

Aspalathin, a flavonoid in *Aspalathus linearis* (rooibos), is absorbed by pig intestine as a C-glycoside

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Abstract

Aspalathin, a dihydrochalcone and C-glycoside, is the most abundant flavonoid in rooibos (*Aspalathus linearis*), which is well known as an herbal tea in many countries. Aspalathin appears to have in vitro antioxidative and antimutagenic effects. To understand the effects of aspalathin in the body, research on the absorption in the intestinal tract, metabolism in the body, and identification of circulating metabolites in vivo is required. We investigated the metabolism of aspalathin to identify the parent compound and related metabolites in urine and plasma after orally administering a rooibos extract (16.3% aspalathin by 96 g rooibos extract, which equates to 1.1 kg dried rooibos material), produced from unfermented rooibos plant material, to pigs over a period of 11 days (oral dosage, 157–167 mg aspalathin per kg body weight daily). On days 7 and 11 of the study and days 1 and 2 after termination, urine was collected in 24-hour fractions, and plasma samples were collected at various time points. To our knowledge, this is the first time aspalathin metabolites have been identified in vivo, by presenting evidence of the absorption of aspalathin. Six substances identified in the urine by liquid chromatography–mass spectrometry were identified; these represent aspalathin and the metabolites methylated aspalathin, glucuronidated aspalathin glucuronidated and methylated aspalathin, a glucuronidated aglycone of aspalathin, as well as a metabolite of eriodictyol. The latter compound was methylated and contained 2 glucuronic acid moieties. This study showed that aspalathin can be absorbed by the intestine as C-glycoside as well as being cleaved in an aglycone and sugar moiety. The major metabolite in the enzymatically treated samples was methylated aspalathin. Between 0.1% and 0.9% of the administered dose of aspalathin could be detected in the urine on days 7 and 11 of the feeding study. No metabolites or aspalathin were found in plasma samples. The identification of the metabolites in vivo enables investigations to determine the biological potential of rooibos extracts.

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Abbreviations: CV, coefficient of variation; LC/MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; [M-H]⁻, deprotonated molecular ion; MSⁿ, multistage MS full-scan experiment; MW, molecular weight; RT, retention time; SPE, solid phase extraction

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1. Introduction

Several studies have shown that plant-based diets containing beverages such as tea and food such as fruits and vegetables are associated with a lower risk of chronic diseases, such as cardiovascular disease and some forms of cancer [1–3]. Epidemiologic studies suggest a strong protective effect of fruits and vegetables against strokes, and a weaker protective effect against coronary heart disease [4]. It is assumed that plant-based foods contain phytochemical constituents or “secondary metabolites” such as polyphenols and vitamins responsible for these actions. Demonstration of a possible relationship between a diet rich in flavonoids and a proposed positive health effect requires an understanding of the manner of absorption, metabolism, and transport of the flavonoids.

Aspalathin is a unique flavonoid present in relatively high quantities in the rooibos plant, *Aspalathus linearis*, native to the mountainous area of the western Cape Province of South Africa. Unfermented rooibos plant material contains between 4% and 12% aspalathin. Used as herbal tea, rooibos is very famous in South Africa, as well as in Germany and many other countries. Rooibos tea is also well known for its constitutional effects in various health conditions such as calming digestive disorders and stomach problems, reducing nervous tension, and alleviating allergies [5].

In vivo studies examining the effects of rooibos show different benefits. Inami et al [6] found that rooibos prevented age-related accumulation of lipid peroxidase in rat brain. Marnewick et al [7] observed enhanced phase II enzyme activities and also later described [8] that applying a rooibos extract to mouse skin resulted in a reduced number and volume of skin tumors. This could be due to the main flavonoid, aspalathin, found in rooibos. An aqueous infusion of unfermented rooibos contains 49.9 mg/g aspalathin. However, rooibos contains a great variety of other flavonoids: nothofagin, isoorientin (3.6 mg/g), orientin (2.3 mg/g), vitexin (0.5 mg/g), rutin (1.3 to 1.7 mg/g), isovitexin (0.7 mg/g), isoquercitrin und hyperoside (0.3 to 0.4 mg/g), luteolin (0.02 to 0.03 mg/g), qercetin (0.04 to

0.11 mg/g), and chrysoeriol (0.01 to 0.02 mg/g) [9]. Data on the quantification of nothofagin are rare. In the rooibos plant 10% to 15% compared with the aspalathin amounts are known [10].

However, to date, no in vivo studies with aspalathin and its bioactivity have been carried out. Aspalathin is characterized as a C-glycosyldihydrochalcone [11]. In flavonoid C-glycosides, the sugar is directly linked to the flavonoid nucleus via an acid-resistant and largely enzyme-resistant C-C bond. The structure of aspalathin is shown in Fig. 1A. Its antioxidant activity against the α,α -diphenyl- β -picrylhydrazyl radical is comparable to butylhydroxytoluene, butylhydroxyanisole, and α -tocopherol [12]. Some literature has described the antimutagenic activity of aspalathin [13].

Increasing consumption of rooibos tea is attracting notice to this unique flavonoid. However, absorption from the diet is a prerequisite for a causal relation between aspalathin, and its described physiologic effect as an antioxidative and antimutagenic substance. To appreciate the bioavailability of aspalathin and its biological effect, it is necessary to know what is in blood and its active forms in vivo. Complete identification of the circulating metabolites of aspalathin should help to clarify their actual biological properties. Physiologic similarities between humans and pigs make the latter an excellent model for studying flavonoid metabolism [14].

Flavonoids consumed as glycosides are supposed to be mainly deglycosylated to their respective aglycones in the small intestine by β -glucosidase present on the luminal side of the brush border in the small intestines [15]. During transfer across the small intestine and uptake by the liver, flavonoids undergo *O*-methylation of catechol containing phenolics and glucuronidation [16,17]. Conjugated forms can pass into the bile through enterohepatic circulation and reach the colon. Degradation of the conjugated forms by colonic microorganisms and absorption of the breakdown products (formed via hydrolysis, ring cleavage, and/or dehydroxylation) by the colon can also occur [18]. These flavonoid modifications circulating in the body may change their biological activity [19]. Therefore, regarding the biological effects, it is important to know the circulating forms in vivo. In contrast to the common *O*-glycosides, there is not much known about the absorption and metabolism of C-linked glycosides such as aspalathin. Although the C-C bond between the sugar and flavonoid structure is very resistant to acids and enzymes, Sanugul et al [20] demonstrated cleavage of the C-linked glycoside mangiferin by some intestinal bacteria. The stability of this bond could be one of the limiting factors for the absorption of aspalathin. Concerning the bioavailability of aspalathin, to the best of our knowledge, no studies have been published.

We developed an analytical method for quantifying aspalathin and its metabolites in plasma and urine after oral intake of rooibos extract. This would enable the estimation of metabolism and bioavailability of aspalathin and demonstrate the potential biological activities in vivo.

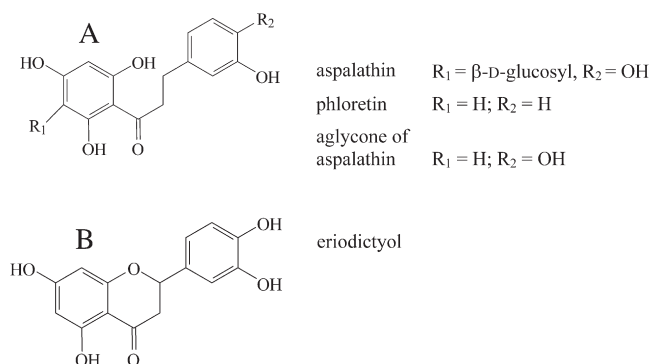


Fig. 1. Chemical structures of aspalathin (MW: 452 g/mol), phloretin (MW: 274 g/mol), aglycone of aspalathin (MW: 290 g/mol) and eriodictyol (MW: 288 g/mol).

This method could be useful for further investigation on the metabolism of aspalathin in humans.

2. Methods and materials

2.1. Animals and diets

The protocol for the animal treatment was approved by the appropriate authority (protocol no. 05-A-13.05) and its conduct under the supervision of the Animal Welfare Officer of the University of Veterinary Medicine Hannover. The feeding experiment was carried out in cooperation with the Clinic of Swine, Small Ruminants, Forensic Medicine and Ambulatory Service of the University of Veterinary Medicine Hannover.

The rooibos extract (16.3% aspalathin) was administered daily to healthy female pigs ($n = 3$, aged 6 months, weighing 94–99 kg) via their feed for 11 days. The amount of the extract (daily dose of the extract, 96 g) was calculated to achieve a daily intake of 157 to 167 mg aspalathin per kg body weight/daily or 15.3 ± 1.2 g (means \pm SD) aspalathin per animal/daily. The rooibos extract was given in addition to the habitual diet of 500 to 800 g of diet. Via long-term exposure, an equilibration between intake of aspalathin and excretion of the metabolites should be reached.

Preparation of the rooibos extract for the administration was achieved as follows: the rooibos extract was dispensed and stirred in warm water for 15 minutes. To avoid oxidation, 3 g of ascorbic acid was added. Afterwards, the dispensed extract was mixed with part of the daily amount of the diet (daily amount, 500–800 g). The daily diet consisted of 79% barley groats, 15% soy groats, 3% mineral diet, and 3% soy bean oil.

To guarantee complete extract intake, the extract-containing feed was offered first thing in the morning after overnight starvation. Water was given ad libitum. The urine was collected completely on days 7 and 11 of the study by using a bladder catheter. The urine was also collected on days 12 and 13 (1 and 2 days after the termination of the feeding study) to obtain data about the excretion duration of aspalathin. Urine blanks were collected via a bladder catheter for 24 hours 3 days before the experiment commenced. Urine volume was determined and an aliquot was frozen at -60°C immediately until needed. Prevention of oxidation was achieved by adding 2% ascorbic acid to each sample (40 mg ascorbic acid per 2 mL of urine).

Blood samples from *vena jugularis* were collected in heparinized tubes after 6 and 10 hours on days 7 and 10 and after 2, 6, and 10 on day 11 after feeding. Additional blood samples were taken 1 day after termination of the study and 26 and 30 hours after last ingestion of the extract. Three days before feeding started, blood samples were taken and used as blanks.

2.2. Chemicals

Rooibos extract, made of unfermented *A linearis* plant material and manufactured by RAPS GmbH and Co KG

(Kulmbach, Germany), according to a patent process (WO 2006/081989 A1), was used for the feeding experiments. The extract was manufactured by solvent extraction with ethanol/water (80:20) of unfermented rooibos plant material (1.2 kg dried rooibos plant material equates to 100 g rooibos extract). After filtration, the aqueous phase was rotary evaporated. The residue was dissolved in ethylacetate and stirred. After filtration, the filter cake was dried and the extract enriched with aspalathin is obtained. This extract contains $16.3\% \pm 1.3\%$ aspalathin as quantified in our laboratory. In comparison, content of aspalathin in unfermented rooibos tea quantified in our laboratory was 1.7 g per 100 g tea material.

Aspalathin was available from the collection of reference materials of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa). β -D-Glucuronidase solution, type HP-2 from helix pomatia with 104 800 U/mL and phloretin were purchased from Sigma (St Louis, Mo). Eriodictyol was purchased from Extrasynthese (Genay, France).

Water was of high-performance liquid chromatography (HPLC) grade and prepared using a Milli-Q-System from Millipore (Eschborn, Germany). Polystyrol divinylbenzol (3 mL/500 mg) cartridges were obtained from Macherey-Nagel (Dueren, Germany). Sample preparation was carried out by using a phosphate buffer (pH 2.4, 75 mmol/L) [21].

L-Ascorbic acid was purchased from Carl Roth GmbH (Karlsruhe, Germany). High-performance liquid chromatography grade methanol and HPLC grade acetonitrile were purchased from Acros Organics (Geel, Belgium). All other chemicals used were of analytical grade.

2.3. Determination of the aspalathin content of the rooibos extract

The aspalathin content of the extract was determined in triplicate: 45 to 65 mg of the extract was dissolved in slightly less than 20 mL warm water, stirred for 10 min, cooled to room temperature, and made up to volume. After centrifugation at 8000g, supernatant was appropriately diluted and injected into the liquid chromatography–mass spectrometry (LC/MS). The determination was achieved via external linear calibration curve ($R^2 = 0.99$). A stock solution of aspalathin was prepared by dissolving the aspalathin standard in methanol, which was then stored at -20°C . Methanolic working solutions were prepared in the range of 1 to 40 $\mu\text{g/mL}$ and used as external standard for quantification. Nothofagin could also be detected. It was not quantified because of the lack of standard.

2.4. Sample preparation

Each collected urine sample was prepared twice: without enzymatic treatment and with enzymatic treatment, to cleave off glucuronic acid from the metabolites. The sample volume, incubation time, and volume of glucuronidase at 36°C were optimized in our laboratory. The enzymatic treatment was optimized by using urine samples after ingestion of the rooibos extract. Optimized conditions were

achieved when no metabolites were present anymore. These conditions should also include an acceptable recovery of aspalathin. For this purpose, blank urine samples were spiked with aspalathin.

Enzymatic treatment of 1 mL urine was carried out by adding 200 μL of sodium acetate buffer (0.78 mol/L, pH 4.8), 20 μL β -D-glucuronidase (2096 U/mL urine), followed by mixing and incubation for 3 hours at 36°C in water bath. After incubation, the urine sample was centrifuged for 2 min at 8000g, and the supernatant further cleaned by solid phase extraction (SPE). Solid phase extraction is very common for sample preparation of urine and plasma [21,22]. This kind of preparation is qualified for separating the metabolites from proteins. The experimental conditions were optimized by testing various solvent systems. A polystyrol divinylbenzol cartridge was conditioned with 4 mL of methanol and 4 mL phosphate buffer before applying the supernatant. The cartridge was subsequently washed with 10 mL phosphate buffer and 2 mL water, and elution was carried out with 5 mL methanol. The methanolic solution was rotary-evaporated to dryness at 50°C and 200 mbar. The residue was dissolved in 250 μL methanol by using an ultrasonic bath. The solution was centrifuged, and the supernatant was stored at -60°C until measurement.

Sample preparation without enzymatic treatment entailed centrifugation of 2 mL urine, whereafter, the supernatant was cleaned by SPE and further treated following the sample procedure as described. The final volume was 500 μL methanol. The recovery for aspalathin after enzymatic treatment was 68.7%, while its coefficient of variation (CV) for aspalathin after enzymatic treatment was $\pm 8.4\%$. The recovery of aspalathin in samples without enzymatic treatment was 76.9%, and the CV was $\pm 9.8\%$. For the preparation of plasma samples, the conditions were based on published methods [15,22,23]. Furthermore, an acceptable recovery of aspalathin was implicated by spiking a plasma blank sample. Experimental conditions were varied to optimize the conditions of enzymatic treatment.

The blood samples collected in heparinized tubes were centrifuged at 8000g to obtain plasma. Ascorbic acid (2%) was added directly before sample preparation. The plasma preparation was carried out following the method published by Erlund et al [21]. Each collected plasma sample was prepared twice: without enzymatic treatment and with enzymatic treatment, to cleave off glucuronic acid from the metabolites. Enzymatic treatment was carried out by incubating 450 μL plasma with 100 μL sodium acetate buffer, 20 μL β -D-glucuronidase (4611.2 U/mL plasma) for 1 hours at 36°C in water bath. After incubation, the sample was passed through a polystyrol divinylbenzol cartridge that had been preconditioned with 4 mL methanol and 4 mL phosphate buffer.

The SPE cartridge was washed with 10 mL phosphate buffer and 2 mL water. The sample was then eluted with 5 mL methanol into a pointed flask and rotary-evaporated to dryness. The residue was dissolved in 225 μL methanol by

using an ultrasonic bath. The solution was centrifuged and the supernatant stored at -60°C until measurement. Plasma (1 mL) without enzymatic treatment was added to a polystyrol divinylbenzol cartridge and cleaned following the same procedure as for the enzyme-treated plasma. The final sample volume was 500 μL methanol.

The recovery of aspalathin after enzymatic treatment for plasma at a concentration of 5 $\mu\text{g}/\text{mL}$ was 102.6%. The CV for aspalathin after enzymatic treatment was $\pm 11.9\%$. Without enzymatic treatment, the CV for aspalathin at a concentration of 5 $\mu\text{g}/\text{mL}$ was 3.3%, and the recovery was $>85.6\%$.

2.5. High-performance liquid chromatography and mass spectrometry

Mass spectrometry was carried out using an LCQ Ion trap with an electrospray ionization source from Thermo Finnigan (San Jose, Calif). The electrospray ionization source was optimized by using a 50- $\mu\text{g}/\text{mL}$ standard solution of aspalathin. Before being introduced into the MS detector, the tuning solution delivered by a syringe pump was combined through a peek tee union with the eluent in the initial constitution and a flow of 0.25 mL/min. The source polarity was set negatively, and the spray needle voltage was 4.5 kV. Sheath gas was nitrogen-generated from pressurized air in an Ecoinert 2 ESP nitrogen generator from DWT GmbH (Gelsenkirchen, Germany). The conditions were as follows: sheath gas 60 U, auxiliary gas 20 U, and capillary temperature 230°C. The divert valve was set to introduce the eluent flow from 1 to 29.6 min to the MS detector, with the other eluent flowing to waste. The deprotonated molecules exhibiting the same molecular mass as the detected metabolites were selected with an isolation width of m/z 1.

The HPLC system was a Waters system (Milford, Mass), consisting of a 616 Pump, a 600S Controller, and a 717 plus Autosampler. Separation was achieved on an Ascentis RP-Amide column (5 μm , 150 \times 2.1 mm, Supelco, Bellefonte, Pa) operated at 23°C. The flow was set at 0.25 mL/min. The mobile phase consisted of methanol (solvent A), acetonitrile (solvent B), and 0.2% acetic acid in water (solvent C). The gradient was 86% solvent C and 11% solvent B. Solvent A was 3% during the 38 min of the HPLC run. The amount of B was linearly increased to 22% within 5 min, then linearly increased within the next 3 min to 38% and finally raised to 58% within 7 min. Afterward, the amount of solvent C was kept constant for 7 min and then further raised to 74% within 3 min. Finally, solvent B content was lowered to the initial 11% over 3 min and the column equilibrated for 10 min.

The limit of detection (LOD) for aspalathin in urine without enzymatic treatment for aspalathin was 1.5 $\mu\text{g}/\text{mL}$ and the limit of quantification (LOQ) was 3 $\mu\text{g}/\text{mL}$ (CV 10.6%). The LOD in urine after enzymatic treatment was 0.3 $\mu\text{g}/\text{mL}$ and the LOQ was 2 $\mu\text{g}/\text{mL}$ (CV 5.0%). The LOD in plasma with and without enzymatic treatment was 0.3 $\mu\text{g}/\text{mL}$. The LOQ was 0.5 $\mu\text{g}/\text{mL}$ (CV 8.4%) for both. The calibration curve of aspalathin was linear in the range of 0.5 to 50 $\mu\text{g}/\text{mL}$ ($R^2 = 0.99$).

2.6. Mass spectrometric experiments

The prepared samples were measured both in full scan mode and by using a data-dependent mode. The experimenting cycle in the ion trap consisted of three scan events. In the first scan event (MS), the mass spectrometer performed a full scan (180 to 1300 m/z). The second scan event (MS²) was a full scan of the most intense ion created by the first scan event.

This precursor ion produced a product ion mass spectrum. In the third scan event (MS³), a full scan on the most intense ion of the second scan event was performed. This cycle was continuously repeated during the HPLC run. The normalized collision energy was set to 40% for the second and third scan events. By carrying out MS, MS², and MS³ experiments together with determination of retention time (RT) and molecular weight (MW), significant structural information was obtained.

2.7. Quantification of metabolites in urine

Quantitative estimation of the metabolites was carried out to calculate the absorption of aspalathin. For this purpose, the urine was treated with β -D-glucuronidase. Glucuronidated aspalathin could be converted to unconjugated aspalathin and be determined as the latter one. References for the methylated metabolites, which were not converted via enzymatic treatment and for the aglycon, were not available. Aspalathin was used to quantify the detected metabolites due to the lack of standard substances. Regarding structural similarities, similar response can be assumed. The samples were processed as reported in Section 2.4. After injecting the final solution in the LC/MS, qualification was carried out as described in Section 3.2. The quantification of the metabolites was carried out via an external linear 5-point calibration curve of aspalathin ($R^2 = 0.99$). Errors were calculated by the confidence limits of the regression line.

2.8. Statistical analysis

The results were expressed as their means \pm SDs and the CVs were presented. The data for determination of the aspalathin content in the extract and the recoveries were exposed to Grubbs outlier test ($P < .05$) using Microsoft Excel 2003 (Microsoft Germany, Unterschleißheim, Germany). The errors (Tables 4 and 5) were estimated from the 95% confidence intervals of the calibration curves.

3. Results

Important phase II metabolic reactions in which many xenobiotics are converted to more hydrophilic compounds also result in an increase in the MW of the substances. Mass shifts for some common biotransformations are glucuronidation ($M + 176$), methylation ($M + 14$), sulfation ($M + 80$), and hydroxylation ($M + 16$). Because a similar metabolism for aspalathin compared with other flavonoids can be expected, identification of the metabolites was realized

with a self-produced list showing all masses of aspalathin, its aglycone, eriodictyol, eriodictyolglucopyranosid, and phloretin, combined with the possible biotransformation mass shifts resulting from conjugation in the body. This list includes biotransformation mass shifts, which were assumed for the conjugation of the hydroxylic groups of the aglycon. Further metabolic reaction could be the cleavage of aspalathin in the small intestine into the sugar moiety and the aglycone. After absorption from the intestine the aglycone of aspalathin (289 m/z in full scan) could also undergo further biotransformation in the liver cells.

3.1. Standard substances

Based on of the analytical method described above, the standards were analyzed and measured in both full scan and in the data-dependent mode to characterize metabolites. In full scan mode, the standard and the metabolites appeared as deprotonated ion $[M-H]^-$. $[M-H]^-$ 451 m/z was measured as the deprotonated molecule ion of aspalathin. The fragmentation pattern for aspalathin determined in our laboratory has already been reported in the literature [24].

Table 1 presents the fragmentation patterns of the standard substances used. The collision energy was set at 40%. The collision of the aspalathin parent ion $[M-H]^-$ 451 m/z with helium in the ion trap leads to the characteristic fragments 331 m/z and 361 m/z . These fragments are characteristic product ions formed by cross ring cleavages of flavonoid C-C glucosides in the hexose residue [25]. Loss of 120 m/z and 90 m/z in MS² mode is the typical fragmentation for C-glycosides. Eriodictyol-6-C- β -D-glucopyranoside is formed via the oxidative cyclization of the dihydrochalcone aspalathin under fermentation conditions (oxidative process) [26]. It can be assumed that aspalathin undergoes oxidation in the body as well. Therefore, it was of interest to investigate the possible presence of eriodictyol-6-C- β -D-glucopyranoside and its metabolites in the samples. Eriodictyol is shown in Fig. 1B. Shimamura et al [27] demonstrated the presence of eriodictyol glucosides in the rooibos plant. Therefore, the assumed presence of eriodictyol cannot be exclusively related to aspalathin, although no eriodictyol-glucosides were determined in the extract.

Table 1

Results of the data dependent experiments carried out with the standard substances; the collision energy was set at 40%

Standard substance	Aspalathin	Eriodictyol	Phloretin
RT (min)	11.9 \pm 0.6 ^a	19.5 \pm 0.2 ^b	22.5 \pm 0.2 ^c
means \pm SD			
$[M-H]^-$ m/z	451	287	273
MS ² spectrum m/z	331, 361	151, 135	167, 123
MS ³ spectrum m/z	209, 167	107	123

The most intense ion in the MS² mode is the m/z in bold which produces the MS³ spectra.

^a n = 6.

^b n = 8.

^c n = 14.

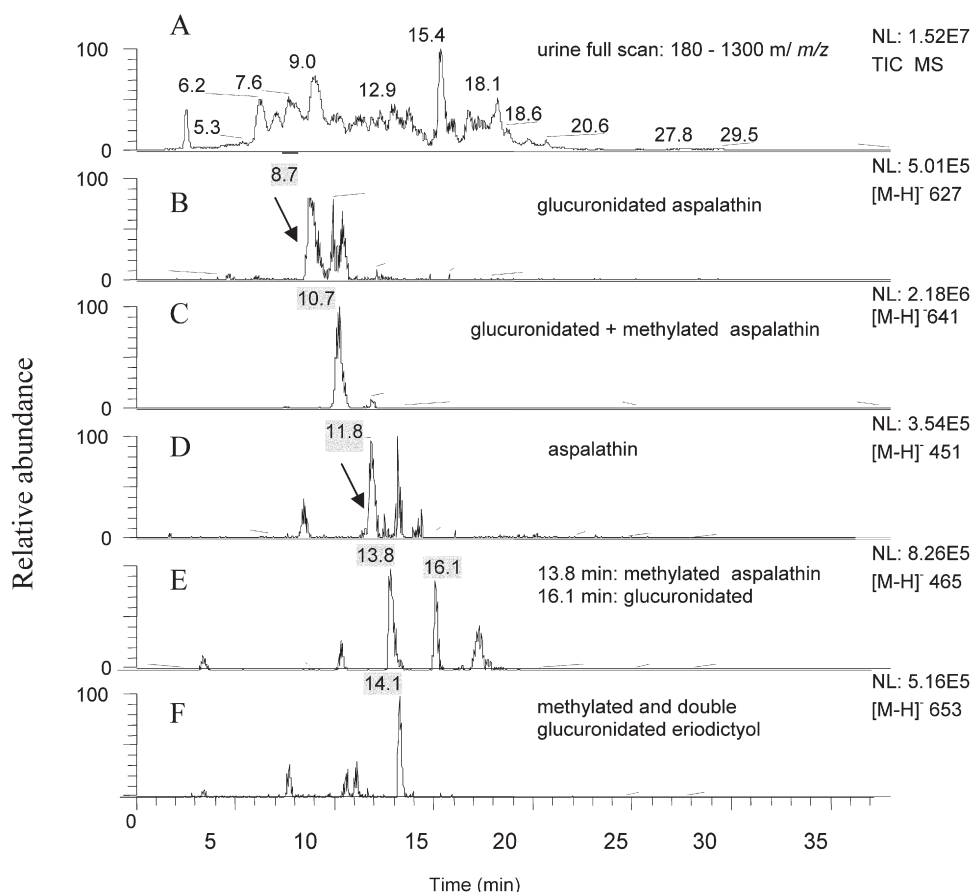


Fig. 2. A typical urine sample after oral ingestion of the rooibos extract (16.3 % aspalathin) to pigs collected on day 7 of the study. Total ion current (TIC) of the urine sample without enzymatic treatment (A) and the extracted ion chromatograms of the six metabolites (B – F) are presented: (B) [M-H]⁻ m/z 627 glucuronidated aspalathin, (C) [M-H]⁻ m/z 641 glucuronidated and methylated aspalathin, (D) [M-H]⁻ m/z 451 aspalathin, (E) [M-H]⁻ m/z 465 glucuronidated aglycone and methylated aspalathin, (F) [M-H]⁻ m/z 653 double glucuronidated and methylated eriodictyol. The relative abundance is normalized (NL) to the most abundant peak in the chromatogram.

The characteristic ion for the [M-H]⁻ of eriodictyol is 287 m/z. The collision of the parent ion [M-H]⁻ 287 m/z with helium in the ion trap leads to characteristic fragments, as shown in Table 1. Phloretin, a dihydrochalcone aglycone, was used as a reference substance to confirm the assumptions about the aglycone of aspalathin. Unfortunately, the latter substance was not available, but information was obtained on phloretin, which had only one hydroxyl group less on the

B-ring. The structures of both compounds are shown in Fig. 1A. The [M-H]⁻ for phloretin was measured as 273 m/z and its fragmentation pattern is summarized in Table 1.

3.2. Samples

Fig. 2 shows the total ion current of a typical urine sample without enzymatic treatment (A). The selected ion monitoring (SIM) chromatograms derived from the total ion current

Table 2

Data dependent results of the urine sample without enzymatic treatment, collected on day 7 of the feeding experiment, 0 to 24 hours after ingestion of a rooibos extract (16.3% aspalathin)

Metabolite number	1	2	3	4	5	6
RT (min) (means ± SD)	9.4 ± 0.7 ^a	10.6 ± 0.5 ^a	12.1 ± 0.5 ^a	14.0 ± 0.3 ^a	16.0 - 16.2 ^b	14.3 ± 0.4 ^a
[M-H] ⁻ m/z	627	641	451	465	465	653
MS ² spectrum m/z	537, 507	551, 521	331, 361	345, 375	289	477
MS ³ spectrum m/z	361	375, 345	209, 167	289, 167	209, 167	301

The most intense ion in the MS² mode is the m/z in bold which produces the MS³ spectrum.

The metabolites are 1 glucuronidated aspalathin, 2 glucuronidated and methylated aspalathin, 3 aspalathin, 4 methylated aspalathin, 5 glucuronidated aglycone, 6 double glucuronidated and methylated eriodictyol.

^a n = 4.

^b n = 2.

Table 3
Urinary excretion after feeding a rooibos extract (16.3% aspalathin) to three pigs for 11 days

Metabolite <i>m/z</i>	Day 7			Day 11			Day 12			Day 12			Day 13		
	0-24 h			0-24 h			0-12 h			12-24 h			0-12 h		
	ind 1	ind 2	ind 3	ind 1	ind 2	ind 3	ind 1	ind 2	ind 3	ind 1	ind 2	ind 3	ind 1	ind 2	ind 3
451	p	p	p	p	p	p	p	p	p	p	p	p	nd	nd	nd
465 ¹	p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
465 ²	p	p	p	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
627	p	p	p	p	p	p	p	p	p	nd	p	p	nd	nd	nd
641	p	p	p	p	p	p	p	p	p	nd	p	p	nd	nd	nd
653	p	p	p	p	p	p	p	p	p	nd	p	p	nd	nd	nd

The occurrence of aspalathin and its metabolites on days 7 and 11 is presented. Days 12 and 13 represent the excretion over 24 to 60 hours after the last ingestion of the extract.

ind, individual; p, present; nd, not detectable; 451 *m/z*, aspalathin; 465¹ *m/z*, methylated aspalathin; 465² *m/z*, glucuronidated aglycone; 627 *m/z*, glucuronidated aglycone; 641 *m/z*, glucuronidated and methylated aglycone; 653 *m/z*, double glucuronidated and methylated eriodictyol.

of the identified metabolites are shown in Fig. 2B to F. Six metabolites (glucuronidated aspalathin, glucuronidated and methylated aspalathin, aspalathin, methylated aspalathin [13.8 min], glucuronidated aglycone of aspalathin

[16.1 min], and methylated eriodictyol with two glucuronic acid groups) were detected in the urine samples.

The metabolites with numbers 1 to 4 (Table 2) appeared in the MS² mode cleavage of 90 *m/z* and 120 *m/z* mass units. It

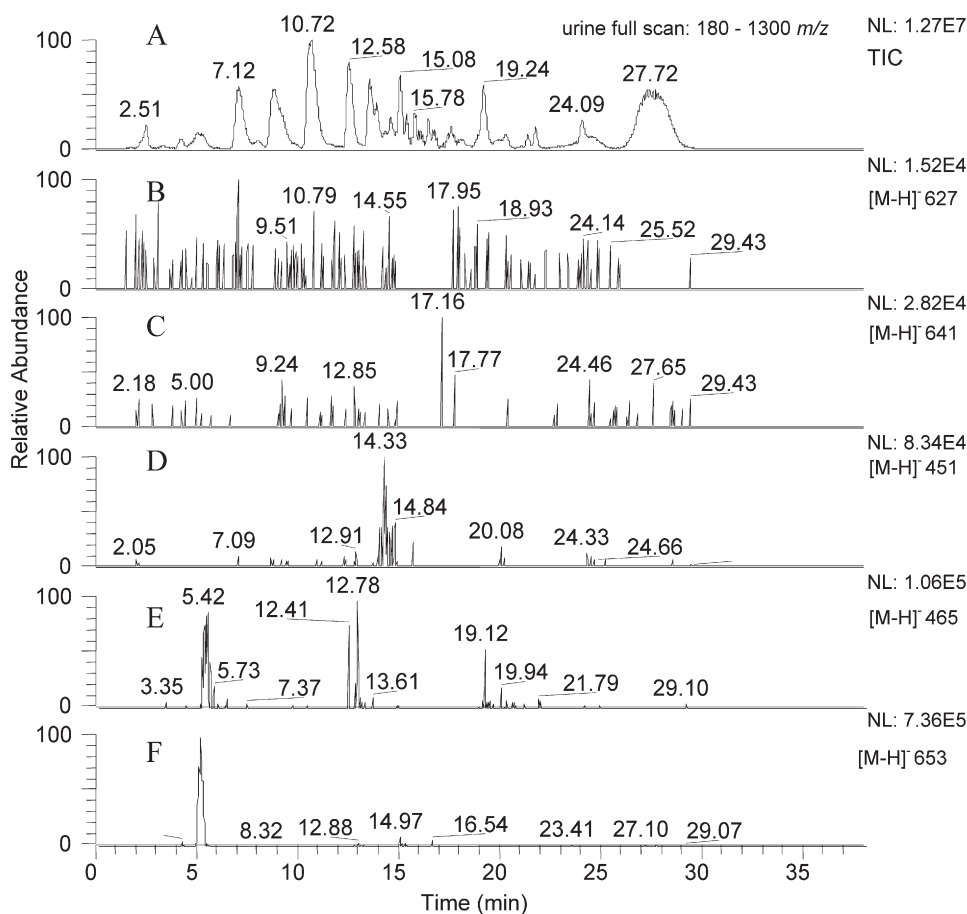


Fig. 3. A typical urine blank sample without enzymatic treatment is presented. The total ion current (A) and (B) - (F) show the extracted ion chromatograms. The [M-H]⁺ of the metabolites which were detected in urine samples without enzymatic treatment after ingestion of a rooibos extract (16.3 % aspalathin) are presented in (B) - (F). No metabolites which were present after rooibos extract intake (compare Fig. 2) could be detected; (B) [M-H]⁺- 627 *m/z* glucuronidated aspalathin, (C) [M-H]⁺- 641 *m/z* glucuronidated and methylated aspalathin, (D) [M-H]⁺- 451 *m/z* aspalathin, (E) [M-H]⁺- 465 *m/z* glucuronidated aglycone and methylated aspalathin, (F) [M-H]⁺- 653 *m/z* double glucuronidated and methylated eriodictyol. The relative abundance is normalized (NL) to the most abundant peak in the chromatogram.

can be proposed that the structure is related to flavonoid C-glycosides. The metabolites with number 5 and 6 did not show this cleavage, indicating a structure without a hexose moiety.

Metabolite 1 with $[M-H]^-$ 627 m/z and a RT of 9.7 min showed a mass shift of 176 m/z in the MS^3 mode. A fragment in the MS^3 was already reported to be from the aspalathin spectrum (Table 1). This molecule $[M-H]^-$ 627 m/z can be referred to as a metabolite of aspalathin and characterized as glucuronidated aspalathin. The molecule was also less retentive than aspalathin (RT 11.9 min vs 9.7 min).

Metabolite 4 with the $[M-H]^-$ 465 m/z and a RT of 13.7 min (Table 2) is characterized as methylated aspalathin. The stronger retention of 465 m/z in relation to aspalathin supports this. Furthermore, the MS^2 spectrum, which showed the same fragments as aspalathin but with a mass shift of 14 m/z : 345 m/z and 375 m/z , also supported the metabolite structure. These fragments seem to be the methylated fragments 331 m/z and 361 m/z shown by the aspalathin spectrum. It does not seem to be easy to split methylated groups off under the MS conditions used. Metabolite 2 with $[M-H]^-$ 641 m/z gave fragments of 120 m/z and 90 m/z in the

MS^2 mode as well. In the MS^3 spectrum the fragments, given by methylated aspalathin ($[M-H]^-$ 465 m/z), appeared. This leads to the supposition that the metabolite with the 641 m/z is a methylated and glucuronidated aspalathin.

Unconjugated aspalathin was found as metabolite in urine as well. This was confirmed by comparing the spectra of compound $[M-H]^-$ 451 m/z with the standard spectra and with the retention time of the standard. The urine samples with enzymatic treatment were used to confirm these results. After deglucuronidation, methylated aspalathin and free aspalathin could be found in the urine. The substances that did not show the loss of 90 m/z and 120 m/z mass units are assumed to be metabolites of the aglycone of aspalathin.

Metabolite 5 with $[M-H]^-$ 465 m/z and a RT of 16 min (Table 2) showed a loss of 176 m/z in the MS^2 mode and occurred at 289 m/z in the spectrum. This points to 465 m/z as being the glucuronidated aglycone of aspalathin. The results of the enzymatic treated urine sample confirmed this: $[M-H]^-$ 289 m/z with a RT of 20 min and a collision energy of 50% shows a fragmentation pattern of 167 m/z , 125 m/z in the MS^2 mode. This compound, which is only present in enzymatic treated urine samples, is assumed to be the

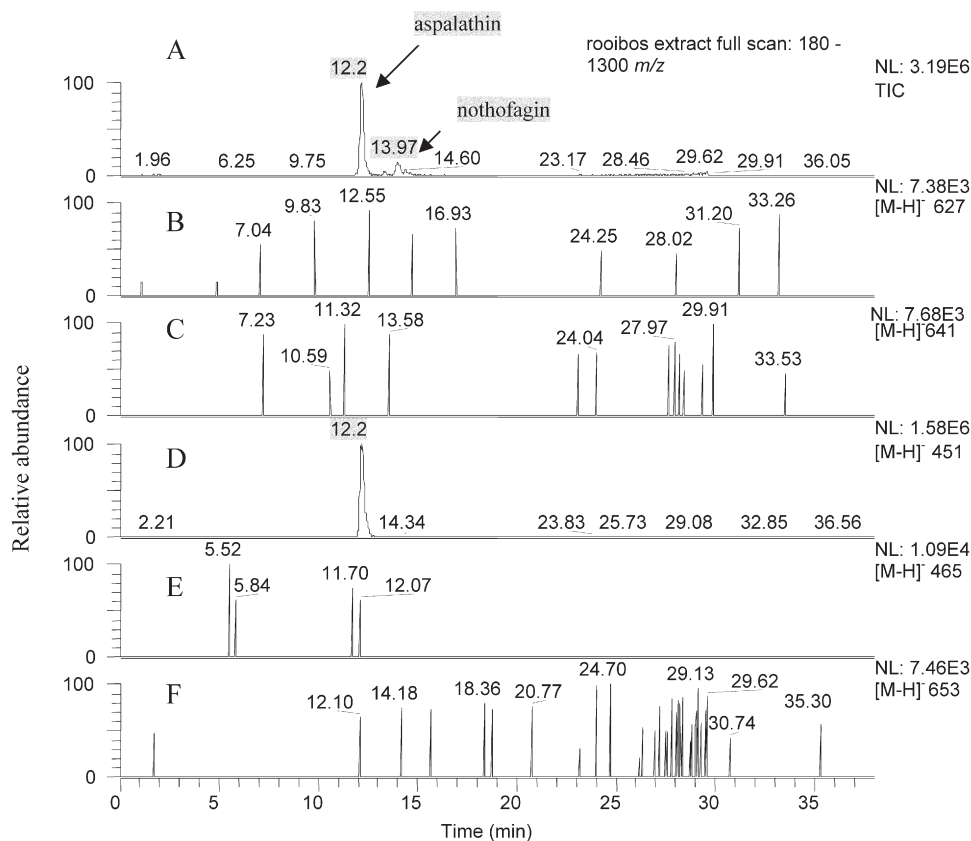


Fig. 4. A typical sample of rooibos extract (16.3 % aspalathin) is presented. (A) shows the total ion current (TIC) and (B) - (F) show the extracted ion chromatograms of the metabolites which were detected in the urine samples without enzymatic treatment after ingestion of a rooibos extract (16.3 % aspalathin). No metabolites which were present after extract intake, could be detected except aspalathin (compare Fig. 2); (B) $[M-H]^-$ 627 m/z glucuronidated aspalathin, (C) $[M-H]^-$ 641 m/z glucuronidated and methylated aspalathin, (D) $[M-H]^-$ 451 m/z aspalathin, (E) $[M-H]^-$ 465 m/z glucuronidated aglycone and methylated aspalathin, (F) $[M-H]^-$ 653 m/z double glucuronidated and methylated eriodictyol. The relative abundance is normalized (NL) to the most abundant peak in the chromatogram.

aglycone of aspalathin. Unfortunately, there was no standard available, but phloretin was used to obtain data on characteristic fragments of the aglycone of aspalathin that could be detected. Characteristic fragments of phloretin (RT 22.5 min) are shown in Table 1. In the MS² spectrum of phloretin 167 *m/z* appeared as shown in the MS² spectrum of 289 *m/z*, the assumed aglycone of aspalathin (data not shown). Compound [M-H]⁻ 289 *m/z* and phloretin had similar retention times with the more hydrophilic 289 *m/z* a bit less retentive than phloretin because of the additional hydroxyl group.

Metabolite 6 with 653 *m/z* showed a cleavage of 176 *m/z* in the MS² as well as in the MS³ mode (Table 2). The metabolite seems to contain two glucuronic acid groups.

In the urine samples treated with enzymes, two metabolites with the 301 *m/z* could be found, suggesting that their formation is a result of enzymatic removal of both glucuronic acid groups of [M-H]⁻ 653 *m/z*. The compounds are assumed to be methylated forms of eriodictyol.

The metabolite with the RT of 20.9 min showed 151 *m/z* and 177 *m/z* in the MS² mode. An MS² spectrum in the negative ion mode with collision-induced dissociation of homoeriodictyol, a methylated form of eriodictyol, was found in literature: 177 *m/z* and 151 *m/z* are created by the dissociation of 301 *m/z* [28]. This metabolite 301 *m/z* with the retention time of 20.9 min found in the enzymatic treated urine samples after rooibos extract intake is assumed to be homoeriodictyol.

The metabolite with the RT of 22.0 min and the 301 *m/z* shows fragments in the MS² of 286 *m/z*, 257 *m/z*. This spectrum is described for hesperetin measured with LC/MS

in negative ion mode and was measured in our laboratory. Thus, methylation could also occur on either of the hydroxyl groups of the B-ring.

The metabolite with 653 *m/z* found in urine without enzymatic treatment is assumed to be a methylated eriodictyol with two glucuronic acid groups. There is no information about the position of the methyl group of the molecule. It is possible that the metabolite with 653 *m/z*, obviously one metabolite, consists of two substances that are methylated in different positions and co-elute in the developed HPLC-method. This can explain the two different 301 *m/z* metabolites in the enzymatic treated samples corresponding to 653 *m/z*.

From the data-dependent experiment, structural information about the substances occurring in the urine was collected. These results are summarized in Table 3. Except for aspalathin itself, the masses of the metabolites did not occur in the mass spectra of the urine blank sample (Fig. 3) as well as of the rooibos extract (Fig. 4) used in the feeding study. On a reversed-phase column with aqueous/organic mobile phase glucuronidated and sulfated compounds, the hydrophilic ones are generally less retentive than their parent compounds. Methylation has the opposite effect [25]. Structural information obtained from the chromatographic retention times supports the information from the LC/MS measurements.

3.3. Excretion duration of the metabolites

Table 3 shows the urinary excretion of the metabolites on the different days. Day 11 was the last feeding day with the rooibos extract made from unfermented raw material. The excretion of aspalathin and its metabolites finished 36 hours

Table 4

Total amounts of aspalathin, methylated aspalathin, aglycone of aspalathin and methylated eriodictyol in urine samples with enzymatic treatment after ingestion of a rooibos extract (16.3% aspalathin) to pigs for 11 days

	Metabolite	Aspalathin [M-H] ⁻	Methylated aspalathin [M-H] ⁻	Aglycone [M-H] ⁻	Methylated eriodictyol [M-H] ⁻
		451 <i>m/z</i>	465 <i>m/z</i>	289 <i>m/z</i>	301 <i>m/z</i>
		μmol/urine fraction	μmol/urine fraction	μmol/urine fraction	μmol/urine fraction
Day 7 0-24 h	ind 1	52.0 ± 21.0	222.4 ± 21.75	<LOQ	29.08 ± 13.6
	ind 2	11.8 ± 3.3	34.7 ± 3.3	<LOQ	8.2 ± 4.9
	ind 3	20.5 ± 4.1	49.0 ± 4.1	<LOQ	<LOQ
Day 11 0-24 h	ind 1	30.21 ± 15.5	140.1 ± 11.3	nd	<LOQ
	ind 2	5.3 ± 1.76	14.0 ± 1.7	nd	9.17 ± 2.6
	ind 3	16.6 ± 3.3	49.68 ± 3.3	nd	14.5 ± 4.9
Day 12 0-12 h	ind 1	<LOQ	55.5 ± 14.2	nd	<LOQ
	ind 2	<LOQ	14.6 ± 4.1	nd	<LOQ
	ind 3	<LOQ	<LOQ	nd	<LOQ
Day 12 12-24 h	ind 1	<LOQ	12.1 ± 6.5	nd	nd
	ind 2	5.6 ± 1.4	20.96 ± 1.5	nd	<LOQ
	ind 3	3.6 ± 1.0	12.5 ± 1.2	nd	0.9 ± 0.3
Day 13 0-12 h	ind 1	nd	<LOQ	nd	nd
	ind 2	nd	<LOQ	nd	nd
	ind 3	nd	<LOQ	nd	nd
Day 13 12-24 h	ind 1	nd	nd	nd	nd
	ind 2	nd	nd	nd	nd
	ind 3	nd	nd	nd	nd

Results are presented for day 7 and day 11 of the feeding study and for day 1 and day 2 after discontinuing the administration. The errors were calculated from the 95% confidence intervals of the calibration curves.

Table 5

Percentage of aspalathin in urine referring to the ingested dose of rooibos extract (16.3% aspalathin) administered to pigs for 11 days

	% Ingested dose of aspalathin	
	Day 7	Day 11
Pig 1	0.87 ± 0.32	0.49 ± 0.02
Pig 2	0.16 ± 0.02	0.08 ± 0.02
Pig 3	0.20 ± 0.02	0.23 ± 0.02

The errors were calculated from the 95% confidence intervals of the calibration curves.

after the last feed. On day 13 (2 days after termination of the study) 36 to 48 hours after the last ingestion of the rooibos extract, no metabolites could be detected. The results presented in Table 3 should support and confirm the quantitative data obtained from the enzymatic treated urine samples shown in Table 4.

3.4. Quantification

Aspalathin, methylated aspalathin, the aglycone of aspalathin, and methylated eriodictyol were identified as urinary metabolites after β -D-glucuronidase treatment. The qualification was confirmed by comparison with the identified metabolites in urine samples without enzymatic treatment, as shown in Table 3. The amounts of these metabolites are shown in Table 4. The percentage of the ingested dose is shown in Table 5. All 3 individuals show the majority of urinary metabolites excreted via C-linked-glycoside, namely, aspalathin and methylated aspalathin.

3.5. Analysis of the plasma samples

The plasma samples taken on days 7 and 10 at 6 and 10 hours after the feeding did not show any metabolites, nor did the samples taken 2, 6, and 10 hours after the feed on day 11. Additional blood samples taken 1 day after termination of the study, 26 and 30 hours after last ingestion of rooibos extract, plasma samples show neither metabolites nor aspalathin.

Preliminary studies indicated that when pigs were given a three times higher single dose of rooibos extract, aspalathin could be detected in the plasma, but its concentration was under the LOQ (data not shown).

4. Discussion

The metabolism of aspalathin was investigated by feeding three pigs rooibos extract made from the unfermented rooibos plant *A. linearis*. To date, no metabolites of aspalathin have been described in vivo. In this study, we identified six different metabolites in the urine. Four of these metabolites were conjugated C-glycosidic forms of aspalathin: aspalathin, glucuronidated aspalathin, methylated aspalathin, and aspalathin that was both glucuronidated and methylated. One metabolite was referred to the aglycone of aspalathin, glucuronidated aglycone (2,3,4,4',6-pentahydroxydihydrochalcon), and one compound could be determined as methylated eriodictyol metabolite, a flavanone, also containing two glucuronic acid groups. These qualified metabolites confirmed the assumption about glucuronidation and methylation of aspalathin in the body. Sulfated metabolites were not detected, bearing in mind the fact that pigs are not able to form sulfated metabolites [29]. The position of glucuronic acid groups and methyl groups could not be obtained from the data. This could be the subject of further characterization studies, including methods such as nuclear magnetic resonance spectroscopy.

It has been reported that eriodictyolglucopyranosid is formed in the fermentation process of rooibos tea via an oxidation of aspalathin [26]. The results of this study led to the assumption that oxidation of the aglycone of aspalathin could generate eriodictyol, known as potent antioxidant in vitro [30], which could undergo further metabolism in the body. Another possibility could be the oxidation of aspalathin to the flavanone glucoside structure, which could be deglycosylated in the intestine. However, it cannot be ruled out that the eriodictyol metabolite could be created by the metabolization of eriodictyol glucosides present in the rooibos plant [27], although this glycoside was not detected in the rooibos extract used in the feeding experiments.

The elimination of aspalathin metabolites was completed 36 hours after the last ingestion of the rooibos extract had been made. The metabolite excreted for the longest time was methylated aspalathin. All three individuals showed the same metabolite composition on feeding days. On the second half of day 12, the metabolite composition showed that individual 1 differed from the other individuals (i.e., only aspalathin and methylated aspalathin were found). This could be due to individual differences in intestinal flora but also to the different habits of the animals due to their unlimited water access. The pattern of metabolites on day 13 was the same for all individuals. The glucuronidated aglycone could only be measured on day 7 for all 3 individuals, showing that the excretion via the aglycone does not play an important role in the aspalathin metabolism. Methylation as well as glucuronidation of the C-linked glycoside seems to be a very important conjugation pathway. This was confirmed by the obtained quantitative data, whereas the majority of metabolites are excreted as aspalathin and methylated aspalathin derivatives. Concerning their known biological properties, aspalathin can act as antioxidant and antimutagen in the body [12,13]. The properties of methylated aspalathin should be investigated in further studies. Between 0.1% and 0.9% of the administered dose of aspalathin could be detected in urine. Individual differences were found between the 3 individuals in the amounts and in the resulting percentage of the ingested aspalathin in the urine samples. This could be due to individual variations of the intestinal bacteria as well as different enzyme activities in the small intestine.

Although no metabolites could be found in plasma as evidence for circulating aspalathin, substances found in the

urine samples can be assumed to be important in the body. The recovery of the identified substances can be estimated as comparable to known excretion rates in urine of quercetin (0.12%) and quercetin-rutinoside (0.07%) in humans [31]. It should be kept in mind that the excretion rate in urine is not necessarily the same as the absorption rate. This can be seen in literature. Hollmann et al [31] investigated quercetin and quercetin-3-rutinoside absorption in healthy ileostomy volunteers. The absorption rate, measured by investigating the ileostomy effluent, showed 17% for quercetin-3-rutinoside and 24% for its aglycone. Walle et al [32] found values for oral absorption of ¹⁴C-quercetin based on measuring the radioactivity (36.4%–53.0%) and urinary excretion (3.3%–5.7%).

However, the recovered dose in urine confirmed the absorption and conjugation of aspalathin in the small intestine as C-glycoside. Sanugul et al [20] demonstrated cleavage of the C-linked glycoside mangiferin by some intestinal bacteria. This can be assumed for the metabolism of aspalathin as well as the glucuronidated aglycone and metabolite of eriodictyol present in urine. Based on the relatively small amounts of aspalathin in urine and the lack of detected metabolites in the plasma, an intensive metabolism in the colon to breakdown products forming lower-molecular-weight phenolics is assumed. These breakdown products can undergo absorption from the colon and undergo metabolization in the liver as well. We did not detect any breakdown product with the introduced LC/MS method. Studies on the absorption of such products with lower MW are needed. For this purpose, gas chromatography coupled with mass spectrometry should be the method of choice, as described in literature [15].

The failed detection of aspalathin in plasma can be for different reasons. Because of an aromatic nucleus and hydroxyl substituents, flavonoids have a great affinity to proteins, particularly albumin (e.g., quercetin is bound to albumin), as found in in vitro studies [23,33]. This can be assumed for in vivo as well [33]. The assumed protein-polyphenol complex circulating in the body presents a depot capable of gradually releasing the polyphenol [34]. The phenomenon of the polyphenol-protein complex could be an important consideration for the failed detection of aspalathin in plasma. Although the determined LOD in spiked and prepared plasma samples of aspalathin amounted to 0.3 µg/mL, low amounts in the plasma of metabolites could have escaped LC/MS detection. This hypothesis is supported by the detection of aspalathin in a three times higher single dose of the extract given to pigs in an earlier study. Aspalathin could be measured under the LOD in plasma samples 5 and 8 hours after ingestion of the extract. Considering that a single administration cannot represent reliable data about metabolism, because of the inexistent equilibration, aspalathin was measured coincidentally. The formation of an aspalathin-protein complex is in agreement with the relatively low amounts detected in urine. However, the obtained results should present the first insight into the fate

of aspalathin in vivo. The restricted number of individuals in this study allows no general statement. Further investigations about possible degradation of products in the body, the excretion via feces, the fate of aspalathin in the blood, and the determination in organs are needed.

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