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ARTICLE

Bioavailability of *C*-Linked Dihydrochalcone and Flavanone Glucosides in Humans Following Ingestion of Unfermented and Fermented Rooibos Teas

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High-performance liquid chromatography−mass spectrometry (HPLC-MS^n) detected aspalathin and nothofagin, *C*-glycosides of apigenin and luteolin, and four eriodictyol-*C*-glycoside isomers in unfermented and fermented rooibos teas. The fermented drink contained 10-fold higher levels of aspalathin and nothofagin and a 4-fold lower eriodictyol-*C*-glycoside content than the fermented tea. The total flavonoid contents in 500 mL servings of the teas were 84 (fermented) and 159 μmol (unfermented). Following the ingestion of 500 mL of the teas by 10 volunteers, 0−24 h urine and plasma samples were collected for analysis. HPLC-MS^n identified eight metabolites in urine. These were *O*-linked methyl, sulfate, and glucuronide metabolites of aspalathin and an eriodictyol-*O*-sulfate. The main compound excreted was an *O*-methyl-aspalathin-*O*-glucuronide (229 nmol) following ingestion of the unfermented drink and eriodictyol-*O*-sulfate (68 nmol) after ingestion of the fermented beverage. The overall metabolite levels excreted were 82 and 352 nmol, accounting for 0.09 and 0.22% of the flavonoids in the fermented and unfermented drinks, respectively. Most of the aspalathin metabolites were excreted within 5 h of tea consumption, suggesting absorption in the small intestine. Urinary excretion of the eriodictyol-*O*-sulfate occurred mainly during the 5−12 h collection period, indicative of absorption in the large intestine. Despite exhaustive searches, no flavonoid metabolites were detected in plasma.

KEYWORDS: Rooibos tea; flavonoid metabolites; urine; absorption; bioavailability; humans

INTRODUCTION

Rooibos tea is a caffeine-free alternative to tea and coffee, with infusions being prepared from the leaves of *Aspalathus linearis* belonging to the Fabaceae family. Traditionally, the beverage is made from either oxidized leaves (also referred to as fermented) or unfermented "green" leaves. The main compounds identified in rooibos tea are 2′,3,4,4′,6′-pentahydroxydihydrochalcone-3′-*C*-glucoside (aspalathin) and 2′,4,4′,6′-tetrahydroxydihydrochalcone-3′-*C*-glucoside (nothofagin), as well as luteolin-6-*C*-glucoside (isoorientin aka homoorientin), luteolin-8-*C*-glucoside (orientin), apigenin-6-*C*-glucoside (isovitexin), and apigenin-8-*C*-glucoside (vitexin) (*1*, *2*). The oxidation process has been shown to influence the amount of aspalathin, because of its conversion to eriodictyol-6-*C*-glucosides, which are further degraded to isoorientin and orientin (*3*).

A few studies have reported beneficial effects associated with rooibos drinking. Aspalathin and other flavonoids in rooibos were reported to exert antioxidant (*4*, *5*) and antimutagenic activities in vitro (*6*). Recently, McKay and Blumberg (*7*) reviewed animal and human studies investigating the effects of rooibos in vivo, and the only two human trials to date did not report any effect. Results from animal studies, however, suggested antioxidant, chemopreventive, and immune-modulating effects associated with rooibos extracts in some instances.

Nonetheless, if rooibos compounds are to have beneficial effects in vivo, they have to be absorbed and metabolized in such a way that they can reach and target specific sites. To date, there are no reports in the literature on the bioavailability of aspalathin or nothofagin in humans. This study, therefore, analyzed urine and plasma samples for possible metabolites of aspalathin, nothofagin, and/or other *C*-linked glycosylated flavonoids following a single 500 mL intake of these compounds in bottled drinks of unfermented and fermented rooibos teas. The analyses were carried out using high-performance liquid chromatography−photodiode array−mass spectrometry (HPLC-PDA-MS^n).

MATERIALS AND METHODS

Chemicals. Quercetin, luteolin-6-*C*-glucoside (homoorientin), luteolin-8-*C*-glucoside (orientin), apigenin-6-*C*-glucoside (isovitexin), apigenin-8-*C*-glucoside (vitexin), eriodictyol-7-*O*-glucoside, and quercetin-3-*O*-galactoside (hyperoside) were purchased from Extrasynthèse

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Table 1. Identification of Flavonoids in Fermented and Unfermented Rooibos Teas

peak no.	R_t (min)	compounds	$[M-H]^-$ (m/z)	MS^2 (m/z)	λ_{max} (nm)
1	20.4	eriodictyol-*C*-glucoside	449	359, 329	290
2	22.9	eriodictyol-*C*-glucoside	449	359, 329	290
3	25.1	eriodictyol-*C*-glucoside	449	359, 329	290
4	25.9	eriodictyol-*C*-glucoside	449	359, 329	290
5	29.7	luteolin-6-*C*-glucoside	447	429, 357, 327	350, 255
6	31.6	luteolin-8-*C*-glucoside	447	357, 327	350, 255
7	35.5	aspalathin	451	361, 331	290
8	39.0	quercetin-*O*-rutinoside	609	301, 300, 271, 255	355, 255
9	39.4	apigenin-8-*C*-glucoside	431	341, 311	340, 270
10	41.0	apigenin-6-*C*-glucoside	431	413, 341, 311	340, 270
11	41.1	quercetin-3-*O*-rutinoside	609	301, 300, 271, 255	355, 255
12	42.0	quercetin-3-*O*-galactoside	463	301	355, 255
13	43.9	quercetin-3-*O*-glucoside	463	301	355, 255
14	50.8	nothofagin	435	345, 315	285
15	71.8	quercetin	301	301, 179, 151	380, 255
16	71.8	luteolin	285	285, 241	350, 255

(Genay, France). Apigenin and quercetin-3-*O*-rutinoside (rutin) were obtained from Sigma Aldrich Co. Ltd. (Poole, Dorset, United Kingdom). Luteolin and quercetin-3-*O*-glucoside (isoquercitrin) were from AASC Ltd. (Southampton, United Kingdom), and aspalathin (2′,3,-4,4′,6′-pentahydroxy-dihydrochalcone-3′-*C*-glucoside) was from Phyto-Lab GmbH & Co. KG (Germany). HPLC grade solvents were obtained from Rathburn Chemicals (Walkerburn, Pebbles, Scotland, United Kingdom).

Rooibos Teas. Five hundred milliliter bottles of "ready-to-drink" rooibos tea were supplied by Beverage Partners Worldwide (Zürich, Switzerland). Two types of tea were tested; one was produced from unfermented leaves, and the other was produced from fermented leaves.

Human Feeding Study. The feeding study was carried out with five male and five female volunteers. The study was approved by the ethics committee of the S. Camillo-Forlanini Hospital in Rome, and all participants gave their written consent. For 2 days prior to and 24 h after the ingestion of rooibos teas, the subjects followed a diet low in flavonoids and phenolic compounds by avoiding fruits, nuts, vegetables, tea, coffee, fruit juices, wine, and dietary antioxidant supplements. On the day of the study, after an overnight fast, each subject drank 500 mL of either fermented or unfermented rooibos tea. Basal venous blood samples were collected at 0 h, and after drinking the tea, further blood samples were collected at 0.5, 1, 2, and 5 h time points. The blood was collected in heparin tubes and centrifuged immediately at 3000*g* for 15 min at 4 °C, after which the plasma was divided into 1 mL aliquots to which was added 30 μL of 50% aqueous formic acid and 100 μL of 10 mM ascorbic acid before being stored at −80 °C prior to extraction and analysis. Urine was collected before the volunteers drank the tea and 0−5, 5−12, and 12−24 h after consumption. The volume of urine collected during each period was measured, and aliquots were stored at −80 °C prior to analysis by HPLC-PDA-MS^3 without further processing.

Samples Preparation. *Rooibos Beverages*. The rooibos beverages were kept refrigerated at 2 °C. Prior to analysis, 2 mL aliquots were centrifuged at 16110*g* for 5 min at 4 °C, prior to triplicate 10 μL samples being analyzed by HPLC-PDA-MS^n.

Plasma. The extraction of plasma was based on the method by Day et al. (*8*) and consisted of adding 450 μL of plasma dropwise to a 2.5 volume of acetonitrile in a 2 mL Eppendorf. The plasma was spiked with 1 μg of ethyl gallate used as an internal standard. Samples were vortexed for 30 s every 2 min and centrifuged at 1500*g* for 20 min at 4 °C. The supernatants were then dried under a flow of nitrogen in a heated block at 35 °C. The remaining pellets were re-extracted in the same condition using a 2.5 volume of methanol. The two supernatants were combined and dried under nitrogen. The residues were resuspended in 250 μL of mobile phase containing 10% of methanol and centrifuged at 16110*g* for 10 min at 4 °C in a 0.2 μm Micro-Spin Eppendorf filter. Extracts (200 μL) were injected in duplicate into the HPLC-MS^n system.

Urine. Urine samples were defrosted and centrifuged for 5 min at 4 °C at 16110*g* prior 200 μL aliquots being analyzed in triplicate by HPLC-MS^n.

HPLC-PDA-MS^n Analyses. Flavonoids and their metabolites in rooibos beverages and plasma and urine samples were analyzed using a Surveyor HPLC with a PDA detector system and a LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). Separations were performed at 40 °C using a Synergi 4 μm RP-MAX 80 Å 250 mm × 4.6 mm (i.d.) reverse phase column (Phenomenex, Macclesfield, United Kingdom) using an autosampler maintained at 4 °C. The mobile phase, pumped at a flow rate of 1 mL/min, was a 75 min gradient of 10−20% acetonitrile in 0.1% aqueous formic acid for 60 min, followed by a 20−50% acetonitrile gradient over 15 min (analysis of the teas). A 35 min 10−30% acetonitrile gradient was used to analyze aspalathin metabolites in urine, and a 35 min 20−38% acetonitrile gradient was employed to analyze eriodictyol metabolites. The column eluent passed through the flow cell of the PDA and was then split, and 0.2 mL/min was directed to the mass spectrometer with electrospray ionization operating in full scan negative ionization mode (100−1000 m/z). Analyses of samples were initially carried out using full scan, data-dependent MS scanning from m/z 100 to 1000. The tuning of the mass spectrometer was optimized by infusing a standard of aspalathin into the source along with the 10% acetonitrile in 0.1% aqueous formic acid, the initial HPLC mobile phase, at a flow rate of 0.2 mL/min. The capillary temperature was 320 °C, the sheath gas and auxiliary gas were 60 units/min, respectively, and the source voltage was 4.80 kV, with the collision energy set at 35%.

Following HPLC separation, flavonoids in the teas were detected using selective ion monitoring (SIM) targeting negatively charged molecular ions ($[M-H]^-$). Compounds were quantified as mean values ± standard errors (SEs) ($n = 3$) in micromoles of the standard of reference, with the exception of nothofagin, which was quantified as aspalathin equivalents, and eriodictyol-*C*-glucosides, which was quantified as eriodictyol-7-*O*-glucoside equivalents. Identification of aspalathin and eriodictyol derivatives and metabolites in urine was based on HPLC with mass spectrometry using consecutive reaction monitoring (CRM), while HPLC with mass spectrometry in SIM mode was utilized for quantification and expressed as mean values ± SEs ($n = 3$) in nanomoles of aspalathin and eriodictyol-7-*O*-glucoside equivalents. The limits of quantification for aspalathin using SIM at m/z 451 were 0.5 (1.1 pmol) and 0.1 ng (0.2 pmol) for eriodictyol-7-*O*-glucoside (m/z 449).

RESULTS

Analysis of Rooibos Teas. The compounds identified in the rooibos teas are listed in **Table 1**, which presents the retention times, MS^n fragmentation patterns, as well as the λ_{max}. Typical HPLC-SIM traces of the compounds identified in the teas are presented in **Figure 1** with the identifications being based on the following criteria.

Peaks 1−4 (R_t 20.4, 22.9, 25.1, and 25.9 min) had a $[M-H]^-$ at m/z 449, which produced MS^2 ions at m/z 359 and 329, characteristic of the fragmentation of a *C*-linked glycoside, with losses

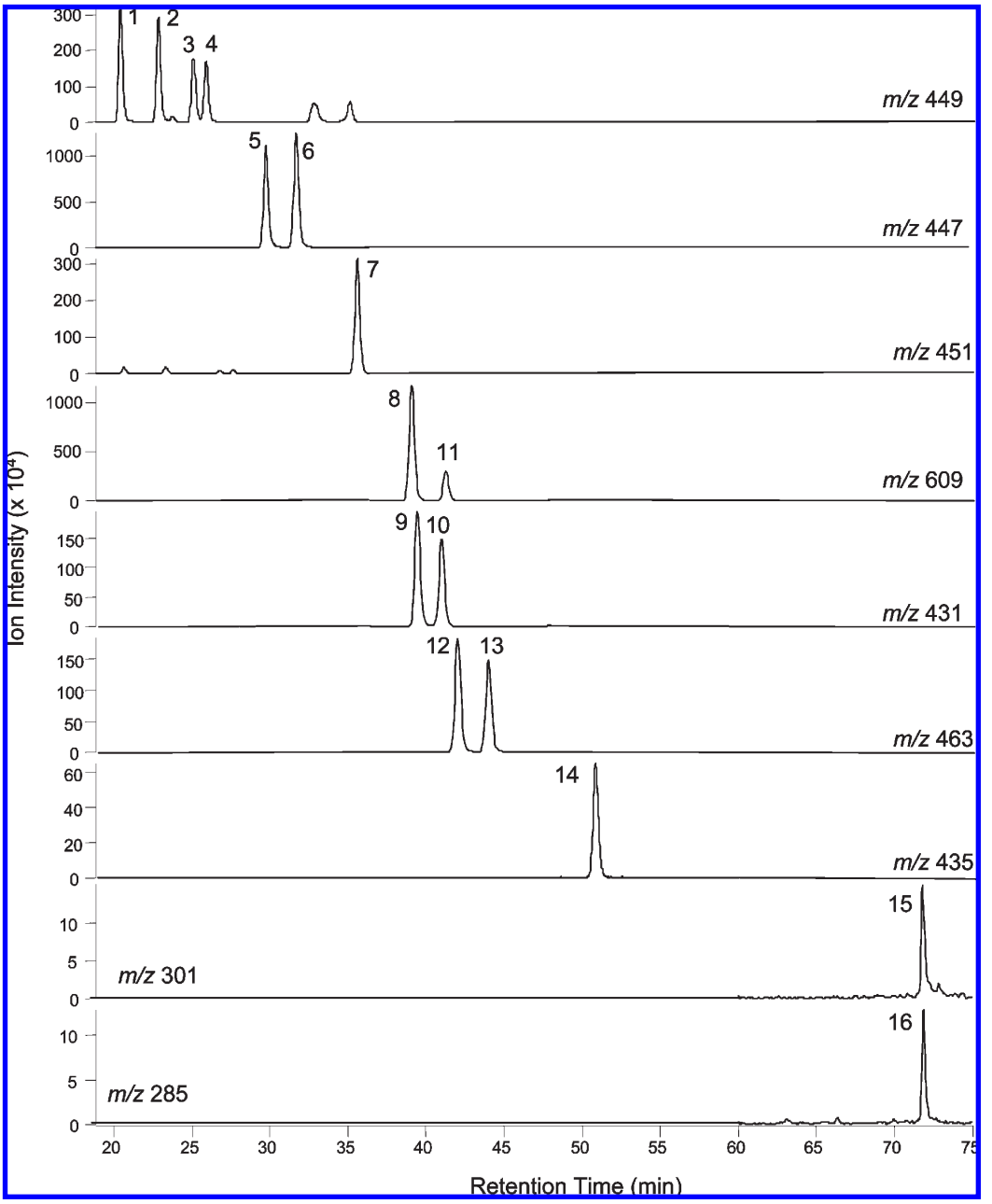

Figure 1. HPLC-SIM traces obtained with the analysis of fermented rooibos tea. Ions monitored as follows: *m/z* 449, eriodictyol-*C*-glucosides; *m/z* 447, luteolin-*C*-glucosides; *m/z* 451, aspalathin; *m/z* 609, quercetin-*O*-rutinosides; *m/z* 431, apigenin-*C*-glucosides; *m/z* 463, quercetin-3-*O*-galactoside/glucoside; *m/z* 435, nothofagin; *m/z* 301, quercetin; and *m/z* 285, luteolin. For peak numbers, see **Table 1**.

of 90 (*m/z* 359) and 120 amu (*m/z* 329) (*9*). In keeping with this fragmentation pattern, peaks 1−4 were tentatively identified as diastereomeric isomers of eriodictyol-*C*-glucosides. The reversed-phase HPLC elution order reported by Krafczyk and Glomb (*3*) when analyzing rooibos tea was first (*S*)-eriodictyol-6-*C*-glucoside (**I** in **Figure 2**) followed by (*R*)-eriodictyol-6-*C*-glucoside (**II**), (*S*)-eriodictyol-8-*C*-glucoside (**III**), and finally (*R*)-eriodictyol-8-*C*-glucoside (**IV**). However, as a C_{12} rather than a C_{18} reversed-phase HPLC support from a different manufacturer was used in the current study, the elution sequence is not necessarily the same as that obtained by Krafczyk and Glomb (*3*).

Peak 5 (R_t 29.7 min) cochromatographed with luteolin-6-*C*-glucoside (isoorientin) (**V**) and had the same MS^2 fragmentation pattern as the reference compound, with a $[M-H]^-$ at *m/z* 447 producing MS^2 ions at *m/z* 429, 357, and 327, in keeping with the data of March et al. (*10*). Peak 5 was therefore identified as luteolin-6-*C*-glucoside.

Peak 6 (R_t 31.6 min) cochromatographed with a standard of luteolin-8-*C*-glucoside (orientin) (**VI**) and had an identical MS^2 fragmentation pattern with a $[M-H]^-$ at *m/z* 447 yielding MS^2 ions at *m/z* 357 and 327. Peak 6 is therefore luteolin-8-*C*-glucoside.

Peak 7 (R_t 35.5 min) had a $[M-H]^-$ at *m/z* 451, which upon fragmentation produced ions at *m/z* 361 and 331, characteristic of fragmentation of *C*-linked glycoside (*11*), with a loss of 120 amu (*m/z* 331) and a loss of 90 amu (*m/z* 361). On the basis of cochromatography with reference compound and a matching MS fragmentation pattern, peak 7 was identified as 2′,3,4,4′,6′-pentahydroxy-dihydrochalcone-3′-*C*-glucoside (aspalathin) (**VII**).

Peaks 8 and 11 (R_t 39.0 and 41.1 min) both had a $[M-H]^-$ at *m/z* 609 that produced daughter ions at *m/z* 301, 300, 271, and 255. Peak 11 had the same retention time as a standard of

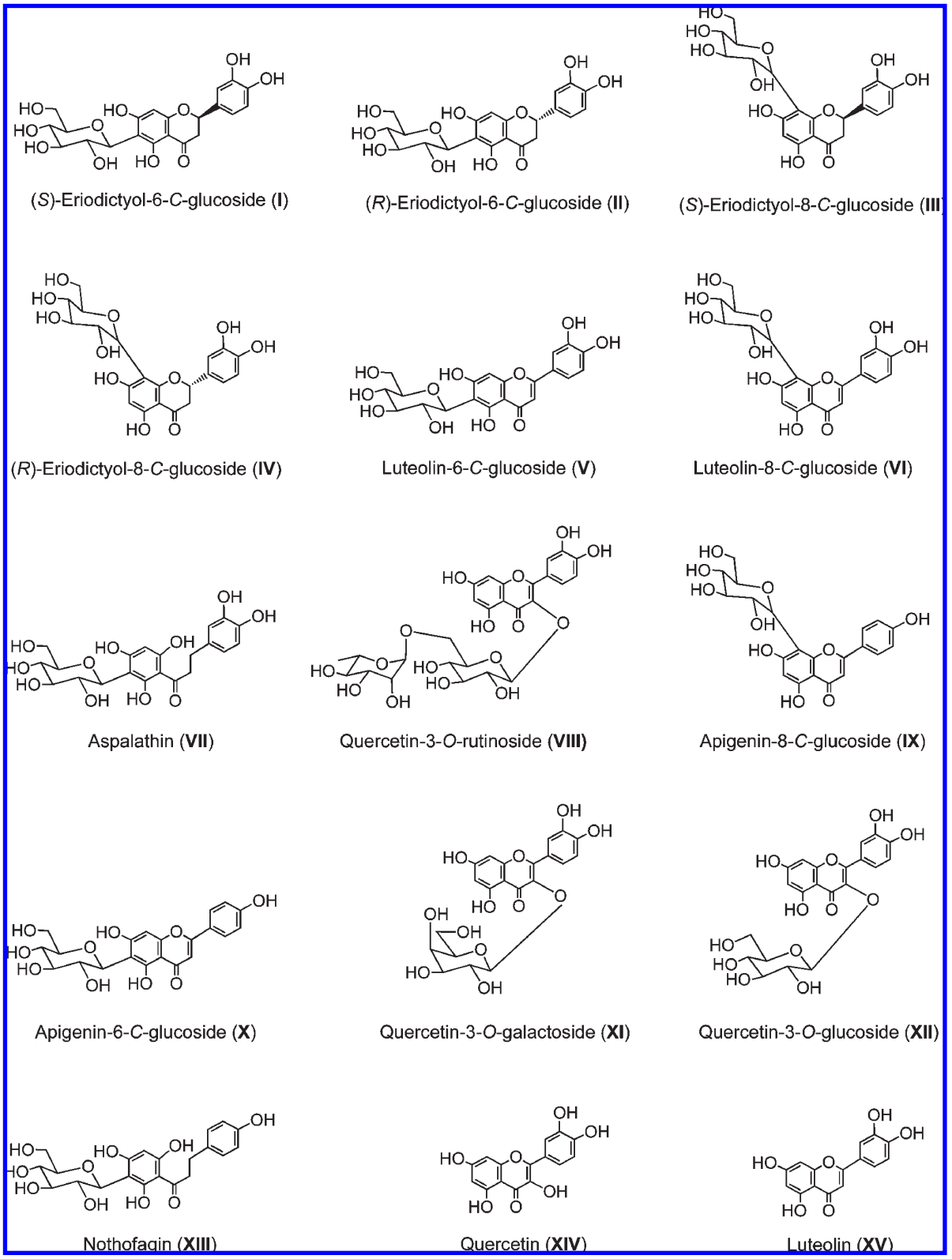

Figure 2. Structures of rooibos tea flavonoids and their urinary metabolites.

quercetin-3-*O*-rutinoside (**VIII**). Peak 8, however, did not chromatograph with a quercetin-3-*O*-rutinoside standard but had the same MS fragmentation pattern. Therefore, peak 8 was identified as an isomer of quercetin-3-*O*-rutinoside.

Peak 9 (R_t 39.4 min) had a $[M-H]^-$ at m/z 431, which gave daughter ions at m/z 341 and 311. Peak 9 was identified as apigenin-8-*C*-glucoside (vitexin) (**IX**) on the basis of a matching MS fragmentation pattern and cochromatography with a standard.

Peak 10 (R_t 41.0 min) cochromatographed with a standard of apigenin-6-*C*-glucoside (isovitexin) (**X**), with fragmentation of the $[M-H]^-$ at m/z 431 producing ions at m/z 413, 341, and 311. Peak 10 was therefore identified as apigenin-6-*C*-glucoside.

Peak 12 (R_t 42.0 min) was quercetin-3-*O*-galactoside (**XI**) as it had a $[M-H]^-$ at m/z 463, which upon MS^2 produced an ion at m/z 301 in keeping with a reference compound with which it cochromatographed.

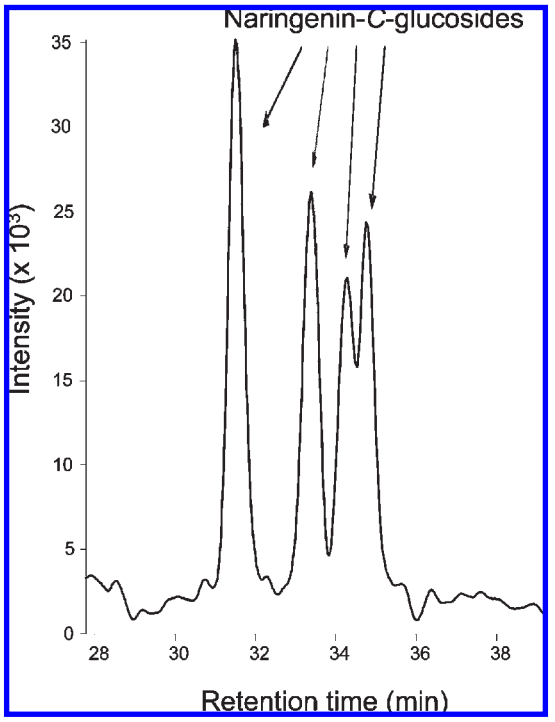

Figure 3. HPLC-SRM trace of the analysis of fermented rooibos tea obtained by monitoring the ion at *m/z* 433, which upon MS^2 produced ions at *m/z* 343 and 313, thereby tentatively identifying naringenin-*C*-glucoside isomers, potential oxidation products of nothofagin.

Peak 13 (R_t 43.9 min) had a $[M-H]^-$ at *m/z* 463, which fragmented upon MS^2 yielding a daughter ion at *m/z* 301. On the basis of this mass spectrum and cochromatography with a standard, peak 13 was identified as quercetin-3-*O*-glucoside (**XII**).

Peak 14 (R_t 50.8 min) had a $[M-H]^-$ at *m/z* 435, which upon MS^2 yielded ions at *m/z* 345 and 315, indicative of the fragmentation of a *C*-linked glycoside (*11*), with a loss of 120 amu (*m/z* 315) and a loss of 90 amu (*m/z* 345), as described by Kazuno et al. (*9*). Peak 14 was therefore tentatively identified as 2′,4,4′,6′-tetrahydroxydihydrochalcone-3′-*C*-glucoside (nothofagin) (**XIII**), a known component of rooibos tea (*1*, *2*).

Peak 15 (R_t 71.8 min) cochromatographed with a standard of quercetin (**XIV**) and had an identical MS fragmentation pattern with a $[M-H]^-$ at *m/z* 301, which gave daughter ions at *m/z* 179 and 151. Peak 15 is therefore the flavonol aglycone quercetin.

Peak 16 (R_t 71.8 min) cochromatographed with a standard of luteolin (**XV**), with a fragmentation pattern of the $[M-H]^-$ at *m/z* 285 producing a MS^2 ion at *m/z* 241. Peak 16 was therefore identified as the flavone aglycone luteolin.

Other compounds with an $[M-H]^-$ at *m/z* 433 producing MS^2 ions at *m/z* 343 and 313 were detected at R_t 31.5, 33.4, 34.3, and 34.8 min (**Figure 3**). As with peaks 1−4, these ions are characteristic of the MS^2 fragmentation of a *C*-linked glycoside (*9*), with losses of 90 (*m/z* 343) and 120 amu (*m/z* 313), and were tentatively identified as naringenin-*C*-glucoside diastereomeric isomers, which would be produced by the oxidation of nothofagin (*3*). These compounds were not quantified as they were present in only trace quantities.

The quantities of flavonoids identified in the rooibos teas are presented in **Table 2**. The concentration of *C*-glycosylated dihydrochalcones, *C*- and *O*-glycosylated flavonoids, as well as free flavonoids (luteolin and quercetin) was found in the range of 84 ± 2.9 μmol in the fermented beverage to 159 ± 6.5 μmol in the unfermented beverage. The amounts of most of the glycosylated flavonoids in the two drinks were similar with the main difference lying in the amounts of aspalathin and nothofagin, which in the fermented tea was ca. 8% of the content in the unfermented drink. The eriodictyol-*C*-glucosides, oxidation products of aspalathin, were 4-fold higher in the fermented tea. The analysis of urinary excretion products and the search for plasma metabolites focused primarily on the detection of aspalathin, nothofagin, eriodictyol, and luteolin-*C*-6- and *C*-8-glucoside metabolites.

Table 2. Quantification of Flavonoids in 500 mL of Unfermented and Fermented Rooibos Teas[a]

	unfermented		fermented	
flavonoids	μmol	mg	μmol	mg
aspalathin	90 ± 4.4	41 ± 2.0	8.0 ± 0.6	3.6 ± 0.3
nothofagin	16 ± 0.9	7.0 ± 0.4	1.9 ± 0.1	0.8 ± 0.0
eriodictyol-*C*-glucoside	2.0 ± 0.0	0.9 ± 0.0	7.4 ± 0.1	3.3 ± 0.0
eriodictyol-*C*-glucoside	1.8 ± 0.0	0.8 ± 0.0	7.1 ± 0.1	3.2 ± 0.0
eriodictyol-*C*-glucoside	0.9 ± 0.0	0.4 ± 0.0	4.4 ± 0.1	2.0 ± 0.1
eriodictyol-*C*-glucoside	0.8 ± 0.0	0.4 ± 0.0	4.2 ± 0.0	1.9 ± 0.0
luteolin-8-*C*-glucoside	15 ± 0.5	6.6 ± 0.2	16 ± 0.6	7.3 ± 0.3
luteolin-6-*C*-glucoside	13 ± 0.5	5.6 ± 0.2	12 ± 0.5	5.3 ± 0.2
apigenin-8-*C*-glucoside	2.2 ± 0.1	1.0 ± 0.0	2.7 ± 0.1	1.2 ± 0.0
apigenin-6-*C*-glucoside	2.3 ± 0.1	1.0 ± 0.0	2.2 ± 0.1	1.0 ± 0.0
quercetin-3-*O*-galactoside	1.5 ± 0.0	0.7 ± 0.0	2.4 ± 0.1	1.1 ± 0.1
quercetin-3-*O*-glucoside	2.5 ± 0.1	1.2 ± 0.0	2.2 ± 0.1	1.0 ± 0.1
quercetin-3-*O*-rutinoside	4.0 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	1.6 ± 0.1
quercetin-*O*-rutinoside isomer	7.1 ± 0.2	4.3 ± 0.1	10 ± 0.4	5.9 ± 0.2
luteolin	0.5 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.1 ± 0.0
quercetin	0.1 ± 0.0	0.0 ± 0.0	1.0 ± 0.1	0.3 ± 0.0
total	159 ± 6.5	73 ± 3.0	84 ± 2.9	40 ± 1.4

[a] Data are expressed as mean values ± SE (n = 3).

Analysis of Urine. *Identification of Metabolites*. The identification of the urinary metabolites excreted 0−24 h after the ingestion of the rooibos teas was carried out using full scan data-dependent MS^2 and targeted MS^n analysis.

Table 3 summarizes the retention time, $[M-H]^-$, as well as MS^2 fragmentation pattern of metabolites detected in urine samples. Typical HPLC-SRM traces are illustrated in **Figure 4**. On the basis of the data in **Figure 4** and **Table 3**, the urinary metabolites were partially identified as follows.

Peaks 1 and 4 (R_t 17.5 and 20.0 min) both had a $[M-H]^-$ at *m/z* 627, which upon MS^2 fragmentation produced ions at *m/z* 537 and 507. This fragmentation pattern corresponds to the loss of 90 and 120 amu, respectively, in keeping with that of a *C*-linked glycoside. The neutral loss of 176 amu ($[M-H-176]^-$) is due to cleavage of a glucuronide moiety to *m/z* 451. Therefore, in keeping with the data of Kreuz et al. (*12*), peaks 1 and 4 were tentatively identified as aspalathin-*O*-glucuronides.

Peaks 2, 3, and 5 (R_t 18.0, 20.0, and 23.8 min) all had a $[M-H]^-$ at *m/z* 641 but produced different ions upon MS^2 fragmentation. Peak 2 had MS^2 ions at *m/z* 551, 521, 465, 345, and 375, whereas peaks 3 and 5 only produced MS^2 ions at *m/z* 551 and 521. The ions at *m/z* 551 and 521 are consistent with the fragmentation of a *C*-glycoside. The ion at *m/z* 465 is from the neutral loss of 176 amu, typical of a glucuronide moiety. These ions are consistent with those seen in peaks 1 and 4, although 14 amu higher, which suggests a methyl derivative of these peaks. Further fragmentation ions, only seen in peak 2, are losses of 90 and 120 amu, again typical fragments seen from *C*-glycoside compounds. This fragmentation pattern has been described by Kreuz et al. (*12*). The difference in the MS fragmentation between peak 2 and peaks 3 and 5 may be attributed to a different positioning of the methyl and glucuronide moieties on the aspalathin molecule, resulting in different MS^2 fragmentations of the parent ion at *m/z* 641. Peaks 2, 3, and 5 were therefore identified as *O*-methyl-aspalathin-*O*-glucuronides.

Table 3. Tentative Identification of Flavonoid Metabolites in 0−24 h Urine Following the Ingestion of 500 mL of Rooibos Tea[a]

peak no.	R_t (min)	$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	metabolites
1	17.5	627	537, 507	aspalathin-*O*-GlcA
2	18.0	641	551, 521, 465, 375, 345	Me-*O*-aspalathin-*O*-GlcA
3	20.0	641	551, 521	Me-*O*-aspalathin-*O*-GlcA
4	20.1	627	537, 507	aspalathin-*O*-GlcA
5	23.8	641	551, 521	Me-*O*-aspalathin-*O*-GlcA
6	28.6	367	287 (MS^3 151)	eriodictyol-*O*-sulfate
7	29.5	545	465 (MS^3 375, 345)	Me-*O*-aspalathin-*O*-sulfate
8	30.3	531	513, 451, 441, 411, 361, 331	aspalathin-*O*-sulfate

[a] GlcA, glucuronic acid; and Me, methyl.

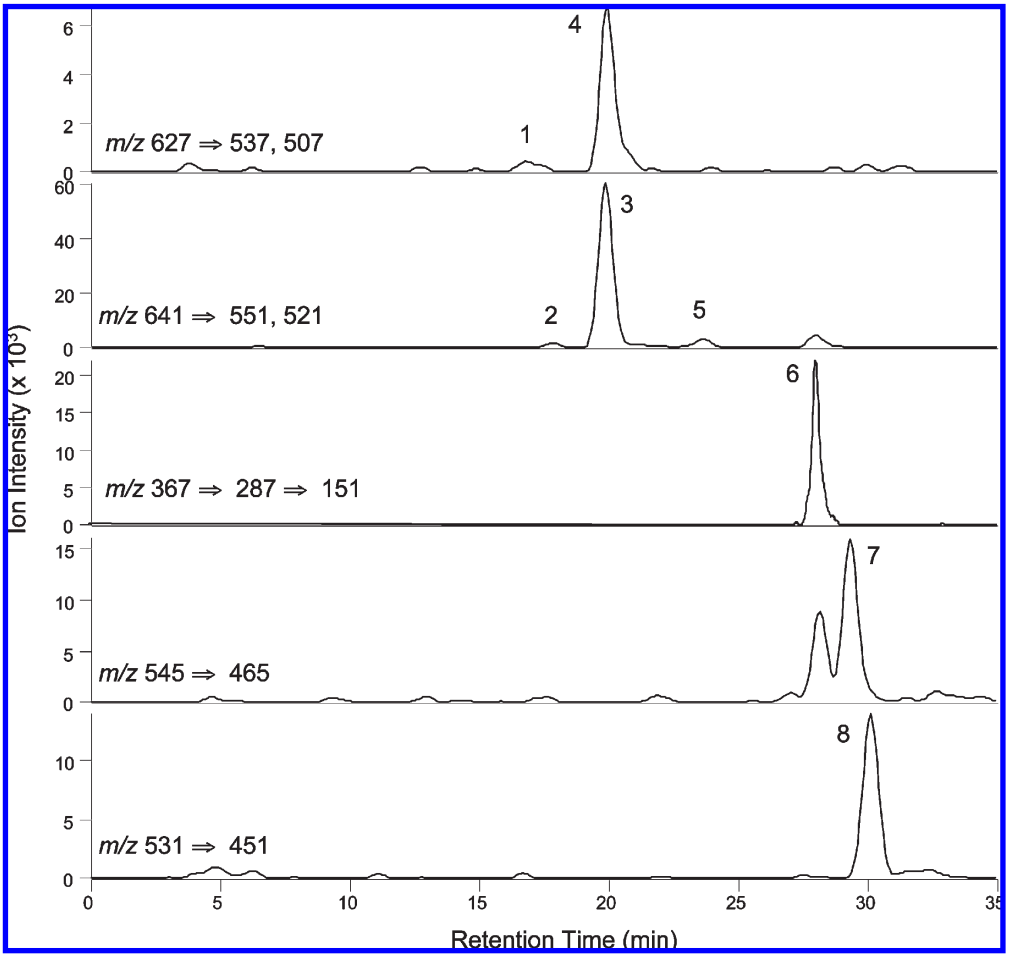

Figure 4. HPLC-SRM traces obtained with the analysis of urine collected after the ingestion of 500 mL of fermented rooibos tea. Ions monitored as follows: *m*/*z* 627 → *m*/*z* 537 and 507, aspalathin-*O*-glucuronides; *m*/*z* 641 → *m*/*z* 551 and 521, methyl-*O*-aspalathin-*O*-glucuronides; *m*/*z* 367 → *m*/*z* 287 and 151, eriodictyol-*O*-sulfates; *m*/*z* 545 → *m*/*z* 465, methyl-*O*-aspalathin-*O*-sulfates; and *m*/*z* 531 → *m*/*z* 451, aspalathin-*O*-sulfates. For peak numbers, see **Table 3**.

Peak 6 (R_t 28.6 min) was characterized by its $[M-H]^-$ at *m*/*z* 367, which upon a loss of 80 amu (sulfate moiety) produced a daughter ion at *m*/*z* 287 and MS^3 ion at *m*/*z* 151, corresponding to the MS^2 profile of a standard of eriodictyol. Peak 6 was thus identified as an eriodictyol-*O*-sulfate. In some volunteers, peak 6 was accompanied by a secondary peak, with a similar MS^3 profile. The separation between the two peaks was difficult to achieve, suggesting that they may be diastereomers, as occurs in the rooibos tea (*3*).

Peak 7 (R_t 29.5 min) was characterized by its $[M-H]^-$ at *m*/*z* 545, which produced a main MS^2 ion at *m*/*z* 465 and MS^3 ions at *m*/*z* 375 and 345, characteristic of methylated aspalathin. The 80 amu loss ($[545-80]^-$) corresponds to a sulfate moiety, indicating that peak 7 is an *O*-methylated aspalathin-*O*-sulfate.

Peak 8 (R_t 30.3 min) was characterized by its $[M-H]^-$ at *m*/*z* 531, which upon a loss of 80 amu (sulfate moiety) produced a daughter ion at *m*/*z* 451. Peak 8 also displayed MS^2 fragments at *m*/*z* 441 and 411 ($[531-90]^-$ and $[531-120]^-$, respectively), as well as the MS^2 ions at *m*/*z* 361 and 331, characteristic of aspalathin. Peak 8 is, therefore, an aspalathin-*O*-sulfate.

Other minor compounds were identified in urine samples of volunteers following the ingestion of the fermented tea. These compounds were tentatively identified as an eriodictyol-*O*-glucuronide (*m*/*z* 463) and eriodictyol-*O*-glucuronide-*O*-sulfate (*m*/*z* 543), as MS^3 and MS^4 fragmentation profiles corresponded to that of MS^2 of a standard of eriodictyol. These compounds were detected in trace amounts and could not, therefore, be accurately quantified. No derivatives or metabolites of nothofagin were detected in urine samples, despite a limit of detection for aspalathin of 1.1 pmol. Likewise, the trace amounts of other glycosylated flavonoids present in the teas did not yield detectable

Table 4. Summary of the Urinary Excretion of Aspalathin and Eriodictyol Metabolites in Urine Collected from 10 Human Volunteers after Drinking 500 mL of Unfermented Rooibos Tea Containing 159 μmol of Flavonoids[a]

metabolites	0−5 h	5−12 h	12−24 h	0−24 h
aspalathin-*O*-GlcA	6.2 ± 1.1	5.0 ± 1.1	1.3 ± 0.6	12 ± 1.6
O-Me-aspalathin-*O*-GlcA	180 ± 22	42 ± 9.1	7.2 ± 2.5	229 ± 26
aspalathin-*O*-sulfate	40 ± 6.6	11 ± 2.3	1.3 ± 1.1	52 ± 6.8
O-Me-aspalathin-*O*-sulfate	20 ± 3.0	2.7 ± 0.9	ND	23 ± 3.2
eriodictyol-*O*-sulfate	10 ± 4.4	22 ± 4.2	3.0 ± 1.3	35 ± 7.3
total	256 ± 29	83 ± 14	13 ± 3.6	352 ± 34 (*0.22%*)

[a] Data are expressed as mean values in nmol ± SEs (n = 10). Italicized data in parentheses indicate 0−24 h excretion as a percentage of flavonoid intake. ND, not detected; GlcA, glucuronic acid; and Me, methyl.

Table 5. Summary of the Urinary Excretion of Aspalathin and Eriodictyol Metabolites in Urine Collected from 10 Human Volunteers after Drinking 500 mL of Fermented Rooibos Tea Containing 84 μmol of Flavonoids[a]

compounds	0−5 h	5−12 h	12−24 h	0−24 h
aspalathin-*O*-GlcA	ND	ND	ND	ND
O-Me-aspalathin-*O*-GlcA	8.8 ± 1.7	1.6 ± 0.6	ND	10 ± 2.0
aspalathin-*O*-sulfate	2.6 ± 0.9	0.2 ± 0.2	ND	2.8 ± 0.9
O-Me-aspalathin-*O*-sulfate	1.5 ± 1.0	ND	ND	1.5 ± 1.0
eriodictyol-*O*-sulfate	3.5 ± 2.4	52 ± 14	12 ± 4.2	68 ± 16
total	16 ± 4.2	54 ± 14	12 ± 4.2	82 ± 18 (*0.09%*)

[a] Data are expressed as mean values in nmol ± SEs (n = 10). Italicized data in parentheses indicate 0−24 h excretion as a percentage of flavonoid intake. ND, not detected; GlcA, glucuronic acid; and Me, methyl.

levels of urinary metabolites. Following identification, 0−24 h urine samples collected after the ingestion of the fermented and unfermented rooibos teas were quantitatively analyzed for aspalathin and eriodictyol metabolites.

Quantification of Metabolites. Excreted compounds were quantified using HPLC-SIM, in urine collected 0−5, 5−12, and 12−24 h following ingestion of 500 mL of unfermented and fermented rooibos drinks. Quantifications from individual volunteers are summarized in **Tables 4** and **5**.

The main metabolites excreted 0−24 h after drinking 500 mL of unfermented rooibos drink were *O*-methyl-aspalathin-*O*-glucuronides, followed by aspalathin-*O*-sulfate, eriodictyol-*O*-sulfate, *O*-methyl-aspalathin-*O*-sulfate, and aspalathin-*O*-glucuronides. It is of note that all four aspalathin metabolites retained their *C*-glucoside structure. A total of 352 nmol were excreted over the 24 h period, accounting for 0.22% of the 159 μmol of flavonoid intake (**Table 4**).

Following the ingestion of 500 mL of fermented tea containing 84 μmol of flavonoids, a total of 82 nmol of metabolites were excreted in 0−24 h urine samples, accounting for 0.09% of intake (**Table 5**). The main metabolites were the eriodictyol-*O*-sulfate (68 nmol), *O*-methylated aspalathin-*O*-glucuronides (10.4 nmol), aspalathin-*O*-sulfate (2.8 nmol), and *O*-methyl-aspalathin-*O*-sulfate (1.5 nmol). No aspalathin-*O*-glucuronide was detected in urine, in contrast to urine collected after drinking the unfermented tea.

Analysis of Plasma. All of the plasma samples were thoroughly analyzed by HPLC-MS^n, but no flavonoids or their metabolites were present in detectable quantities after drinking either the fermented or the unfermented beverages.

DISCUSSION

Analyses of the two rooibos teas, made from unfermented and fermented leaves, revealed the presence of *C*-linked glycosides of luteolin and apigenin, which were in similar concentrations in the two beverages. Other compounds present in low concentrations in both beverages such as quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, an isomer of quercetin-*O*-rutinoside, and free quercetin and luteolin were also quantified. The main difference linked to the fermentation process was in the aspalathin and nothofagin contents, which in the unfermented drink were found to be ca. 10-fold higher than in the fermented beverage (**Table 2**). Krafczyk and Glomb (*3*) reported the presence of four diastereomeric isomers of eriodictyol-*C*-glucosides in a rooibos tea extract, produced from the oxidation of aspalathin. In the current study, the amount of eriodictyol-*C*-glucosides in the fermented tea (23 μmol) was ca. 4-fold higher than in the unfermented drink (5.5 μmol) (**Table 2**).

The very low quantities of luteolin-*C*-glucosides and apigenin-*C*-glucosides and the quercetin glycosides in the beverages (**Table 2**) did not allow the detection of either flavone or flavonol metabolites in urine. However, glucuronides, sulfates, methyl, methyl sulfates, and methyl glucuronides metabolites of aspalathin were detected in urine (**Table 3**). The majority of urinary excretion of the aspalathin metabolites occurred during the first 5 h following intake (80−90% of the total amounts excreted), indicative of small intestine absorption. In total, 317 ± 30 nmol of aspalathin metabolites was excreted following an intake of 90 μmol with unfermented tea (**Table 4**). This represents a recovery of 0.35%. Following the ingestion of 8 μmol of aspalathin with the fermented tea, there was urinary recovery of 15 ± 3.5 nmol of metabolites corresponding to 0.18% of intake (**Table 5**). Aspalathin, therefore, has very limited bioavailability. Incubation of aspalathin with artificial gastric juice for up to 2 h led to the recovery of ca. 100% (data not shown), suggesting that aspalathin is not subject to degradation in the stomach. The very low urinary recoveries of aspalathin metabolites, and their failure to accumulate in detectable quantities in plasma, are probably a consequence of the *C*-glucoside moiety not being readily cleaved by either lactase phloridzin hydrolase or cytosolic β-glycosidase as the dihydrochalcone passes through the small intestine.

Urinary excretion of eriodictyol-*O*-sulfate was also relatively low with recoveries of 0.3 and 0.6%, following consumption of 23 and 5.5 μmol of eriodictyol-*C*-glucosides contained in the fermented and unfermented beverages, respectively. The majority of eriodictyol-*O*-sulfate excretion occurred during 5−12 h following ingestion. This is indicative of low level absorption occurring in the large rather than the small intestine, in which case some of the eriodictyol may have been derived from colonic microflora-mediated biotransformation of aspalathin.

This is the first study to date that investigated the bioavailability of aspalathin in humans. A recent study by Kreutz et al. (*12*) reported urinary excretion of aspalathin in the form of glucuronidated, methyl glucuronidated, methylated, and free aspalathin, as well as dimethyl eriodictyol glucuronide in pigs, following intakes of 15.3 g of aspalathin for 11 days (daily intake equivalent to ca. 400 times that given as a single dose in this study). They reported urinary excretion levels ranging from 0.16 to 0.87% in three pigs after 7 days of treatment, which is in broad agreement with the urinary excretion observed in the current human study. This suggests that limited absorption occurs, regardless of the dose ingested.

In summary, the data obtained in the current study indicate that the dihydrochalcone and flavanone *C*-glucosides in unfermented and fermented rooibos tea are poorly bioavailable with only trace quantities of metabolites being excreted in urine up to 24 h after consumption. The absence of metabolites in plasma in detectable quantities also reflects the low bioavailability of these compounds, coupled with a rapid rate of turnover and removal from the circulatory system. Most of the rooibos flavonoids

almost certainly pass from the small to the large intestine where, when subjected to the action of the colonic microflora, they undergo cleavage of the sugar moiety and ring fission producing low molecular weight phenolic acids. These catabolites are typically absorbed into the portal vein and, after passing through the body, are excreted in substantial amounts (*13*).

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