 2 h incubations.

Values are mean ±SD. -: not determined.

intracellular-based mechanism, thus further studies during the course of the investigation were performed only in the presence of Caco- 2 cells.

Transport and metabolism of methyl ferulate after 2 hour incubation. A 1mM solution of methyl ferulate in HBS was incubated directly with Caco- 2 cells for 2 hours in a humidified incubator of 5% CO_2 at 37^{o}C. This study was performed to determine the concentration of methyl ferulate and its primary metabolite, ferulic acid, in the apical and basal chambers of the Transwell insert after 2 hour incubation in order to calculate the percentage hydrolysis that occurred (see Table 1). Post incubation, 11.1 ±0.4% (RSD = 3.2%) of methyl ferulate had been hydrolysed to ferulic acid and detected in the apical chamber. In the basal chamber 8.7 ±0.4% (RSD = 5.0%) of methyl ferulate incubated with the cells had been hydrolysed to ferulic acid. Therefore in total, after direct incubation with Caco- 2 cells for 2 hours 19.8% of methyl ferulate had been hydrolysed to ferulic acid and detected by HPLC-DAD. Recovery of the compounds detected was 88%.

Transport and metabolism of methyl caffeate after 2 hour incubation. A 1mM solution of methyl caffeate in HBS was incubated directly with Caco-2 cells for 2 hours in a humidified incubator of 5% CO_2 at 37^{o}C. Post incubation, the results showed that in the apical chamber, it was detected that 1.9 ±0.1% (RSD = 6.4%) of methyl caffeate was hydrolysed to its metabolite, caffeic acid. In the basal chamber it was detected that 9.5 ±0.3% (RSD = 2.7%) of methyl caffeate had been hydrolysed to caffeic acid during incubation with Caco- 2 cells. Therefore in total, 11.4% of the methyl caffeate incubated with the Caco-2 cells for 2 hours was hydrolysed to caffeic acid and detected by HPLC–DAD. Recovery of the compounds was 96%.

Transport and metabolism of 5-O-caffeoylquinic acid. A 1mM solution of 5-O-caffeoylquinic acid in HBS was incubated directly with Caco-2 cells for 2 hours in a humidified incubator of 5% CO_2 at 37^{o}C. Post incubation, the results showed that in the apical chamber, no metabolites of 5-O-caffeoylquinic acid were detected after taking into account any caffeic acid formed due to degradation. In the basal chamber it was detected that 0.02 ±0.0012% (RSD = 5.7%) of 5-O-caffeoylquinic acid had been hydrolysed to caffeic acid during incubation with Caco-2 cells. Therefore in total, 0.02 ±0.0012% of the test compound incubated with the Caco-2 cells for 2 hours was hydrolysed to caffeic acid and detected by HPLC–DAD. Recovery of the compounds was 98%.

Transport and metabolism of 3-O-caffeoylquinic acid. A 1mM solution of 3-O-caffeoylquinic acid in HBS was incubated directly with Caco-2 cells for 2 hours in a humidified incubator of 5% CO_2 at 37^{o}C. Post incubation, the results showed that in the apical chamber, it was detected that 0.18 ±0.013% (RSD = 7.6%) of 3-O-caffeoylquinic acid was hydrolysed to its
metabolite caffeic acid. In the basal chamber, after taking into account any caffeic acid formed due to degradation, no metabolite of 3-O-caffeoylquinic acid was detected by HPLC-DAD after incubation with Caco-2 cells for 2 hours.

Therefore in total, 0.18 ±0.013% of the test compound incubated with the Caco-2 cells for 2 hours was hydrolysed to caffeic acid and detected by HPLC–DAD. Recovery of the compounds was 90%.

Rat intestinal extract hydrolysis of methyl ferulate and 5-O-caffeoylquinic acid. A 2mM solution of methyl ferulate was incubated with an enzyme extract, made from rat intestinal acetone powder, for 1 and 2 hours in a shaking water bath at 37°C. After 1 hour incubation, 3.1 ±0.4% (RSD = 12.5%) of the methyl ferulate had been hydrolysed to ferulic acid, taking into account any ferulic acid formed due to degradation of the test compound. After 2 hour incubation with the enzyme extract, 4.7% ±0.3% (RSD = 6.8%) of methyl ferulate had been hydrolysed to ferulic acid. The same procedure was also carried out for the compound 5-O-caffeoylquinic acid, and after 1 and 2 hour incubations with the enzyme extract, no caffeic acid was detected after taking into account any formed via degradation, therefore 0% of 5-O-caffeoylquinic acid was hydrolysed by esterase enzymes present in the rat intestinal powder extract.

Discussion

The purpose of this study was to investigate whether phenolic acid esters, caffeoylquinic acid in particular, can be hydrolysed to their constituent HCA and quinic acid molecules by intracellular and extracellular esterase enzymes from Caco-2 cells, and by esterase enzymes found in rat intestinal tissue. The test compounds of interest during this investigation were methyl ferulate, methyl caffeate, 5-O-caffeoylquinic acid and 3-O-caffeoylquinic acid. Each indiviaual test compound was incubated with Caco-2 cells or rat intestinal enzyme extract solution, as described in the methods section, in order to determine if the compounds were able to be hydrolysed by the esterase enzymes and what percentage of the compound was hydrolysed from the detection of the metabolite.

Previous reseach investigating the ability of Caco-2 cell esterases to hydrolyse phenolic acid methyl esters (Kern *et al.* 2003) such as methyl ferulate and methyl caffeate provided a basis for this study as phenolic acid esters contain a similar ester linkage as caffeoylquinic acids, so could theoretically also hydrolyse the ester link between quinic acid and caffeic acid. The results of the 24 hour methyl ferulate incubation with Caco-2 cells support the finding of the work by Kern et al. (2003) because methyl ferulate was able to be hydrolysed by intracellular and extracellular esterase enzymes synthesised by Caco-2 cells. The results of this study also provided evidence that hydrolysis of the compound predominantly occurs via intracellular or membrane- associated enzymes as a higher percentage of methyl ferulate (57.1%) was hydrolysed when directly incubated with cells for 24 hours, compared to the percentage hydrolysed (0.25%) when incubated for 24 hours in medium that had been previously incubated with Caco-2 cells then removed, as only extracellular secreted enzymes would have been present in pre-incubated medium. It was due to these findings that only direct Caco-2 cell incubations were performed in subsequent experiments during this study, as the bioavailability of CGA metabolites due to extracellular hydrolysis in the small intestine appears to be small.

The results of the 2 hour incubations of Caco-2 cells with methyl esters, methyl ferulate and methyl caffeate, and the two CGA isomers, 5-O-caffeoylquinic acid and 3-O-caffeoylquinic acid, show that methyl esters were better able to be hydrolysed by esterase enzymes from Caco-2 cells as HPLC-DAD detection of their respective metabolites showed that in total 19.8% and 11.4% of methyl ferulate and methyl caffeate were hydrolysed respectively. In comparison, 0.02% of 5-O-caffeoylquinic acid and 0.18% of 3-O-caffeoylquinic acid were hydrolysed to release a free molecule of their respective HCA. The ability of esterase enzymes to hydrolyse the compounds appears to be methyl ferulate > methyl caffeate > 3-O-caffeoylquinic acid > 5-O-caffeoylquinic acid.

The difference in the ability of the esterase enzymes synthesised by Caco-2 cells to hydrolyse the phenolic acid methyl esters and caffeoylquinic acids could be due to the different lipophilicities of the different compounds which would affect their interaction with the enzymes and the mechanism by which they are transported across the intestinal epithelium.

Table 2. Lipophilicity Log D vaules of phenolic acid esters at pH 7.4

Phenolic acid ester	Lipophilicity Log D at pH7.4
Methyl ferulate	2.05
Ferulic acid	-1.52
Methyl caffeate	1.90
5-*O*-caffeoylquinic acid	-3.58
3-*O*-caffeoylquinic acid	-3.58
Caffeic acid	-1.71

Table 2 shows the different Log D values of the test compounds investigated during this study. The more positive the Log D number, the more lipophilic the

molecule is, and the more negative the Log D value the less lipophilic and more hydrophilic the molecule is. The methyl esters, methyl ferulate and methyl caffeate, have the highest and second highest Log D values respectively out of the compounds investigated, so are the more lipophilic compounds. This means that they are more able to interact with the cell membrane and enter the interior of the cells via a passive transcellular route (Kern *et al.* 2003). The less lipophilic caffeoylquinic acid isomers will not have the same interection with the membrane and so the absorption mechanism across the epithelium is likely to be via paracellular transport, and so would require the esterases to be located on the Caco-2 cell membrane (Farrell *et al.* 2011). This supports the findings that esterase activity is localised predominantly intracellularly as a higher percentage of the more lipophilic compounds that are transported transcellularly were hydrolysed to their HCA constituent.

Of the two caffeoylquinic acid isomers, the percentage of 3-O-caffeoylquinic acid that was hydrolysed was nine times greater than the percentage of 5-O-caffeoylquinic acid hydrolysed. As the Log D values of the isomers are the same, the difference in hydrolysis is not due to lipophilicity. Instead it could be due to the structural conformations of the isomers. The different position of the ester link between the quinic acid molecule and caffeic acid (Fig. 1) in the two molecules suggests that 3-O-caffeoylquinic acid is better able to fit into the active site of the enzyme and undergo hydrolysis than 5-O-caffeoylquinic acid.

The results of the incubation with rat intestinal tissue extract show that methyl ferulate was able to be hydrolysed to release ferulic acid by the esterase enzymes present in the tissue, however after incubation with 5-O-caffeoylquinic acid no metabolites were detected, suggesting that it is not a substrate for rat esterase enzymes. This is likely due to the structure of the molecule not being able to interact with the enzyme active site.

In conclusion, the results of this study support earlier work that found that Caco-2 cell esterases can hydrolyse phenolic acid methyl esters, and identifies that 3-O-caffeoylquinic acid and 5-O-caffeoylquinic acid are also substrates for these enzymes. Hydrolysis was shown to be predominantly intracellularly based. As Caco-2 cells are commonly used as an in vitro model of the human small intestine (Press and Di Grandi 2008), these results suggest that the human small intestine could be a site of the metabolism of CGAs found in coffee and contributes to the bioavailability of the HCA metabolites. With regards, to the incubation of methyl ferulate and 5-O-caffeoylquinic acid with rat intestinal tissue extract, esterase enzymes present in the tissue clearly can hydrolyse ester links as ferulic acid was detected after 1 and 2 hour incubations of methyl ferulate, however 5-O-caffeoylquinic acid appears not to be a substrate for the enzyme. Further work could be undertaken to assess the ability of the rat intestinal tissue esterases to hydrolyse other isomers of caffeoylquinic acid found in coffee.

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