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Effect of edible oils on quercetin, kaempferol and galangin transport and conjugation in the intestinal Caco-2/HT29-MTX co-culture model

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Abbreviations:

CO: coconut oil; DAD: diode array detector; DMEM: Dulbecco's modified Eagle medium; HBSS: Hanks's balanced salt solution; LOD: limit of detection; LOQ: limit of quantification; MRM: multiple reaction monitoring; MS: Mass spectrometry; OO: olive oil; PAPS: 3'-phosphoadenosine 5'-phosphosulphate; SO: soybean oil; SULT: sulphotransferase; TEER: trans-epithelial electrical resistance; UDPGA: uridine-5'-diphospho-glucuronic acid; UGT: Uridine diphosphate glucuronosyl transferase; UV: ultraviolet.

Abstract

Solubility and matrix play an important role in the gut lumen in delivering bioactive compounds to the absorptive surface of enterocytes. The purpose of this study was to determine the effect of certain commonly consumed lipids, soybean, olive and corn oil, on the transport and conjugation of flavonols (myricetin, quercetin, kaempferol and galangin) using the conjugation-competent co-cultured Caco-2/HT29-MTX intestinal cell monolayer model. To enable identification and quantification of conjugates, each flavonol was enzymatically glucuronidated or sulphated, then analysed by HPLC with triple quadrupole mass spectrometric detection. Quantification showed large differences in mass spectrometric peak area response factors between the aglycones and many of the conjugates, with galangin-sulphate for example ionising ~15-fold better than galangin. Flavonol aglycones and conjugates were transported to the basolateral side of Caco-2/HT29-MTX co-cultures. The total amount of methyl, sulphate and glucuronide conjugates was in the order: Galangin > quercetin > kaempferol > myricetin. All oils inhibited the transport and conjugation of galangin, the most hydrophobic flavonol, whereas they increased the sulphation, and to some extent glucuronidation, of quercetin and kaempferol. The results show that the lipid matrix has the potential to modify both transport and conjugation of dietary flavonols, but that the effect depends upon the structure and hydrophobicity.

Introduction

Flavonols are a sub-group of dietary flavonoids consisting mainly of myricetin, quercetin, kaempferol and galangin, containing 3, 2, 1 and 0 hydroxyl groups in the B-ring structure respectively. Galangin is the least soluble of the series in aqueous solution with the highest octanol-water partition coefficient [1]. Galangin is quite rare in most diets, but is found at high levels in oregano [2] and in the food supplement propolis, mostly in the aglycone form. Kaempferol is found in cumin and capers for example, as glycoside but also with the aglycone at high level in capers ([2] and unpublished results). Quercetin is found in many commonly consumed foods, such as onion, apple and tea, predominantly as glycosides and myricetin is found blackcurrants for example, again mostly as the glycoside [2]. Before absorption across the enterocytes in the intestine, any glucosylated flavonols are hydrolysed to free aglycone by brush border lactase phloridzin hydrolase [3], and consequently the aglycone is the main chemical form which passes into the enterocyte, where conjugation with methyl, sulphate or glucuronide groups can occur [4]. Flavonols, particularly quercetin, are reported to be extensively glucuronidated, sulphated and methylated, with 23 identified metabolites of quercetin in human plasma and urine in vivo [5]. These reactions are important since the position and nature of conjugation dramatically affects the potential biological activity in the human body [6].

In epidemiological studies, dietary flavonol intake has been associated with lower risk of cardiovascular diseases [7] and colorectal cancer [8] in certain groups of the population. The biological effects of flavonols are, however, dependent upon the extent to which they are absorbed, metabolised and excreted as well as the possible biological activities of their circulating conjugates and metabolites in the body. Moreover, a proper analysis of flavonol conjugates has been hampered by the lack of availability of standards, making quantification difficult. Previously this has been done by direct comparison to the aglycone, leading to

potential over-estimation (or possible under-estimation) of the conjugates when analysed by mass spectrometry, currently the most commonly used technique.

The presence of other dietary components can also affect absorption, and lipids have been reported to have a major influence on absorption of quercetin in pigs [9] and rats [10]. However, the effect of lipids in the gut lumen on conjugation of flavonoids by enterocytes has not been widely reported. Coconut oil, olive oil and soybean oil contain fatty acids which represent a high proportion of saturated, monounsaturated and polyunsaturated fatty acids, respectively [11], and these could conceivably affect absorption and conjugation in different ways. In pharmaceutical studies, various excipients and additives can enhance the absorption of poorly soluble drugs [12], and so lipids could have a comparable effect on flavonol transport and conjugation. We tested this hypothesis using a well studied conjugation-competent monolayer Caco-2/HT29-MTX co-cultured cell system as a model for intestinal conjugation of flavonoids.

Materials and methods

Chemicals and materials.

Myricetin, quercetin, kaempferol, kaempferol-3-O-glucuronide, isorhamnetin, galangin and daidzein were purchased from Extrasynthese (Genay, France). Quercetin-3'-O-sulphate, quercetin-7-O-glucuronide, quercetin-3-O-glucuronide, quercetin-3'-O-glucuronide and isorhamnetin-3-O-glucuronide were enzymatically synthesised using human liver S9, purified by HPLC, and characterised by Dr C. C. Wong as described previously [13,14] . The cofactor 3'-phosphoadenosine-5'-phosphosulphate (PAPS) was obtained from MP Biochemicals (London, UK). Periodic acid, phosphate buffered saline 1X (PBS1X), HPLC mobile phase acetonitrile (LCMS grade) and Schiff's reagent were obtained from Fisher Scientific (Loughborough, UK). The culturing flasks (T25 cm^2 and T75 cm^2) and Transwell® plates with polycarbonate semi-permeable membranes of 0.4 µm pore size and 4.7 cm^2 surface area were obtained from the Corning Costar Corp. (Cambridge, MA). A Milli-Q water purification system (Millipore UK Ltd, Hertfordshire, UK) was used throughout the experiments to provide ultrapure water (≥18.2 MΩ cm at 25^{o}C). All other chemicals unless otherwise stated were purchased from Sigma-Aldrich.

Glucuronidation and sulphation of flavonols by rat liver S9 homogenates.

Glucuronidation and sulphation of flavonols was performed according to the protocol modified from Wong et al. using pooled S9 homogenate from rat liver [15] . The reaction was started by the addition of flavonols from 100 mM stock solution prepared in DMSO (final concentration of 500 µM) into the solution containing co-factor and homogenate. Samples were incubated in a 37^{o}C water bath for 1 h, except 30 min for myricetin. At the end of the incubation time, the reaction was stopped by the addition of 100 µL of ice cold acetonitrile and centrifuged at 17,000 x g for 5 min. The supernatant was removed and dried under

centrifugal evaporation (Genevac, EZ-2 Plus, Suffolk, UK). Dried samples were reconstituted with 100 µl of 50% ethanol containing 20 µM daidzein as internal standard. Controls were treated under the same condition and consisted of samples without substrates, co-factor or homogenates.

HPLC-DAD-MS^2 optimisation and characterisation

HPLC analyses were performed using an Agilent 1200 series LC system connected to a photodiode array detector and Agilent 6410 triple quadrupole-MS with electrospray ionisation (Dorset, UK). Five µL samples, dissolved in 50% ethanol in water (50:50, v/v), were injected into the column and chromatographic separation was achieved using an Eclipse Plus C18 column (2.1 mm x 100 mm, 1.8 µM) at 30^{o}C. The mobile phase consisted of pre-mixed 5% acetonitrile in water (5:95, v/v) as solvent A and 5% water in acetonitrile (5:95, v/v) as solvent B. Both solvents contained 0.1% formic acid. Samples were eluted at a flow rate of 0.26 ml/min with 80 min gradient initiated at 0% of solvent B and increased linearly to 15% in 10 min. Then, solvent B was gradually increased to 25% over the next 30 min, then up to 60% B over 20 min. At 63 min, solvent B was immediately increased to 100% and maintained at this percentage for 5 min before the column was re-equilibrated to the initial starting condition for 12 min. UV detection was carried out between 240 and 400 nm using a photodiode array detector.

Further identification of various flavonol conjugates was using an Agilent 6410 triple quadrupole -S coupled in-line with the HPLC system described above. The negative electrospray ionisation mode was used to ionise compounds eluting from the column. Nitrogen was used both as drying and nebulising gas at a flow rate of 11 l/h and pressure of 30 psi respectively. The source temperature was maintained at 350^{o}C with capillary voltage set at 4 kV. Fragmentor voltage and collision energy were optimised for each authentic

standard to maximise the sensitivity of the acquisition method. The instrument parameter optimisations were conducted similarly for glucuronidated and sulphated flavonols to determine the best acquisition setting to produce the optimum signal response. A full scan mode was carried out to confirm the precursor ion of the flavonol conjugates prior to the optimisation. Besides the retention time, glucuronidated, sulphated and aglycone forms of flavonols can be characterised by selective precursor ion in negative mode, with the two most intense corresponding product ions used as quantifier and qualifier. Finally, all of the flavonol conjugate MRM transitions were adjusted dynamically throughout the chromatographic run by selecting specific transitions with relevant retention time windows. This implied that in dynamic MRM mode, analytes are only monitored while they are eluting from the column and the MS duty cycle is not wasted by monitoring them when they are not expected.

Linearity was determined by preparing mixtures of flavonol authentic standards in 50% ethanol in water (50:50, v/v) over the concentration range of 25 nM to 25 µM. Two internal standards (20 µM), daidzein (IS1) and sinapic acid (IS2), were added to keep track of the instrument stability over time to ensure good data quality in terms of retention time and ion response. Peak area response of the major MS^2 product ion (base peak) was plotted against the concentration, based on triplicate injections of each concentration, and the resulting correlation coefficient (R^2) was used to judge linearity.

The intraday precision and accuracy were calculated from triplicate injections of flavonol authentic standards on the same day and the inter-day precision and accuracy were determined by analysis of three injections on three separate days. Three quality control concentrations of quercetin and its conjugates (quercetin-3'-O-sulphate and quercetin-3'-O-glucuronide) were chosen at 50, 500 and 5000 nM. Values for precision are expressed as relative standard deviation of the mean at each concentration level and accuracy determined

by comparing the calculated concentration to the known concentration. For each standard, the limits of detection and quantification were determined by calculation of the signal (peak height) to noise ratio at a level of three and ten times respectively using Agilent MassHunter Workstation Software (Qualitative analysis, version B.01.03).

Conjugate response factors on HPLC-DAD and HPLC-DAD-MS^2

Generally, the signal from the instrument detector measured by peak area or peak height is corrected by the response factor to give an indication of the amount of compound detected. In this study, the response factors of available authentic standards were analysed at 5 µM under the conditions detailed above. The peak area ratio of conjugated metabolite to the corresponding aglycone was identified at the maximum wavelength and data are presented as relative peak area. Likewise, the relative response factors in MRM mode were calculated from the peak area of conjugated metabolite MS^2 base peak ion compared to the peak area of aglycone MS^2 base peak ion. For authentic standards, the response factor was calculated directly from a known concentration. The response factor of conjugated flavonols from synthesis experiments was calculated by making the assumption that all isomers of glucuronides, and of sulphates, formed for each flavonol would have an equal response factor, regardless of the conjugation position. Although the response factors for each substitution position will not be exactly the same, they will be relatively close. The validity of this assumption is supported by our previous data [16] on hydroxycinnamic acids analysed by HPLC-MS^2. Here the response factors relative to the aglycone were very similar for the 3 and 4 isomers of caffeic, and of dihydrocaffeic, acids: caffeic-acid-3-O-sulphate and caffeic-acid-4-O-sulphate, 1.9 and 2.5 compared to caffeic acid respectively; dihydrocaffeic-acid-3-O-sulphate and dihydrocaffeic-acid-4-O-sulphate, 10.3 and 10.7 compared to dihydrocaffeic acid respectively; dihydrocaffeic-acid-3-O-glucuronide and dihydrocaffeic-acid-4-O-

glucuronide, 1.8 and 2.2 compared to dihydrocaffeic acid respectively. The total yield of conjugated flavonols in each reaction was carefully estimated compared to parallel controls with no cofactor and homogenate, and these calculations allowed us to estimate the yield and hence response factors for each group of conjugates.

Cell culture

The human colorectal adenocarcinoma cell line, Caco-2 (HTB-37), was obtained from the American Type Culture Collection at passage 25 (LGC Promochem, Middlesex, UK). Caco-2 cells were maintained in 75-cm^2 culture flask in DMEM supplemented with 15% heat inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin and 0.25 µg/ml amphotericin B. The HT29-MTX cells were elaborated and kindly donated by Dr Thecla Lesuffleur (INSERM, France) at passage 17. The medium for cell cultivation consisted of DMEM containing 10% heat inactivated FBS and 100 U/ml penicillin/streptomycin. Both cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37^oC. The passage number used for the experiments was between 38-48 and 25-35 for Caco-2 and HT29-MTX cells respectively.

Caco-2 and HT29-MTX co-cultured cells were used for the experiments. This co-cultured cell monolayer provides both enterocyte and goblet cells which are the two major phenotypes of the human intestinal epithelium and provide a high capacity for glucuronidation and sulphation. Both cells were seeded together on a 6-well Transwell® plate with polycarbonate membrane pore size of 0.4 µm, membrane diameter of 24 mm and a growth area of 4.67 cm^2. About 6 x10^{-4} cells per cm^2 were seeded on the insert with 76% of Caco-2 and 24% of HT29-MTX cells according to previous practice [15]. Co-cultured cells were allowed to grow and differentiate in DMEM supplemented with 10% FBS, 100 U/ml penicillin/ streptomycin and

0.25 µg/ml amphotericin B. The medium was changed three times per week and cells were cultured for 21 days.

The same passage number of cells were seeded together (6 $x10^{-4}$ cells per cm^2: 76% of Caco-2 and 24% of HT29-MTX cells) in one-well Lab-TEK glass chamber slides to confirm the mucus production by HT29-MTX cells. Co-cultured cells were grown for 21 days in 10% FBS supplemented medium as above which was changed every other day. The mucus was detected using Periodic Acid-Schiff's/Alcian-Blue staining protocol as described elsewhere [17].

Transport study

The effect of coconut oil, olive oil and soybean oil was investigated on flavonol transport across the monolayer. The oil "solution" was prepared as follows: An aliquot of flavonol stock solution and 0.1 g oil were added to warm modified HBSS (37^{o}C, pH 7.4) solution. The solution was vortex-mixed for 5 min, homogenised at the highest speed for 5 min followed by sonication for 10 min. The volume was adjusted to 10 ml. The pH was adjusted to 7.4 with 1 M NaOH or HCl. The final concentration of flavonol aglycones in all control and treatment solutions was ~40 µM.

Prior to the experiments, the culture medium was replaced by fresh medium for at least 12-24 hours. Transport experiments were carried out with transport buffer solution consisting of HBSS supplemented with 1.8 mM $CaCl_2$ (to maintain integrity of the tight junction) and 100 µM ascorbic acid (to minimise degradation of flavonols). Before the experiments were started, medium was aspirated and rinsed three times with transport buffer (modified HBSS, pH 7.4). Approximately 2 ml of modified HBSS were placed in both apical and basolateral sides and left in the incubator (37^{o}C with 5% CO_2 / 95% O_2) for 30 min to equilibrate the pH level of the cell monolayers to pH 7.4. The pH of both compartments was adjusted to 7.4.

After pH equilibration, the HBSS was carefully aspirated to waste and the apical compartments were exposed to 2 ml of 40 µM flavonol aglycones which contained mixtures of myricetin, quercetin, kaempferol and galangin at the same concentration prepared with various additional components in transport buffer solution specifically: 1% of coconut oil (CO), 1% olive oil (OO) or 1% soybean oil (SO). Apically loaded 40 µM flavonols prepared in modified HBSS were used as a control. The basolateral compartment was filled with 2 ml of modified HBSS pH 7.4 and an equivalent amount of DMSO (0.4%). For blank transport control, both the apical and basolateral compartments contained transport buffer solution (modified HBSS: 1.8 mM $CaCl_2$, 100 µM ascorbic acid and 0.4% DMSO, pH 7.4). Flavonol transport from apical to basolateral sides was performed in triplicate at 37^{o}C in a humidified atmosphere. Upon termination of the 120 min incubation, samples from the basolateral side were collected and immediately frozen at -80^{o}C until further analysis. The integrity of the cell monolayer was checked before and after the experiments by measuring trans-epithelial electrical resistance (TEER) values using a Millicell ERS volt/ohm meter (Millipore, Zug, Switzerland). To obtain the true resistance value, the readings across the polycarbonate inserts with cells were corrected against the resistance value of blank polycarbonate insert. No statistically significant difference was observed on the TEER value of each test condition and control after 120 min incubation time.

Samples from transport experiments were defrosted at room temperature and solutions (300 µl) were added to the same volume of ethanol containing daidzein (IS1, final concentration 2 µM) as an internal standard. Samples were vortexed and centrifuged at 17000 x g for 10 min. An aliquot of supernatant was transferred to a clean Eppendorf tube and dried under centrifugal evaporation. Dried samples were reconstituted with 50% ethanol in water (50:50, v/v) containing sinapic acid (IS2, final concentration 2 µM), and vortex mixed before being centrifuged for 10 min at 17000 x g. Samples were analysed using the HPLC-DAD-MS^2

method described earlier. The quantification of flavonol metabolites in media solutions was based on the peak area at respective MS^2 base peak ions in MRM mode. The concentration of flavonol aglycones and metabolites were quantified for both of the apical and basolateral compartments, with the exception of apical solutions in the presence of edible oils since flavonols could not be quantitatively extracted from these oils.

Data analysis

The assumption of normality of the data sets and equality of variance was confirmed by Shapiro-Wilk and Levene's test statistics respectively. Statistical differences between control and all treatments of oil on flavonol transport to basolateral compartment were investigated by analysis of variance, followed by a multiple comparison test (Tukey HSD) to compare all treatment conditions to control. The statistical significance was set at the 5% level (SPSS version 18).

Results

Optimisation of MS parameters and characterisation of flavonol conjugates using HPLC-DAD-MS^2

The mass spectrometric behaviour of all authentic standards and enzymatically synthesised metabolites of flavonols using negative-ion ESI are shown in Table 1. Typical HPLC-DAD-MS^2 chromatograms of enzymatically-synthesised flavonol conjugates are shown in Fig. 1. Quercetin-derived peaks Q1, Q2, Q3 and Q4 exhibit [M-H^-] at m/z 477 in MRM mode, corresponding to quercetin monoglucuronides, and each was confirmed by MS^2 product ions at 301and 151. The positional substitution of three isomers was confirmed by the Rt and absorbance spectra of authentic standards: quercetin-7-O-glucuronide (Q1=17.4 min), quercetin-3-O-glucuronide (Q2=17.8 min) and quercetin-3'-O-glucuronide (Q5=24.4 min). Since conjugation at the 5-position is reported to be very unlikely unless it is the only hydroxyl available [18], Q3 is tentatively assigned as quercetin-4'-O-glucuronide. The availability of authentic standards confirmed peak K2 as kaempferol-3-O-glucuronide (20.9 min) and Q6 as quercetin-3'-O-sulphate. The analyses above gave an indication of the nature of the conjugation, and in some cases, the position of conjugation when authentic standards were available. Where the latter were not available, further identification was supported by the use of shift reagents and relative retention orders [19]. Therefore, on the basis of the previous evidence on quercetin Band I λ_{max} shift patterns, one of three conjugates produced by kaempferol and galangin could be identified as kaempferol-3-O-glucuronide and galangin-3-O-glucuronide respectively. Peak Q4 was tentatively assigned as quercetin-7-O-sulphate (Table 1).

Linearity, precision and accuracy of flavonol authentic standards

Calibration curves were constructed at seven concentrations for each available authentic standard and were linear over the concentration range. A correlation coefficient of 0.99 or higher was obtained for the relationship between the peak area ratios of the analyte/internal standard and corresponding calibration concentration. With regard to accuracy, the LC method used was evaluated by both intraday and inter-day performance; the relative error for all quercetin conjugates were good with values obtained less than 5.4, 6.0 and 0.3% for 50, 500 and 5000 nM concentrations respectively. Good precision was demonstrated for all quercetin standards across the range of quality control concentration for both intraday and inter-day. Relative standard deviation values for quercetin were <7.8%, quercetin-3'-O-sulphate was <9.5% and quercetin-3'-O-glucuronide was <14.4%. For each standard, LOD and LOQ values were deduced from the signal to noise ratio of peak height at their MS^2 base peak ion. The flavonols provided good sensitivity in the negative mode ionisation with the LOQ values ranging from 10 to 50 nM for the conjugated flavonols and >50 nM for the aglycones.

Conjugate response factors on HPLC-DAD and HPLC-DAD-MS^2

Table 1 shows the response factors of conjugated flavonols in relation to their parent aglycones. Based on measurement of the peak area at respective maximum wavelength, the majority of the conjugated metabolites gave relative response factors in the range 0.6 to 1.2, when the aglycone response was normalized to 1. Quercetin glucuronides showed comparable relative values to the aglycone when the response from the UV absorption measurement was compared to MS^2. The response factor of quercetin-3'-O-sulphate and kaempferol-3-O-glucuronide compared to aglycones by MS^2, however, increased by 2- and 10-fold respectively. It is important to note that the estimation of relative response factor of

conjugated flavonols based on synthesis experiments revealed significant differences compared to the aglycone, but when the authentic standard was available, the agreement was good, supporting the methodology and validity of the assumptions made above.

Metabolism and transport of flavonols by Caco-2/HT29-MTX co-culture monolayers in the absence of oils

Using the Caco-2/HT29-MTX co-cultured cell monolayer, the metabolism of flavonols was investigated. Apical to basolateral transport of flavonols was evaluated using a mixture of myricetin, quercetin, kaempferol and galangin. Fig. 2 shows the typical MRM chromatograms of flavonol conjugates detected in the basolateral compartment after addition of flavonol aglycones on the apical side. Quercetin-3 ' -O-sulphate, quercetin-3-O-glucuronide, quercetin-3 ' -O-glucuronide and quercetin-4 ' -O-glucuronide together with isorhamnetin, but only trace amounts of isorhamnetin glucuronide, were detected together with kaempferol-7-O-sulphate and kaempferol-3-O-glucuronide (Table 2). Kaempferol-4 ' -O-glucuronide was only detected in the basolateral compartment, and at low concentration. Galangin generated the highest amount of conjugates whereas no conjugated myricetin was detected in any of the compartments, although myricetin aglycone was transported to the basolateral side. Overall, the summed total of aglycone and corresponding conjugates appearing in the apical and basolateral compartments together accounted for ~70%, 80%, 78% and 73% of the initial amount of myricetin, quercetin, kaempferol and galangin applied respectively showing good stability and yield in these experiments.

Effect of oil matrices on the metabolism of flavonols

The oils tested her did not affect the tight junction of the monolayer as measured by TEER. This is in agreement with data on soybean oil which was non-cytotoxic to Caco-2 cells and

the cell monolayer integrity remained intact even after a four hour incubation [20]. The effect of soybean, olive and corn oils at the apical side was investigated on flavonol transport (Fig. 3a). For quercetin, all of the oils significantly increased the production of conjugates in the basolateral compartment, with, for example, a 2-3-fold increase in quercetin-3′-O-sulphate ($p<0.05$). Soybean oil also enhanced the production of quercetin glucuronides, and basolateral kaempferol-7-O-sulphate was also increased up to 4-fold (Fig. 3b). All oils at the apical side hindered the absorption and conjugation of galangin (Fig. 3c). For example, galangin transport was almost completely inhibited when co-incubated with coconut oil with more than 80% reduction of all galangin conjugates.

Discussion

In this study, the generation of multiple glucuronidated and sulphated forms of flavonols was achieved by enzymatic synthesis with rat liver S9 homogenate, with characterisation using HPLC-DAD-MS^2 and shift reagents, supported by previous identification of some of the compounds [13,19,21] and availability of some authentic standards. For the first time, sulphated myricetin was produced, with two isomers detected. Careful estimation of the yield relative to parallel controls allowed estimation of response factors and quantification, which agreed well with authentic standards when available. Previously we found that estimations of conjugated phenolic acids based on the corresponding aglycone calibration curve were inaccurate due to large differences in relative response, especially on HPLC-DAD-MS^2 in the MRM mode [16]. In a similar way, the response factors of flavonol sulphates and glucuronides were between 2-20 and 1-10 times greater than the parent aglycone respectively. Using this information, we were then able to more accurately determine and quantify metabolism in the intestinal cell model. These values are presented in Table 1 and can be used as a guide for future in vivo and in vitro studies.

Co-culture of HT29-MTX and Caco-2 cells together provide a more balanced metabolic system with higher sulphation ability than Caco-2 cells alone. The system has been used previously to examine metabolism of phenolics, where the metabolism of ferulic acid is mainly by sulphation both in vivo [22] and in the co-cultured cellular system [17]. These conjugation properties are due to the distribution and expression of conjugating enzyme isoforms. The human sulphotransferases SULT1A1 and 1A3 are active on phenolics [23] and are both well expressed in HT29 cells [24]. On the other hand, Caco-2 cells highly express UDP-glucuronosyl transferases UGT1A1, 1A3, 1A6 and 2B7 [25] and also UGT1A8 [26]; UGT1A1, 1A8 and 1A9 were particularly active on quercetin [18]. Both flavonol aglycone and

substantial amounts of conjugates were transported across Caco-2/HT29-MTX co-cultures monolayers. The least hydrophilic flavonol, galangin, produced the highest level of conjugates, followed by kaempferol and then quercetin, in general consistent with Caco-2 cells alone [27]. The overall yield of the flavonol aglycone and conjugates was 70-80%, indicating good stability in the medium over the incubation period, similar to that found for the flavonoid hesperetin [28]. No myricetin conjugates could be detected, even though we were able to synthesise enzymatically the conjugates and use them as standards in the analysis. The possible reasons for this may be that the conjugating enzymes in the human intestinal cells are not active on myricetin, that conjugates are formed but are not substrates for the cellular efflux transporters and so do not reach the cell culture medium, or that intracellular myricetin conjugates are further metabolised to other compounds which are then not detected in our system.

In pharmaceutical research, oily dispersions are often used to increase the absorption of hydrophobic drugs. This may be due to an increase in solubility, but also in membrane fluidity facilitating transcellular absorption, opening of tight junctions to allow paracellular transport, inhibition of p-glycoprotein (ABCB1) and/or cytochrome P450 to increase intracellular concentration, and residence time, as well as stimulation of lipoprotein/chylomicron production [29]. However, here we found that transport and conjugation of the most hydrophobic flavonol, galangin, was substantially decreased by all of the oils irrespective of whether they had a high proportion of saturated, monounsaturated and polyunsaturated fatty acids. This might be related to the decrease of the free fraction of galangin available for transcellular permeation. This has a parallel in drug transport. Hydrophobic drugs such as digoxin were entrapped into micelle structures when prepared with solution containing 0.01% - 1% of Tween 80 and Cremophor EL, and so as the

concentration of surfactant increased, the amount of free digoxin was reduced and suggested the apparent permeability coefficient needed to be corrected for potential changes in the free drug concentration [30].

In contrast, the addition of oils showed a significant and substantial increase in the conjugation and transport of quercetin and kaempferol. Several mechanisms could account for this effect, but a possible explanation might be associated with the production of chylomicrons to facilitate the fraction of lipophilic compound being transported, which in vivo would be via the intestinal lymphatic pathway. Previous studies reported that the Caco-2 cell model has the ability to digest Tween 80 in the absence of pancreatin to liberate oleic acid, which was then used by the cells to enhance the basolateral secretion of chylomicrons [31]. Further, co-administration of olive oil and Tween 80 increased the formation of chylomicrons in the rat [32] and quercetin was transported into lymph after being metabolised in the gastrointestinal mucosa of rats [33,34]. In humans, a suspension in pure soybean oil increased the bioavailability of coenzyme Q10 compared to lipid formulations that included Tween 80 and/or phosphatidylcholine as surfactant. It was suggested that the formation of mixed micelles in the intestine might cause a reduction of drug solubilisation with the bile salts, thus decreasing the absorption of coenzyme Q10 [35].

5. Conclusions

In conclusion, the development of MS operating condition for individual flavonol metabolites facilitate the characterisation and quantification of conjugates present in in vitro transport study without prior enzymatic hydrolysis treatment. Data resulting from transport experiments showed that the aglycones were taken up and moderately metabolised, except

myricetin, into a number of sulphate and glucuronide conjugates when incubated with a monolayer of Caco-2/HT29-MTX co-cultured cells. The addition of oils with a high proportion of saturated, monounsaturated and polyunsaturated fatty acids decreased the transport of the most hydrophobic flavonol, galangin, but increased the transport/conjugation of quercetin and kaempferol.

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Legends for figures

Fig. 1: HPLC-MS^2 Chromatograms of flavonol conjugates after enzymatic synthesis. (a) Q1, Q2, Q3, Q5 = quercetin monoglucuronide (b) Q4, Q6 = quercetin monosulphate (c) quercetin aglycone (d) M1, M3, M5 = myricetin monoglucuronide (e) M2, M4 = myricetin monosulphate (f) myricetin aglycone (g) K2, K3, K4 = kaempferol monoglucuronide (h) K1, K5 = kaempferol monosulphate (i) kaempferol aglycone (j) G1, G2, G3 = galangin monoglucuronide (k) G4, G5 = galangin monosulphate (l) galangin aglycone. MRM scans are of flavonols and conjugates generated by incubation of individual flavonol aglycones (500 µM) with rat liver S9 homogenates (2 mg/ml) in potassium phosphate buffer (100 mM, pH 7.4) and UDPGA (1 mM) or PAPS (100 µM) after 30-60 min incubation at 37^{o}C.

Fig. 2: Typical MRM traces of flavonol metabolites detected in the basolateral compartment after incubation of flavonol aglycones applied to the apical compartment. IS1:m/z 223=sinapic acid, IS2:m/z 253=daidzein, P1-P3:m/z 477 for quercetin glucuronide, P4-P6:m/z 461=kaempferol glucuronide, P7:m/z 317=myricetin, P8-P10:m/z 445=galangin glucuronide, P11:m/z 381=quercetin sulphate, P12:m/z 365=kaempferol sulphate, P13:m/z 301=quercetin, P14:m/z 285=kaempferol, P15:m/z 315=isorhamnetin, P16:m/z 349=galangin sulphate, P17:m/z 269=galangin.

Fig. 3 Effect of surfactant and different types of oil in the apical compartment on flavonol transport across Caco-2/HT29-MTX co-cultured cell monolayers. (a) Quercetin (b) kaempferol (c) galangin. Control and various treatment solutions containing 40 µM flavonol mixtures were loaded in the apical compartment and incubated for 2 h at 37^{o}C. Data are

shown for the conjugates transported to the basolateral compartment and are given as a percentage change compared to the control. Control: HBSS transport buffer solution (1.8 mM $CaCl_2$ and 100 µM ascorbic acid); treatment well contained additional constituent(s) specifically CO: 1% of coconut oil, OO: 1% olive oil, and SO: 1% soybean oil. Values are mean ± SD (n=3). Significant difference to control ($p<0.05$) is indicated by an asterisk.

Fig. 4.

Pathways of transport and conjugation of flavonols in intestinal cells. ABCC2 is the efflux transporter MRP2; sul = sulphate; glcA = glucuronide.

Table 1: HPLC-DAD-MS^2 optimisation parameters and characterisation of flavonol conjugates

Flavonol conjugates	Log P_{est}	Rt (min)	λ_{max} (nm)	MS Optimised Parameter		$[M-H]^-$ (m/z)	$MS^{2\,a}$ (m/z)	LOQ (nM)	Relative peak area		
				Fragmentor Value (V)	Collision Energy (V)				λ_{max}	MRM mode	
			Band II/I						Standard [b]	Standard [b]	Synthesis [c]
Myricetin	0.04	22.8	253/372	120	20	317	151, 179	100	1.0	1.0	1.0
Myricetin-x-O-glucuronide (M1)		13.8	250/372	110	25	493	317, 151, 113				2.1
Myricetin-x-O-glucuronide (M3)	-1.9	18.1	250/371	110	25	493	317, 151, 113				2.1
Myricetin-x-O-glucuronide (M5)		23.1	255/371	110	25	493	317, 151, 113				2.1
Myricetin-x-O-sulphate (M2)	-0.3	17.6	250/375	110	25	397	317, 151				5.6
Myricetin-x-O-sulphate (M4)		21.8	253/372	110	25	397	317, 151				5.6
Quercetin	0.35	31.7	253/370	130	18	301	151, 179	50	1.0	1.0	1.0
Quercetin-7-O-glucuronide (Q1)	-1.53	17.4	255/370	130	20	477	301, 151	50	1.1	0.6	1.0
Quercetin-3-O-glucuronide (Q2)	-1.43	17.8	253/355	130	20	477	301, 151	25	1.2	1.0	1.0
Quercetin-4'-O-glucuronide (Q3)	-1.53	21.9	253/366	130	20	477	301, 151				1.0
Quercetin-3'-O-glucuronide (Q5)	-1.53	24.4	250/368	150	20	477	301, 151	50	1.2	0.6	1.0
Quercetin-7-O-sulphate (Q4)*	0.09	23.4	253/372	110	18	381	301, 151				2.1
Quercetin-3'-O-sulphate (Q6)	-0.08	29.3	253/367	110	18	381	301, 151	25	0.6	2.0	2.1
Isorhamnetin	0.61	44.3	255/370	130	18	315	300, 151	25	1.0	1.0	
Isorhamnetin-3-O-glucuronide	-1.17	22.3	255/350	120	18	491	315, 300	10	0.8	0.8	
Kaempferol	0.74	41.7	265/366	130	30	285	93, 187, 151	100	1.0	1.0	1.0
Kaempferol-3-O-glucuronide (K2)	-1.04	20.9	265/346	110	20	461	285, 113	10	0.6	12.2	10.0
Kaempferol-7-O-glucuronide (K3)	-1.14	21.7	265/366	110	20	461	285, 113				10.0
Kaempferol-4'-O-glucuronide (K4)	-1.14	23.0	265/364	110	20	461	285, 113				10.0
Kaempferol-3-O-sulphate (K1)*	0.59	19.3	260/356	100	30	365	285, 151				20.2
Kaempferol-7-O-sulphate (K5)*	0.03	29.2	263/368	100	30	365	285, 151				20.2
Galangin	1.13	54.0	265/360	150	25	269	169, 213	100	1.0	1.0	1.0
Galangin-3-O-glucuronide (G1)*	-0.65	27.0	264/-	110	18	445	269, 113				10.5
Galangin-7-O-glucuronide (G2)*	-0.75	33.9	265/360	110	18	445	269, 113				10.5
Galangin-5-O-glucuronide (G3)*	-0.75	37.5	255/351	110	18	445	269, 113				10.5
Galangin-3-O-sulphate (G4)*	0.98	38.3	255/347	100	25	349	269, 169				15.4
Galangin-7-O-sulphate (G5)*	0.69	45.7	264/360	100	25	349	269, 169				15.4

UV absorbance recorded between 240-400 nm. MS optimisation performed in selective ion monitoring (SIM) mode for the molecular ion $[M-H]^-$ and further detection of MS-MS product ions, [a] MS-MS product ions produced by fragmentation of [M-H]-, the first fragment listed is the major product ion and is assigned as the base peak. LOQ: Limit of quantification based on MRM mode selection of major MS-MS product ion (signal noise ratio>10) [b] Response factor of conjugated flavonols authentic standard in relation to their parent aglycone were calculated based on peak area at respective maximum wavelength and at respective base peak ion in MRM mode. [c] Response factor of synthesised flavonol metabolites in relation to their parent aglycone were calculated based on peak area at respective base peak ion in MRM mode. *G1, G2 and G3 positional assignments based on elution order [19]; K1 and K5 based on previous assignment from Caco-2 cell culture and ^{1}H-NMR [27]; K3 and K4 based on relative yield [36]; Q4 based on assignments by van der

Woude [21]; G4 and G5 based on UV spectra. Estimated log P values were calculated in silico using ChemDraw Pro v13. The values for myricetin conjugates are an average of the different conjugates possible since the exact conjugation position is not known.

Table 2: Conjugation and transport of flavonols by Caco-2/HT29-MTX co-cultured monolayers

Metabolite	Metabolite production (pmol/cm^2.min)	
	Apical	Basolateral
Quercetin	93.6 ± 4.6	17.4 ± 2.1
Quercetin-3′-O-sulphate	0.10 ± 0.01	0.22 ± 0.05
Quercetin-3-O-glucuronide	0.009 ± 0.002	0.09 ± 0.03
Quercetin-4′-O-glucuronide	n.d.	0.03 ± 0.01
Quercetin-3′-O-glucuronide	0.02 ± 0.01	0.06 ± 0.02
Isorhamnetin	1.35 ± 0.38	0.63 ± 0.08
Myricetin	89.6 ± 5.5	10.9 ± 2.8
Kaempferol	88.0 ± 4.9	18.9 ± 1.7
Kaempferol-7-O-sulphate	1.61 ± 0.33	1.41 ± 0.68
Kaempferol-3-O-glucuronide	0.09 ± 0.06	0.82 ± 0.13
Kaempferol-7-O-glucuronide	0.03 ± 0.02	0.05 ± 0.01
Kaempferol-4′-O-glucuronide	n.d.	0.06 ± 0.02
Galangin	83.9 ± 5.5	9.4 ± 2.2
Galangin-7-O-sulphate	1.70 ± 0.26	2.24 ± 0.46
Galangin-3-O-glucuronide	0.45 ± 0.21	2.42 ± 0.83
Galangin-7-O-glucuronide	1.01 ± 0.12	1.24 ± 0.21
Galangin-5-O-glucuronide	0.72 ± 0.22	1.30 ± 0.41

The co-cultures were incubated for 2 h in the presence of flavonol mixtures which contained myricetin, quercetin, kaempferol and galangin, with a loading concentration of 40 µM each at apical compartment. Both apical and basolateral compartment pH values were adjusted to 7.4. Analyses of the cell culture media were performed by HPLC-DAD-MS^2 and conjugates were confirmed by product ion in MRM mode. Values are mean ± SD (n=6). n.d.: not detected.

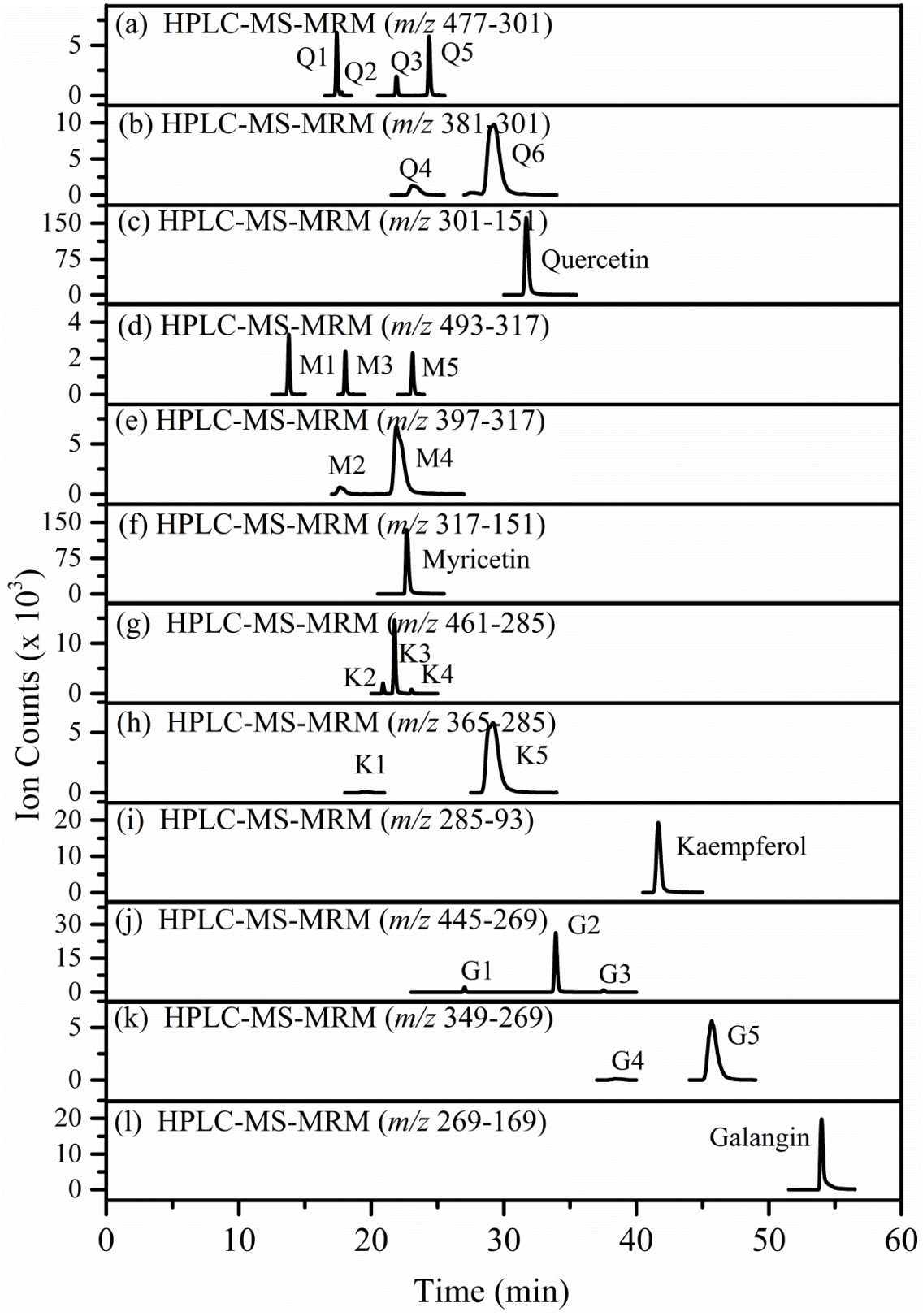
(a) HPLC-MS-MRM (m/z 477-301)
Q1
Q2
Q3
Q5
(b) HPLC-MS-MRM (m/z 381-301)
Q4
Q6
(c) HPLC-MS-MRM (m/z 301-151)
Quercetin
(d) HPLC-MS-MRM (m/z 493-317)
M1
M3
M5
(e) HPLC-MS-MRM (m/z 397-317)
M2
M4
(f) HPLC-MS-MRM (m/z 317-151)
Myricetin
(g) HPLC-MS-MRM (m/z 461-285)
K2
K3
K4
(h) HPLC-MS-MRM (m/z 365-285)
K1
K5
(i) HPLC-MS-MRM (m/z 285-93)
Kaempferol
(j) HPLC-MS-MRM (m/z 445-269)
G1
G2
G3
(k) HPLC-MS-MRM (m/z 349-269)
G4
G5
(l) HPLC-MS-MRM (m/z 269-169)
Galangin
Ion Counts (x 10^3)
Time (min)

Figure 1

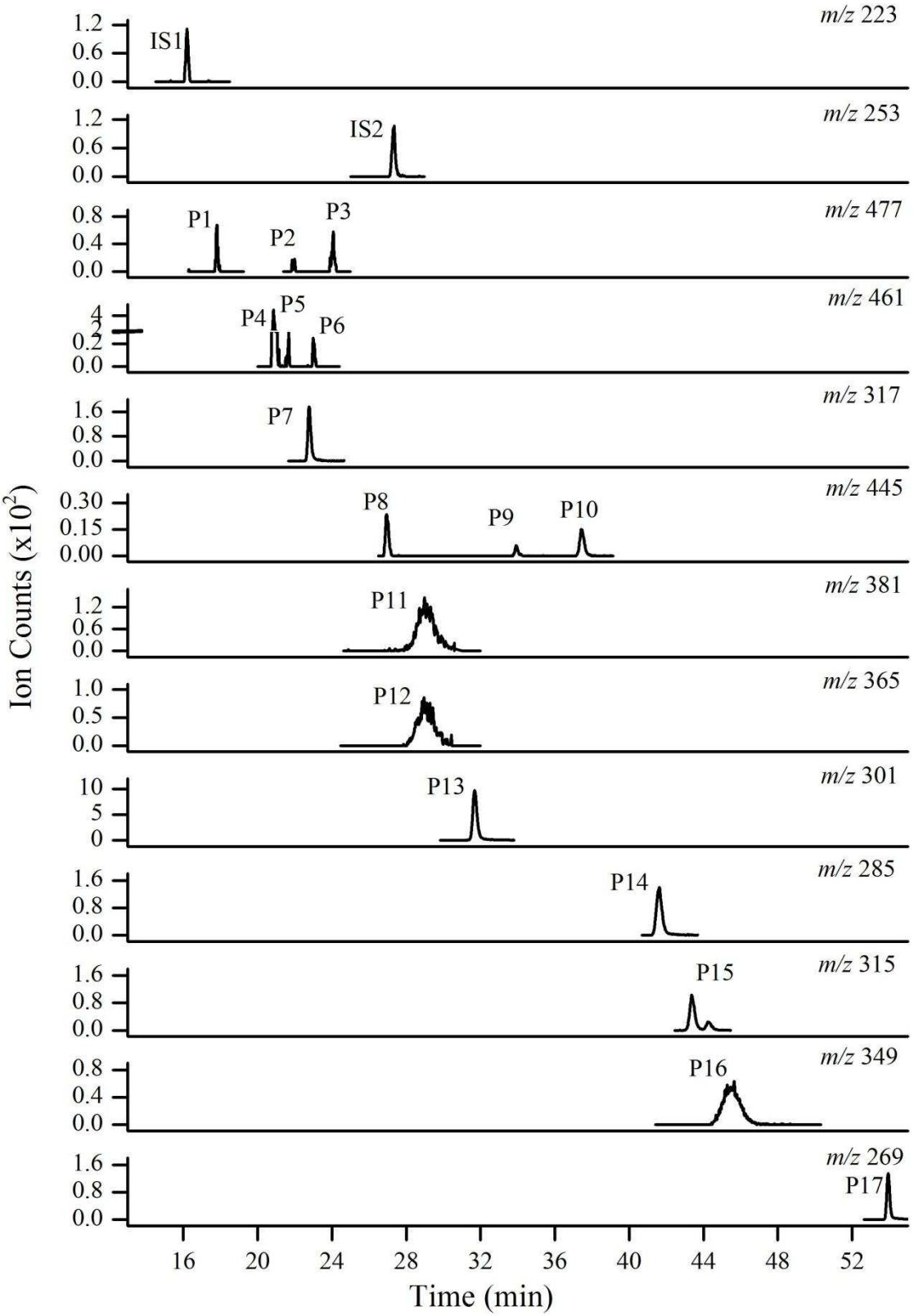
m/z 223
IS1
m/z 253
IS2
m/z 477
P1
P2
P3
m/z 461
P4
P5
P6
m/z 317
P7
m/z 445
P8
P9
P10
m/z 381
P11
m/z 365
P12
m/z 301
P13
m/z 285
P14
m/z 315
P15
m/z 349
P16
m/z 269
P17
Ion Counts (x10²)
Time (min)

Figure 2

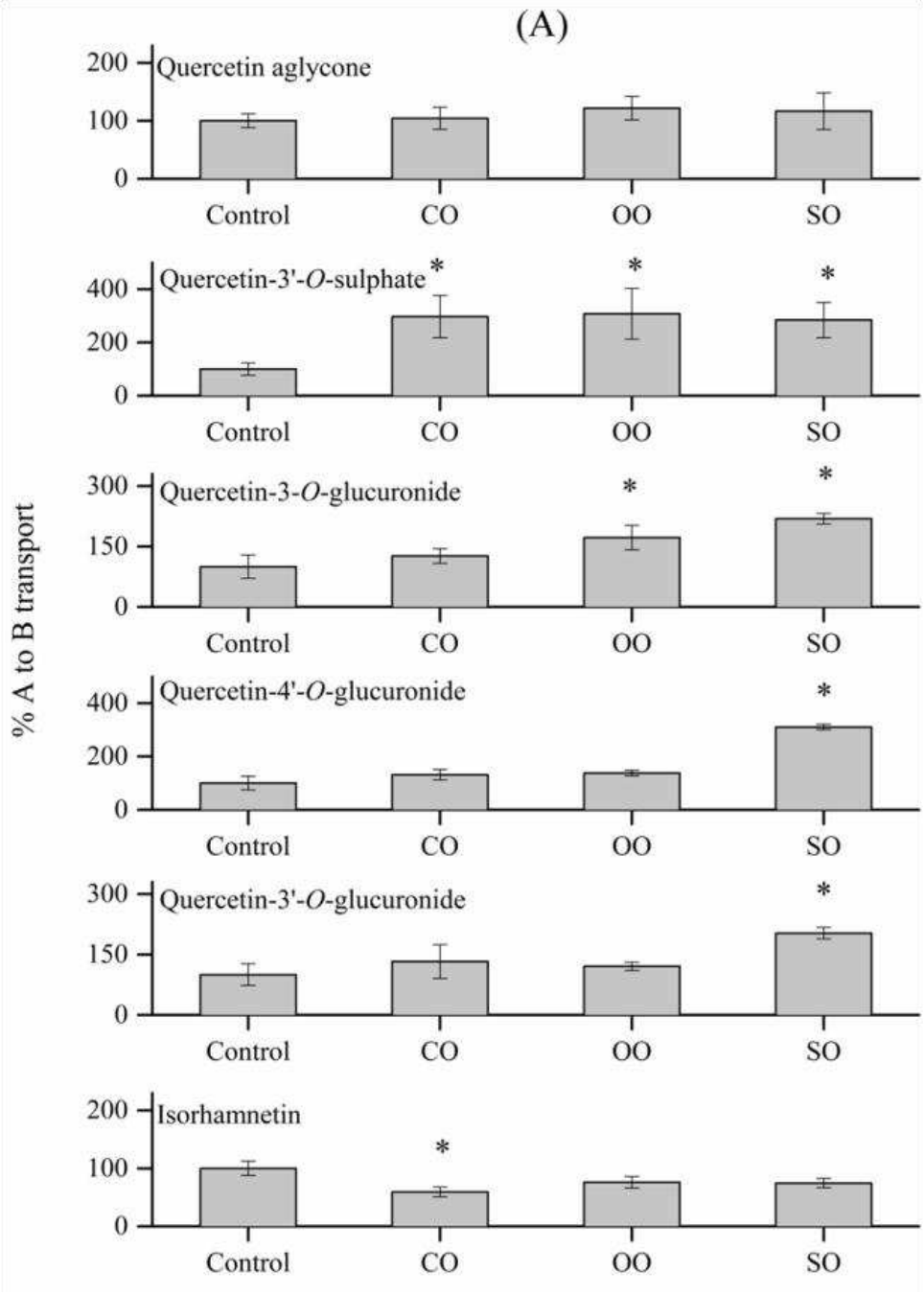
(A)
% A to B transport
Quercetin aglycone
Quercetin-3'-O-sulphate
Quercetin-3-O-glucuronide
Quercetin-4'-O-glucuronide
Quercetin-3'-O-glucuronide
Isorhamnetin
Control
CO
OO
SO
*

Figure 3A.

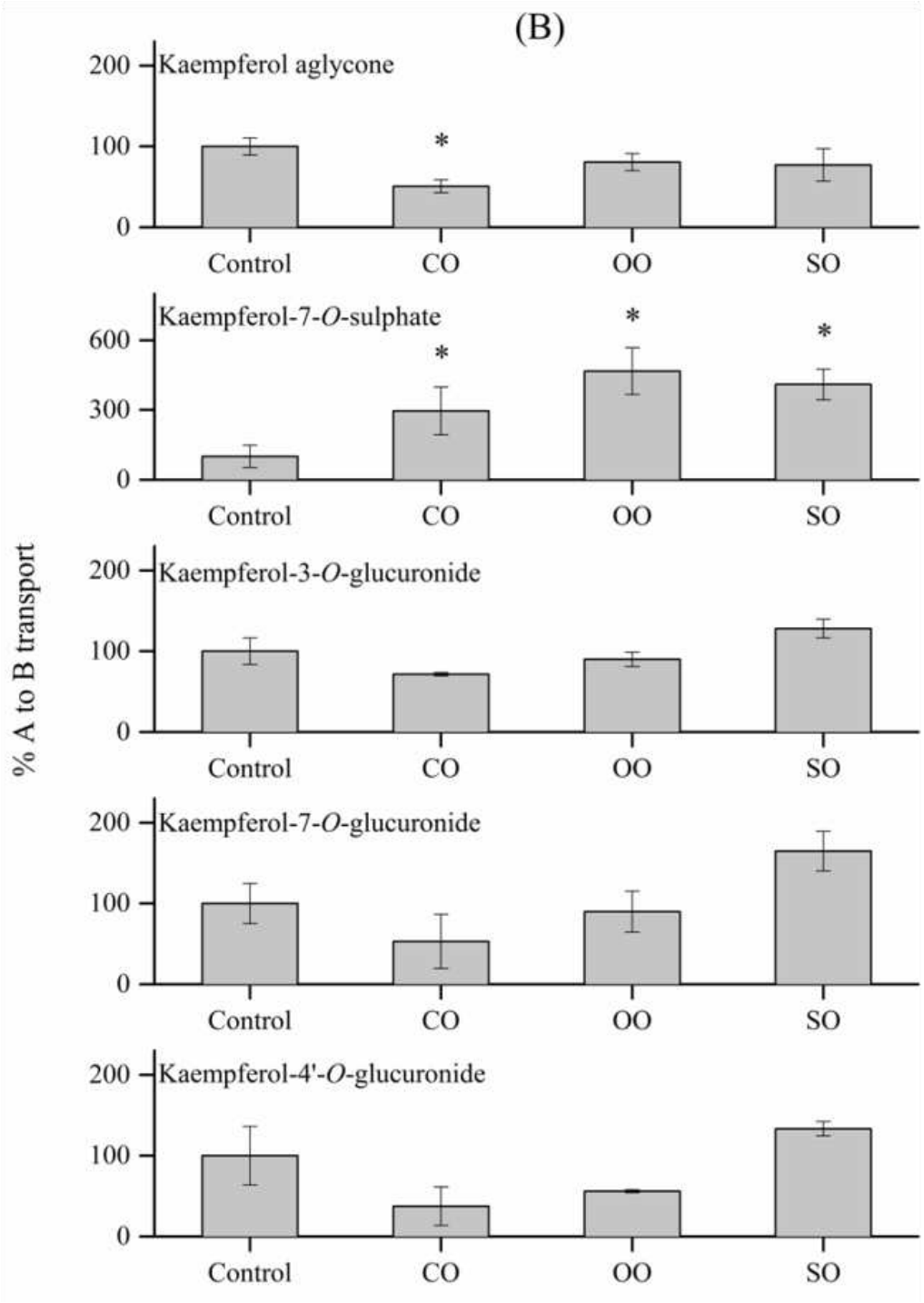
(B)
% A to B transport
Kaempferol aglycone
Kaempferol-7-O-sulphate
Kaempferol-3-O-glucuronide
Kaempferol-7-O-glucuronide
Kaempferol-4'-O-glucuronide
Control
CO
OO
SO
0
100
200
300
600
*

Figure 3B

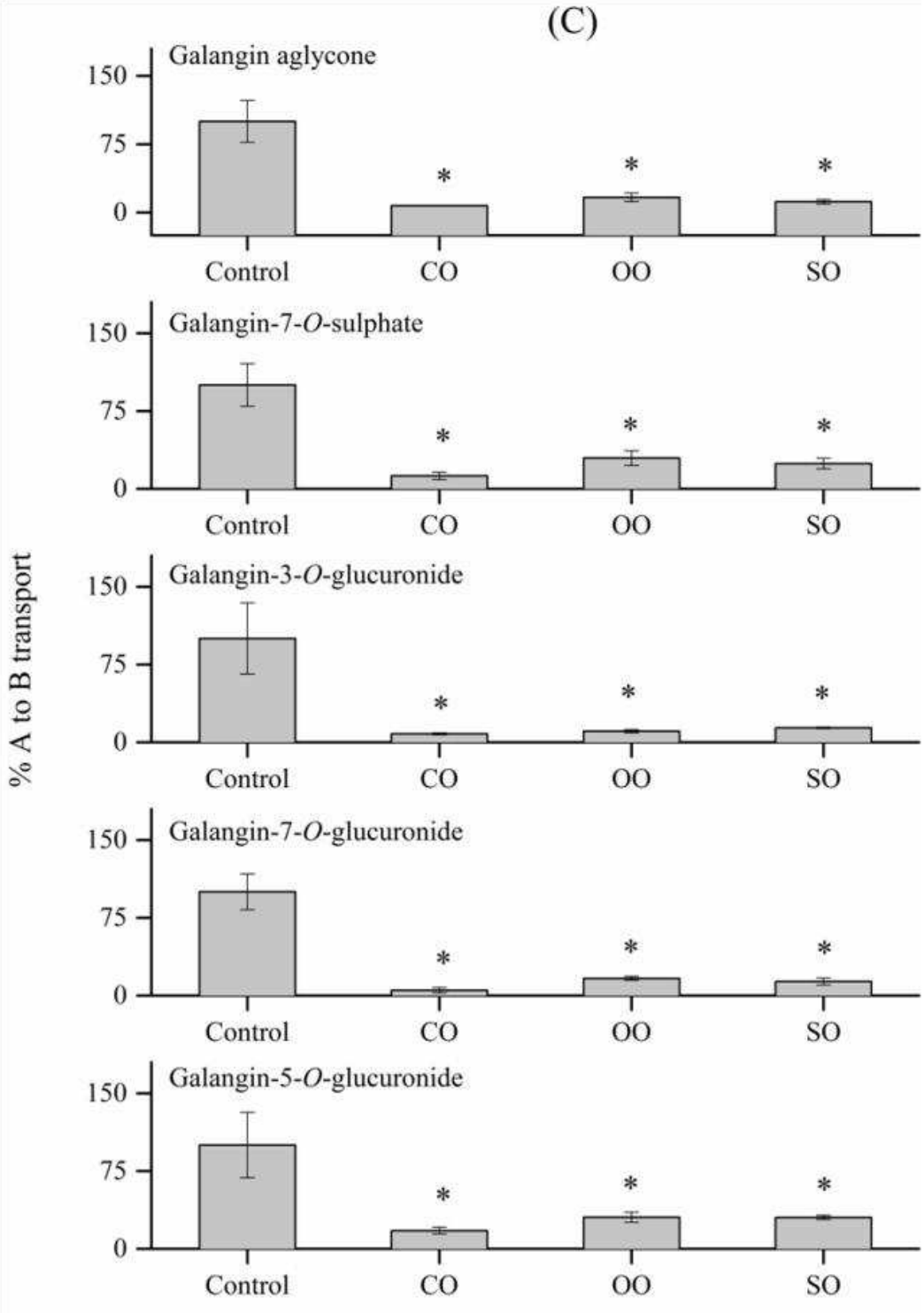
(C)
Galangin aglycone
Galangin-7-O-sulphate
Galangin-3-O-glucuronide
Galangin-7-O-glucuronide
Galangin-5-O-glucuronide
% A to B transport
150
75
0
Control
CO
OO
SO
*

Figure 3C

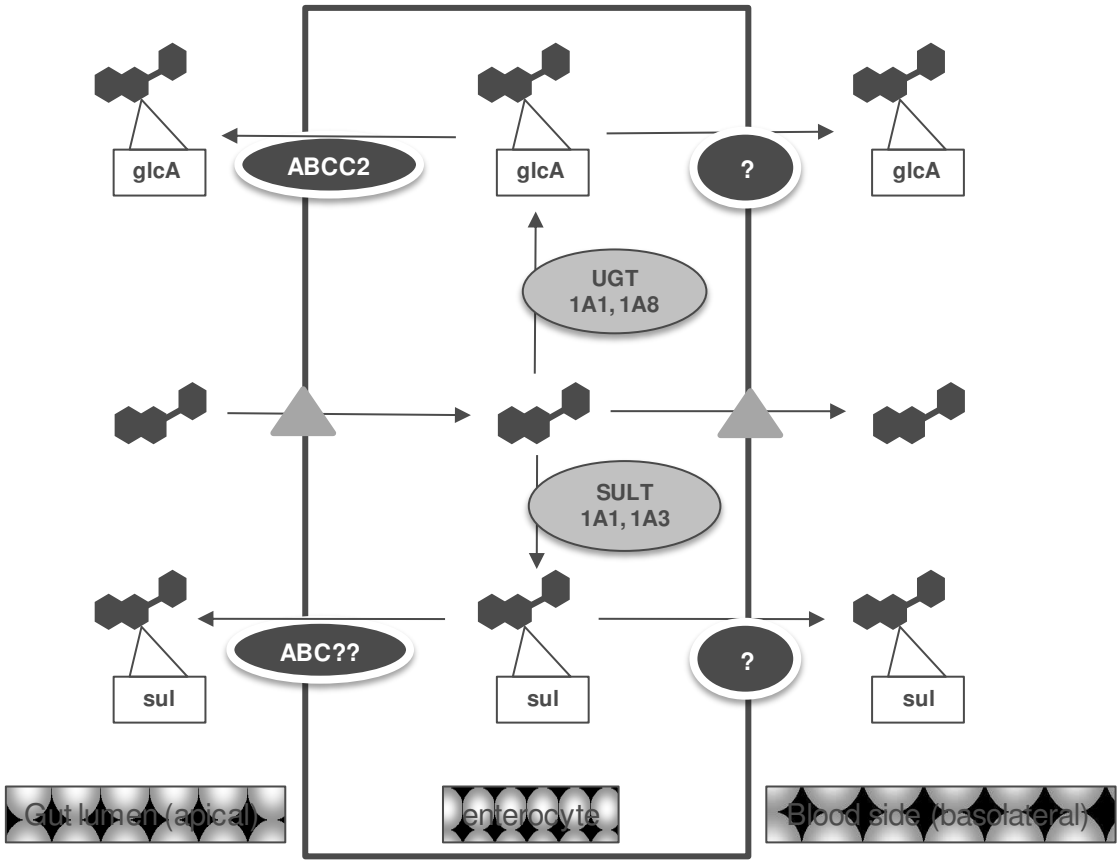
glcA
ABCC2
glcA
?
glcA
UGT
1A1, 1A8
SULT
1A1, 1A3
ABC??
sul
sul
?
sul
Gut lumen (apical)
enterocyte
Blood side (basolateral)

Figure 4.

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