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Research Article

The *C*-glycosyl flavonoid, aspalathin, is absorbed, methylated and glucuronidated intact in humans

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Human bioavailability of the flavonoid dihydrochalcones is little understood, and no evidence exists for *C*-glycosyl flavonoid absorption in humans. The present study uses catechol-*O*-methyltransferase to generate methylated metabolites of aspalathin (a *C*-glycosyl dihydrochalcone from rooibos tea). One of the methylated forms, both with and without glucuronidation, was detected using LC-MS/MS in the urine of human subjects ($n = 6$), demonstrating that deglycosylation is not a prerequisite for *C*-glycosyl flavonoid absorption. Methylation is catalysed by both intestine and liver cytosolic extracts. The results show that flavonoid *C*-glycosides are methylated and glucuronidated *in vivo* in an intact form in humans.

Keywords: Absorption / Aspalathin / *C*-glycoside / Flavonoid / Methylation

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1 Introduction

Aspalathin belongs to the dihydrochalcone sub-group of the flavonoids, dietary polyphenolic compounds which improve biomarkers such as those linked to bone and cardiac health [1]. The dihydrochalcones represent a major polyphenolic constituent of particular foods such as rooibos tea and apples, and are produced by alkaline hydrogenation of flavanones from citrus fruit for use as natural sweeteners by the food and pharmaceutical industries [2]. Their high dietary burden in habitual consumers of dihydrochalcone sources means that human bioavailability of the dihydrochalcones is important, yet remains largely uncharacterised. Aspalathin is of particular interest within the dihydrochalcones as it lacks the *O*-glycosidic linkage commonly attaching single or dimeric sugar moieties to many flavonoids. The aspalathin structure includes a *C*-glycosidic linkage to a single glucose group on the dihydrochalcone A ring, integral to the carbon backbone [3]. While human bioavailability of the dihydrochalcone structure is little understood, no evidence or information exists for *C*-glycosyl flavonoid absorption in humans. The only study to date has been performed on pigs ($n = 3$) which were fed rooibos tea extract for 11 days at an extremely high dose and showed some intact metabolites in collected pig urine [4]. This study therefore aimed to characterise absorption and metabolism of aspalathin in humans using LC-MS/MS to identify and quantify aspalathin metabolites in urine after oral administration of an aspalathin-rich drink, green rooibos tea.

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Abbreviations: COMT, catechol-*O*-methyltransferase; **MA,** methylaspalathin; **MRM,** multiple-reaction monitoring; **SAM,** S-adenosyl methionine

2 Materials and methods

2.1 Materials

Aspalathin was purchased from PhytoLab KG (Vestenbergsgreuth, Germany). Green rooibos tea was purchased from Nothing But Tea (Bedford, Bedfordshire, UK). Distilled water was produced using a Millipore Elix system (Millipore, MA, USA). Drinking water was purchased from WM Morrison Supermarkets PLC (Bradford, UK). Quercitrin, Trizma© tris base, human liver cytosol, β-glucuronidase (type IX-A from *Escherichia coli*), Riedel-de-Haën formic acid and concentrated HCl was purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and absolute ethanol was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). S-adenosyl methionine (SAM) was purchased from New England Biolabs (Ipswich, MA, USA). Catechol-*O*-methyltransferase (COMT, from porcine hepatocytes) was purchased from MP Biomedicals (Santa Ana, CA, USA). Human intestinal

cytosol was purchased from Tebu-Bio (Peterborough, UK). Anhydrous $MgCl_2$ was purchased from Alfa Aesar (Ward Hill, MA, USA). Research-grade N_2 was purchased from BOC (Guildford, UK).

2.2 Subjects

Six individuals (two males, four females) were recruited with a mean age of 26.2 (range 22 – 28) years. All were healthy as determined by a medical questionnaire, had a BMI in the range 19 – 27, and used neither routine nor spontaneous medication. Informed consent was provided in writing by each subject, performed as *per* the protocol approved by the MEEC ethics committee at The University of Leeds, UK.

2.3 Preparation of an aspalathin-rich rooibos tea solution

Dried green rooibos tea leaves (14 g) were added to 1 L drinking water at 100°C and mixed for 10 min. The resulting solution was filtered though muslin. This process was repeated a total of three times, each solution being pooled to produce a final standardised solution which was immediately cooled to 5°C.

2.4 HPLC-DAD quantification of aspalathin

The HPLC system comprised an Agilent 1200 series SL micro degasser, binary pump, high performance autosampler plus with chilled sample compartment, thermostatted column oven compartment, and diode array detector (Agilent Technologies, Santa Clara, CA, USA). The system was controlled and data processed by Agilent MassHunter software (version B.01.03). An Agilent Zorbax Eclipse Plus C18 1.8 μm 2.1 mm × 100 mm rapid-resolution HPLC column provided chomatographic separation at 35°C. The mobile-phase was aqueous 0.2% formic acid with a gradient of methanol increasing from an equilibration concentration of 11% to a plateau of 37.5% from 6.5 to 10.0 min. The concentration returned to 11% by 10.75 min, with an additional 4 min re-equilibration. Mobile-phase flow rate was 0.25 mL/min. All samples were held at 4°C until injection of 8 μL. Aspalathin was quantified by DAD detection at 280 nm, eluting as a single peak at 6.2 min.

2.5 Study design

Subjects followed an aspalathin-free diet for 7 days, and polyphenol- and alcohol-free diet for 3 day prior to study commencement. Baseline urine was collected from each subject immediately prior to oral administration of 300 mL of green rooibos tea at 5°C. Subjects were in the fasted-state, and consumed the solution within 5 min. Each 300 mL portion contained 91.2 mg of aspalathin, measured in duplicate by HPLC-DAD at the point of administration. Urine samples were collected from each subject in acid-washed urine collection vessels every 2 h after aspalathin administration. Three standard polyphenol-free meals were provided to each subject at 1, 5 and 9 h after aspalathin administration. No supplementary food was consumed by the subjects throughout the initial 12 h period, but subjects returned to a nonstandardised polyphenol-free diet for the second 12 h of the study. Drinking water was available to the subjects for *ad libitum* consumption throughout the 24 h duration of the study. Subjects were monitored throughout the study to ensure completeness of urine collection.

2.6 *O*-methylaspalathin (MA) urine sample analysis

The urine samples were immediately treated after measurement of total sample volume. Urine was aliquotted (500 μL) in duplicate, 200 μL methanol added, and 200 μL 50 μM quercetin 3-*O*-β-D-glucoside (quercitrin) in ethanol added as internal standard. Each sample was vortexed and centrifuged at $17\,000 \times g$ for 10 min. The supernatant of each sample was removed for analysis by LC-MS/MS. Samples were kept at 4°C prior to analysis, and analysed no more than 8 h post-excretion to prevent compound degradation.

2.7 *O*-methyl-glucuronide conjugate urine sample analysis

Duplicate 700 μL aliquots of each urine sample were buffered to pH 7.0 with a final concentration of 25 mM potassium phosphate buffer. The glucuronides were cleaved with 166 U β-glucuronidase (type IX-A, *E. coli*) in a final reaction volume of 800 μL. Control samples were prepared for each urine sample in duplicate, replacing the enzyme-enriched buffer with nonenriched buffer. The samples were incubated in a water bath at 37°C for 1 h before the reaction was stopped with 300 μL ice-cold methanol. Quercitrin in ethanol (300 μL, 50 μM) was added to each as an internal standard. Samples were vortexed and centrifuged at $17\,000 \times g$ for 10 min. The supernatant of each sample was removed for individual analysis by LC-MS/MS. Subtraction of the mean MA concentration of the control samples from the mean concentration of the hydrolysed samples after volume correction gave the MA glucuronide concentration.

2.8 3- and 4-*O*-methylaspalathin quantification by LC-MS/MS multiple-reaction monitoring (MRM)

The HPLC system was identical to that used for aspalathin quantification. MS was performed with an Agilent 6410 triple-quad LC-MS with electrospray source (Agilent Technologies) supplied by a N_2 generator (Peak Scientific, Inchinnan, UK). The mobile-phase was aqueous 0.2% for-

mic acid with a gradient of methanol increasing from an equilibration concentration of 11% to a plateau of 62.5% from 8.0 to 14.5 min. The concentration returned to 11% by 15.5 min, with an additional 5.5 min re-equilibration. Mobile-phase flow rate was 0.25 mL/min. All samples were held at 4°C until 10 μL injection. The needle was washed with methanol between injections to prevent sample run-over. MRM enabled mass spectrometric quantification of low-concentrations of MA in complicated matrices due to production of two unique product ions from a single filtered parent ion mass. LC effluent was diverted to waste for the initial 3 min for elution of nonvolatile compounds in the sample that may cause MS/MS contamination, after which the eluent entered the electrospray nebuliser at 0.25 mL/min, ejected with an N_2 flow of 50 psi. into the spray chamber set in positive ionisation mode (ESI+). The solvent was removed with a source gas temperature of 300°C at a flow rate of 6 L/min. The capillary potential was 4 kV, and the fragmentor potential was 120 V. Quad 1 was set to filter an *m*/*z* of 467.2 in unit resolution, the calculated single-ionised mass of MA. The collision cell had a potential of 20 V, and quad 2 alternately filtered an *m*/*z* of 179.1 and 137.1 in unit resolution, the *m*/*z* of the two identified product ions of both 3- and 4-*O*-MA at a collision potential of 20 V. Dwell time of quad 2 for each *m*/*z* was 200 ms. Qualification was determined by detection of both product ions at 14.35 and 15.75 min. Quantification was provided by the integrated peak area of the plot of total product ion abundance over time at each retention time.

2.9 3- and 4-*O*-methylaspalathin standard curve

A stock 904 μM aspalathin standard solution was prepared in 10 mM pH 7.4 Tris-HCl buffer. Aspalathin standard solution (100 μL) was added to duplicate solutions of 50 μg COMT, 1 mM $MgCl_2$ and 110 μM SAM in a final total volume 750 μL 10 mM pH 7.4 Tris-HCl buffer, kept on ice during preparation. The presence of aspalathin substrate initiated the reaction, after which the mixture was vortexed and incubated in a water-bath at 37°C for 20 min. Ice-cold methanol (300 μL) stopped the reaction, and the samples were vortexed. After centrifugation at $17\,000 \times g$ for 10 min, the supernatant was removed for HPLC-DAD analysis. The concentration of 3- and 4-*O*-MA was quantified in duplicate by the aspalathin standard curve at 280 nm, and the mean concentration calculated. Duplicate serial dilution of the supernatant stock with 10 mM pH 7.4 Tris-HCl buffer gave a range of known 3- and 4-*O*-MA concentrations. Quercitrin in ethanol (60 μL, 50 μM) was added to each as an internal standard, and the solutions quantified using the 3- and 4-*O*-MA LC-MS/MS MRM method. The mean values of the duplicate solutions were plotted over concentration and the intercept of the calibration curve forced to 0. The R^2 variance coefficient was 0.999 for both 3- and 4-*O*-MA.

2.10 Kinetic analysis of COMT-catalysed aspalathin *O*-methylation

The reaction mixture was prepared on ice, with all reagents except SAM being prewarmed in a water bath at 37°C prior to SAM addition. Serial dilutions of 596 μM aspalathin solution in 10 mM pH 7.4 Tris-HCl buffer gave a final range of five substrate concentrations; 79.5, 47.8, 31.8, 15.9 and 7.9 μM. The duplicate final mixtures consisted of requisite concentrations of aspalathin substrate, 50 μg COMT, 1 mM $MgCl_2$ and 110 μM SAM in a final volume of 750 μL 10 mM pH 7.4 Tris-HCl buffer. The addition of SAM initiated the reaction and the mixture was incubated in a water-bath at 37°C. Removal of 150 μL aliquots from each sample into 60 μL ice-cold methanol 1, 2, 5, 10 and 20 min after initiation stopped the reaction. Quercitrin in ethanol (60 μL, 50 μM) was added to each as an internal standard. The samples were centrifuged at $17\,000 \times g$ for 10 min, and the supernatant of each removed for LC-MS/MS analysis. The 3- and 4-*O*-MA concentrations were quantified by the standard curve and corrected for matrix dilutions. The resulting duplicate concentrations were plotted in series by mean product concentration over time. All reactions were linear over time, product concentrations at 10 min analysed by the Michaelis – Menten equation to yield the apparent K_M and apparent V_{max}. The M_r of COMT from porcine hepatocytes used for these calculations was determined as 23 kDa by Gulliver and Tipton and was verified by Bertocci *et al*. [5, 6].

2.11 Comparison of aspalathin *O*-methylation in human liver and intestinal cytosolic fractions

The reaction mixture was prepared on ice, with all reagents except SAM being prewarmed in a water bath at 37°C. Final duplicate mixtures consisted of 233 μM aspalathin substrate, 1 mM $MgCl_2$, 110 μM SAM and either 4 mg/mL human liver cytosolic protein or 0.7 mg/mL human intestinal cytosolic protein in a final volume of 750 μL 10 mM pH 7.4 Tris-HCl buffer. SAM addition initiated the reaction and the mixture was incubated in a water-bath at 37°C for 30 min. Ice-cold methanol (300 μL) was added to each sample to stop the reaction, followed by addition of 300 μL quercitrin (50 μM) in ethanol as an internal standard. The samples were centrifuged at $17\,000 \times g$ for 10 min, and the supernatant of each removed for LC-MS/MS analysis. The 3- and 4-*O*-MA concentrations were quantified by the standard curve and corrected for matrix dilutions.

3 Results

3.1 Aspalathin metabolite formation and LC-MS/MS analysis *in vitro*

Incubation of aspalathin with COMT *in vitro* produced two previously unidentified methylated metabolites, identified

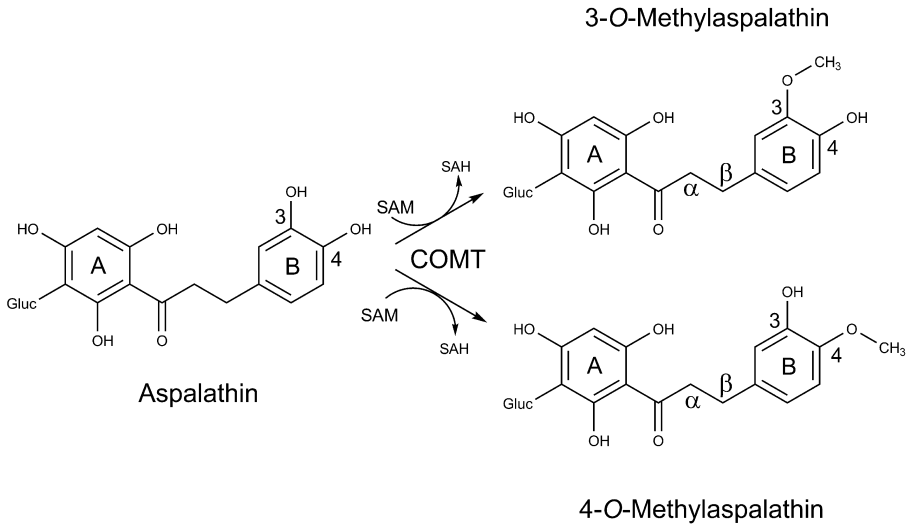

Figure 1. COMT-catalysed formation of two isomeric *O*-methylated aspalathin metabolites, 3-*O*-MA and 4-*O*-MA. The system of carbon numbering on the dihydrochalcone ring structure is reversed from that of ring-closed classes of flavonoids. Positional nomenclature of dihydrochalcone B-ring substituents becomes unprimed, whilst the A-ring carbons are primed.

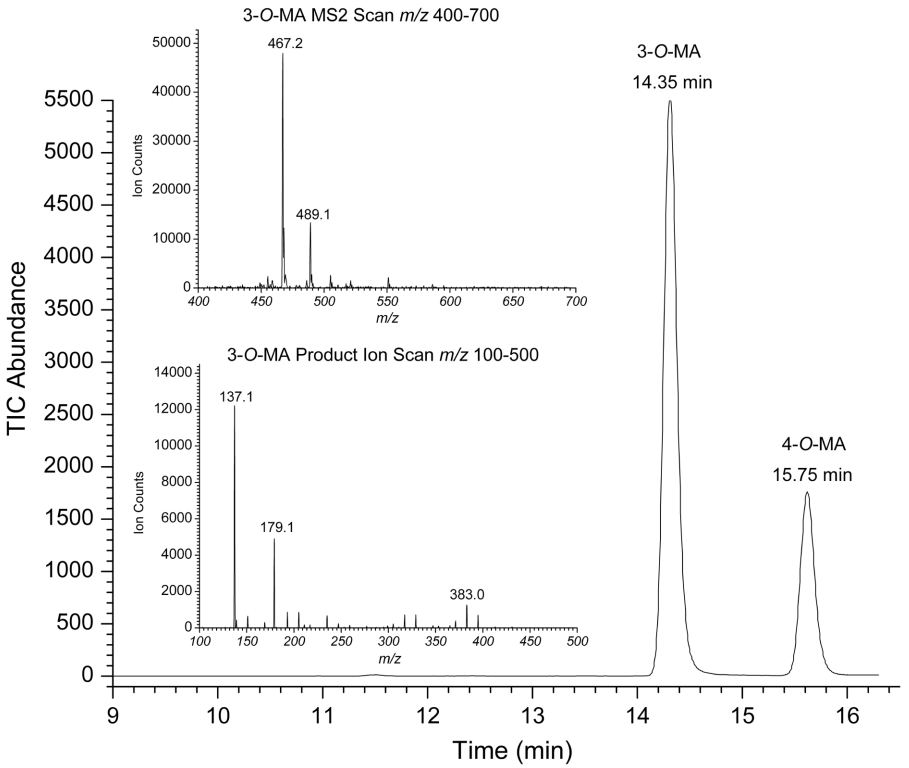

Figure 2. Typical LC-MS/MS MRM chromatogram of 3-*O*-MA and 4-*O*-MA, with 3-*O*-MA MS2 scan and product ion scan spectra (insets).

by MS2 SIM detection as separately-eluting peaks at the predicted single positively-ionised state of the structural isomers (*m*/*z* 467.2). Maximum sensitivity was achieved though optimisation of the fragmentor ion transmission potential (120 V), after which a 0 – 80 V potential range was applied to the collision cell in 10 V increments over nine chromatographic runs. Twenty volt collision potential gave optimum product ion formation, producing identical fragmentation patterns for both metabolites, characterised by the formation of two distinct product ions (Fig. 1). Structural distinction between 3- and 4-*O*-MA by MS was therefore not possible. Fragmentation of the precursor ion appeared to predominantly occur between the α and β carbons of the dihydrochalcone structure, releasing the methylated catechol moiety (*m*/*z* 137.1). While this followed the proposed dihydrochalcone fragmentation pattern of Kazuno *et al*. [7], the structure of the secondary product ion (*m*/*z* 179.1) appeared not to follow this pattern, but is likely to be formed by fragmentation of the carbon linking the A-ring to the carbonyl group. The MRM method for MA quantification was created from these transition ions.

Figure 2 shows the enzymic formation of the two isomeric products, however it must be noted that the position of hydroxyl methylation is unconfirmed and has been

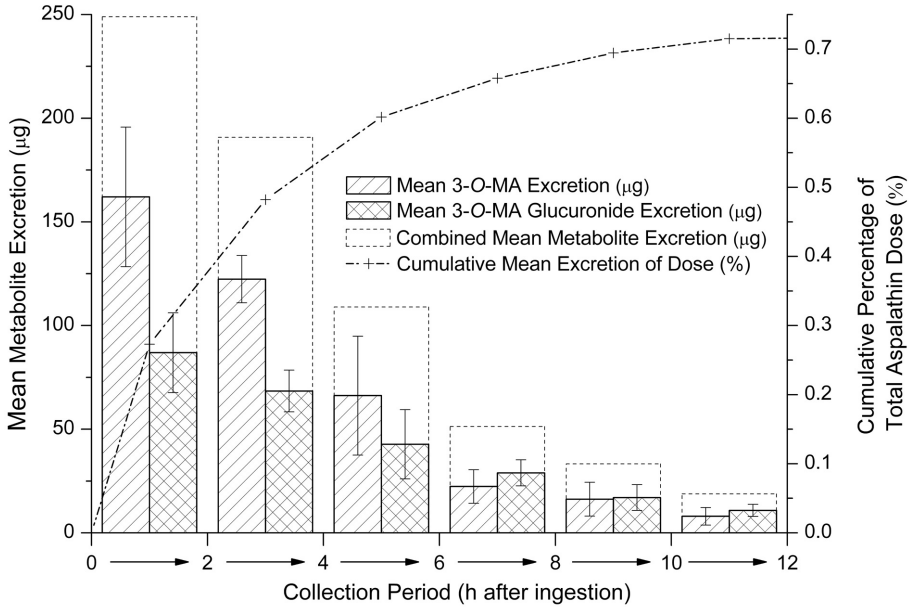

Figure 3. Mean excretion pattern of 3-*O*-MA and 3-*O*-MA glucuronide 12 h after green rooibos tea ingestion, with cumulative mean percentage excretion of total ingested aspalathin dose.

assumed on the basis of work by Creveling *et al*. [8, 9] showing the catechol C3 (meta) hydroxyl group to be increasingly favourably methylated over the C4 (para) hydroxyl group as the polarity of the molecule rises [10]. The ratio of the two reaction products was ~4:1 (retention times 14.35 and 15.75 min, respectively), therefore the two analytes were identified as 3-*O*-MA at 14.35 min and 4-*O*-MA at 15.75 min. This order of retention was in agreement with the chromatographic data of van der Woude *et al*. [11], showing 3′-*O*-methylquercetin (isorhamnetin) to elute before 4′-*O*-methylquercetin (tamarixetin) in a similar RP HPLC system.

3.2 Kinetic analysis of COMT-catalysed aspalathin *O*-methylation

Following identification of the two forms of *O*-MA, the COMT-catalysed kinetics of their formation was measured. The reaction followed Michaelis–Menten enzyme kinetics, with a linear rate of product formation occurring within the range of substrate concentrations over 10 min for both 3- and 4-*O*-MA. Table 1 shows the apparent K_M and V_{max} for the formation of both products.

3.3 Comparison of aspalathin *O*-methylation in human liver and intestinal cytosolic fractions

The *O*-methylating activity of the human liver and small intestine was compared by incubation of their respective cell-free cytosolic extracts with an aspalathin solution at pH 7.4, 37°C for 30 min. The relative *O*-methylation rate of the intestinal extract was slightly higher than the liver extract for both MA forms (Table 2). The 3-*O*-MA production comprised 88% of total MA produced in both cell extracts.

Table 1. Enzyme kinetics of COMT-catalysed 3-*O*-MA and 4-*O*-MA formation at pH 7.4, 37°C

	App K_M (μM)	App V_{max} (Kat/mol COMT)
3-*O*-MA	39 ± 5	6.67 ± 0.40
4-*O*-MA	51 ± 7	1.03 ± 0.08

3.4 *O*-methylated aspalathin metabolite determination in human urine

After consumption of aspalathin by healthy human volunteers, MRM analysis of the urine samples revealed 3-*O*-MA and 3-*O*-MA glucuronide excretion in all subjects ($n = 6$). 4-*O*-MA was not detected, despite being produced by COMT *in vitro*. Baseline urine sample analysis showed the absence of aspalathin metabolites prior to ingestion of the aspalathin solution in all subjects, demonstrating compliance with the wash-out period. Urinary excretion of both aspalathin metabolites was detected in the first sample (0–2 h) and remained in the urine of all subjects for 6 h after oral exposure to aspalathin, disappearing from the urine in a monophasic manner (Fig. 3). Mean excretion of 3-*O*-MA decreased dramatically in the 8 h after aspalathin administration, falling to 14% of the mean maximum metabolite concentration. Conversely, 33% excretion of the mean 3-*O*-MA glucuronide maximum was observed after 8 h. Consequently, 3-*O*-MA glucuronide was present in greater concentrations compared to 3-*O*-MA between 10 and 24 h in five of the six subjects, as shown in Fig. 4. Interindividual variation in the percentage of total to 3-*O*-MA glucuronide to 3-*O*-MA excretion ranged from 34% in subject 6, to 89% in subject 1 (mean, 66%; SD, 22%). The maximum time of excretion of both metabolites occurred in the subjects

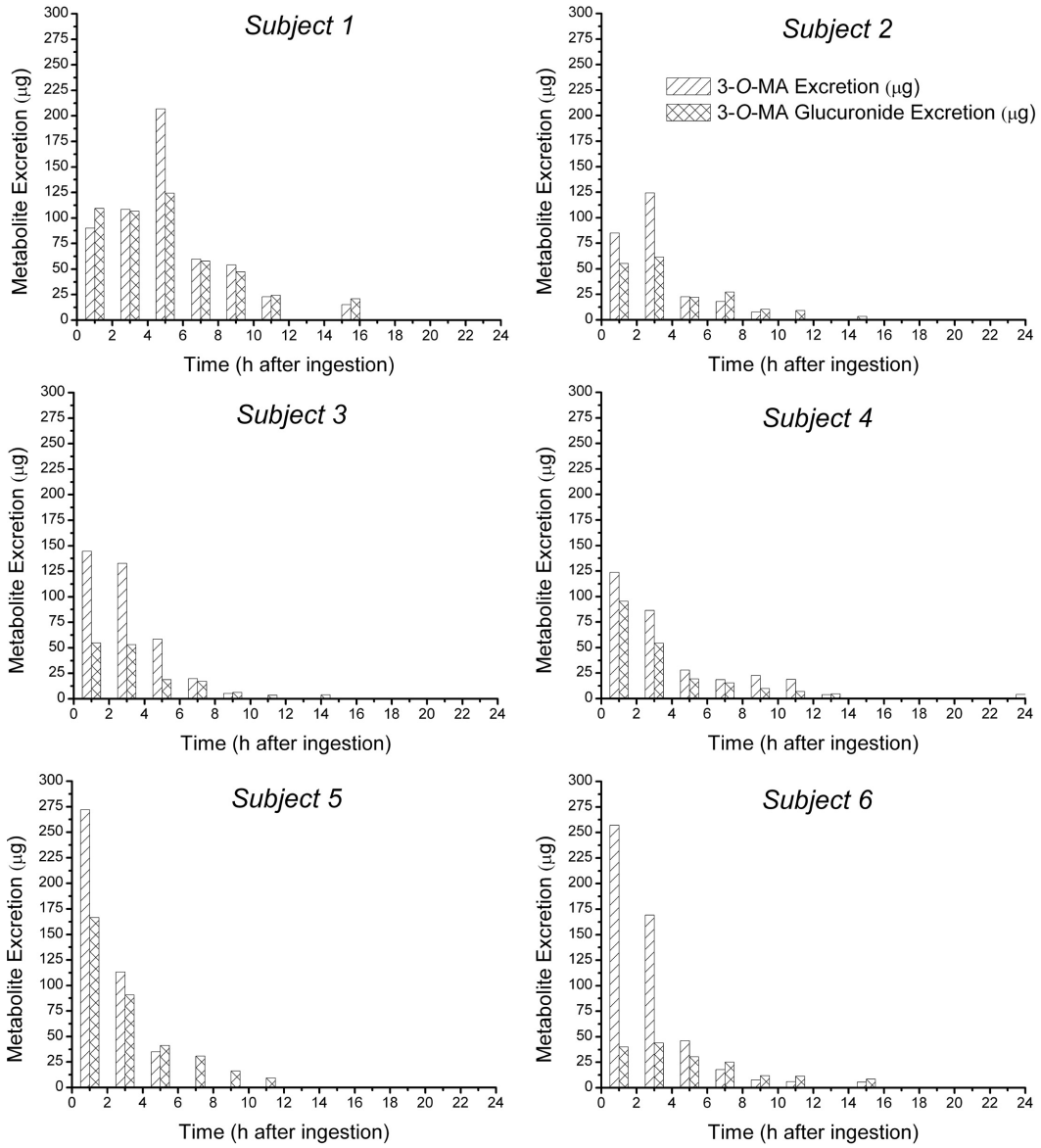

Figure 4. Individual subject excretion patterns of 3-*O*-MA and 3-*O*-MA glucuronide 24 h after green rooibos tea ingestion.

Table 2. Comparison of aspalathin *O*-methylation rates in human liver and intestinal cytosolic fractions

	3-*O*-MA (pmol/min · mg cytosolic protein)	4-*O*-MA (pmol/min · mg cytosolic protein)
Human liver cytosol	99.3 ± 9.21	14.0 ± 1.51
Human intestinal cytosol	116.6 ± 0.06	15.5 ± 0.07

between 0 and 2 h ($n = 4$), 2 and 4 h ($n = 1$) and 4 and 6 h ($n = 1$). This variation is hypothesised to be due to differences in absorption, as the maximum time of metabolite excretion of both metabolic forms was observed simultaneously in all subjects. The mean maximum concentration for both compounds therefore occurred within 2 h after ingestion of aspalathin, at 162 μg (3-*O*-MA) and 87 μg (3-*O*-MA glucuronide) total excretion. Total mean urinary excretion of the ingested dose of aspalathin was 0.74% (SD, ±0.26%; range, 0.57 – 1.2%) as shown in Fig. 3.

4 Discussion

Deglycosylation is known to be fundamental in the intestinal transcellular absorption of *O*-glycosyl flavonoids [12, 13], relying principally on the interaction of the compound with the protein lactase phlorizin hydrolase (LPH) [14], present on the brush-border of the intestinal lumen, and cytosolic β-glucosidase (CBG), present in the enterocyte

cytosol. Once freed, the flavonoid aglycone has been shown to be able to passively diffuse across the intestinal epithelium owing to the increase in structural lipophilicity caused by the loss of the relatively hydrophilic sugar group [12, 13]. Interaction of the *intact* glucose moiety with active transporter proteins has also been cited as an important factor in glycosyl-flavonoid movement across the apical side of the intestinal epithelium; SGLT1 transporting from the lumen to the enterocyte cytosol followed by CBG deglycosylation as mentioned, and multi-drug resistance protein 2 (MRP2) facilitating efflux from the enterocyte cytosol to the intestinal lumen [15, 16].

The specificity of hydrolytic enzymes for the glycosyl linkage is clearly pivotal in this model of transcellular flavonoid absorption, and whilst substrate specificity has only been characterised for flavonoid *O*-glycosides [17], the presence of unhydrolysed methylated metabolites of aspalathin in human urine reveals that deglycosylation is not necessarily a prerequisite for *C*-glycosyl flavonoid absorption in humans. This is supported by a long-term, high-dose study of rooibos tea extract consumption in pigs, which showed some intact aspalathin metabolites in pig urine [4]. It is hypothesised that passive transcellular movement therefore plays a minimal part in the human uptake of this hydrophilic molecule ($\log P_{oct} = -0.347$) [18], implying that transcellular movement of the intact molecule across the intestinal epithelium is predominantly facilitated. While this action can be potentially attributed to SGLT1 on the apical side of the enterocyte, this implies basolateral active transporter specificity towards intact glycosyl-flavonoids, although paracellular diffusion should not be discounted as a potential mechanism of absorption. The increase in log P_{oct} brought-about by methylation may also be a factor in basolateral transport, particularly as this is shown to occur in the enterocyte cytosol whereby the ability of MA to passively cross the basolateral membrane may subsequently increase relative to its precursor. Furthermore, intestinal cell transport studies performed with the adenocarcinoma cell line Caco-2 reported 100% aspalathin transport from an aqueous green rooibos tea solution (concentration of 1.940 mg/mL) across the monolayer [18]. This methodology may be adaptable for future elucidation of active *C*-glycoside transport mechanisms in the intestinal epithelium.

The relative hydrophilicity of aspalathin may be responsible for preferential production of 3-*O*-MA *in vitro* as demonstrated by Creveling *et al*. [8], showing an increase in the meta:para methylation ratio of physiological COMT substrates with substrate polarity. It is possible that a cytochrome enzyme system catalyses the demethylation of 4-*O*-MA *in vivo*, a system hypothesised for the COMT inhibitors BIA 3-202 and tolcapone by Palma *et al*. [19]. The apparent regioselectivity of this system towards the C4 (para) methyl group may therefore explain why 4-*O*-MA was not detected in human urine in the present study, despite its formation by COMT *in vitro*.

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Conflict of interest statement: Prof. Gary Williamson is a part-time employee of Nestlé Research Center.

5 References

[1] Williamson, G., Manach, C., Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies, *Am. J. Clin. Nutr.* 2005, *81*, 243 – 255.

[2] Tomás-Barberán, F. A., Clifford, M. N., Flavanones, chalcones and dihydrochalcones – nature, occurrence and dietary burden, *J. Sci. Food Agric.* 2000, *80*, 1073 – 1080.

[3] Koeppen, B. H., Roux, D. G., *C*-glycosyl flavonoids. The chemistry of aspalathin, *Biochem. J.* 1966, *99*, 604 – 609.

[4] Kreuz, S., Joubert, E., Waldmann, K.-H., Ternes, W., Aspalathin, a flavonoid in *Aspalathus linearis* (rooibos), is absorbed by pig intestine as *C*-glycoside, *Nutr. Res.* 2008, *28*, 690 – 701.

[5] Gulliver, P. A., Tipton, K. F., The purification and properties of pig-liver catechol-*O*-methyl transferase, *Eur. J. Biochem.* 1978, *88*, 439 – 444.

[6] Bertocci, B., Garotta, G., Da Prada, M., Lahm, H. W., *et al*., Immunoaffinity purification and partial amino acid sequence analysis of catechol-*O*-methyltransferase from pig liver, *Biochim. Biophys. Acta* 1991, *1080*, 103 – 109.

[7] Kazuno, S., Yanagida, M., Shindo, N., Murayama, K., Mass spectrometric identification and quantification of glycosyl flavonoids, including dihydrochalcones with neutral loss scan mode, *Anal. Biochem.* 2005, *347*, 182 – 192.

[8] Creveling, C. R., Dalgard, N., Shimizu, H., Daly, J. W., Catechol *O*-methyltransferase III. *m*- and *p*-*O*-methylation of catecholamines and their metabolites, *Mol. Pharmacol.* 1970, *6*, 691 – 696.

[9] Creveling, C. R., Morris, N., Shimizu, H., Ong, H. H., Daly, J., Catechol *O*-methyltransferase IV. *m*- and *p*-methylation of substituted catechols, *Mol. Pharmacol.* 1972, *8*, 398 – 409.

[10] Lotta, T., Vidgren, J., Tilgmann, C., Ulmanen, I., *et al*., Kinetics of human soluble and membrane-bound catechol *O*-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. *Biochemistry* 1995, *34*, 4202 – 4210.

[11] van der Woude, H., Boersma, M. G., Vervoort, J., Rietjens, I. M. C. M., Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II in vitro model systems, *Chem. Res. Toxicol.* 2004, *17*, 1520 – 1530.

[12] Walgren, R. A., Walle, U. K., Walle, T., Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells, *Biochem. Pharmacol.* 1998, *55*, 1721 – 1727.

[13] Murota, K., Shimizu, S., Chujo, H., Moon, J. H., Terao, J., Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2, *Arch. Biochem. Biophys.* 2000, *384*, 391 – 397.

[14] Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., *et al*., Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase, *FEBS Lett.* 2000, *468*, 166 – 170.

[15] Day, A. J., DuPont, M. S., Ridley, S., Rhodes, M., *et al*., Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity, *FEBS Lett.* 1998, *436*, 71 – 75.

[16] Walgren, R. A., Karnaky, K. J., Lindenmayer, G. E., Walle, T., Efflux of dietary flavonoid quercetin 4′-β-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2, *J. Pharmacol. Exp. Ther.* 2000, *294*, 830 – 836.

[17] Berrin, J.-G., Czjzek, M., Kroon, P. A., Mclauchlan, W. R., *et al*., Substrate (aglycone) specificity of human β-glucosidase, *Biochem. J.* 2003, *373*, 41 – 48.

[18] Huang, M., du Plessis, J., du Preez, J., Hamman, J., Viljoen, A., Transport of aspalathin, a rooibos tea flavonoid, across the skin and intestinal epithelium, *Phytother. Res.* 2008, *22*, 699 – 704.

[19] Palma, P. N., Bonifácio, M. J., Loureiro, A. I., Wright, L. C., *et al*., Molecular modeling and metabolic studies of the interaction of catechol-*O*-methyltransferase and a new nitrocatechol inhibitor, *Drug Metab. Dispos.* 2003, *31*, 250 – 258.

