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Urinary metabolites of French maritime pine bark extract in humans

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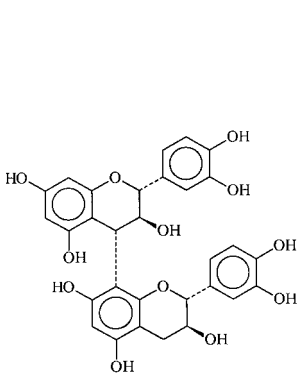
After oral administration of 5.28 g and 1.06 g of French maritime pine bark extract to a human volunteer, metabolites of some of the components of the extract could be detected. Ferulic acid and taxifolin, conjugated as glucuronide/sulphate, were excreted within 18 h. The peak urinary excretion was observed approximately 2–3 h after intake. Recovery of ferulic acid in urine was 36–43% and 7–8% for taxifolin. Two further metabolites could be identified as δ-(3,4-dihydroxy-phenyl)-γ-valerolactone and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone conjugated with glucuronic acid/sulphate. These metabolites could also be detected after intake of 960 mg of a procyanidin fraction of French maritime pine bark extract. Thus, it was shown that procyanidins are metabolised by humans. Both metabolites show maximal urinary excretion 8–12 h after intake and are excreted within 28–34 h.

1. Introduction

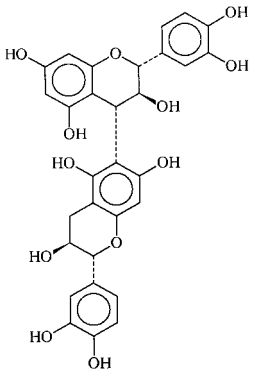
French maritime pine bark extract is the spray-dryed aqueous extract from the bark of the french maritime pine *Pinus pinaster* Ait (Pycnogenol®). This extract is known to contain phenolic acids such as gallic acid, ferulic acid, caffeic acid and their glycosylation products, flavanoids as monomers such as catechin, taxifolin and procyanidins [1]. Procyanidins are oligomers and polymers of (+)-catechin and (−)-epicatechin units linked from the C-4 of one flavanoid unit to the C-8 or C-6 of an adjacent flavanoid unit. By separation of French maritime pine bark extract with Sephadex LH-20 a procyanidin containing fraction could be obtained that contains dimeric, trimeric and a few tetrameric procyanidins [1].

French maritime pine bark extract has been shown to have different pharmacological effects such as radical-scavening activities, immunomodulation, increase of capillary resistance and antiinflammatory actions [1].

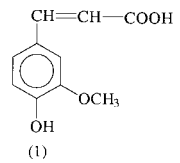
Little information is available on the metabolism of procyanidins and other components of French maritime pine bark extract in man. Pirasteh [2] investigated the urine of volunteers after oral intake of 320 mg French maritime pine bark extract in gelatine capsules. After enzymatic hydrolysis, three new compounds were detected in urine extracts by HPLC. Comparison of the retention times showed that these compounds were not identical with constituents of French maritime pine bark extract and therefore must be metabolites of those. Two of the metabolites were excreted directly after intake, the third metabolite had his maximum concentration 8–10 h after intake. In a second study, 200 mg mixture of the two dimeric procyandins B1 and B3 isolated from French maritime pine bark extract were administered in the same way. Two new compounds were detected that were not identical with the metabolites observed after intake of French maritime pine bark extract nor with components of French maritime pine bark extract. One of these new metabolites had a maximum urinary concentration 4–8 h after intake, the other one 12 h following intake when the first one was completely eliminated. Attempts to identify these metabolites in urine failed. However, that study showed that procyandins and other components of French maritime pine bark extract must be absorbed and metabolised in man. Hecker-Niediek [3] studied the absorption of oligomeric procyandins of *Crataegus monogyna* after oral administration. After peroral administration of a trimeric procyanidin 2.2% of ingested radioactivity could be detected in urine. In that study only total radioactivity was measured, so it remained uncertain, whether absorbed oligomeric procyanidins were intact or metabolised. The metabolism of (+)-catechin in man after oral administration was clarified by Das [4]. Eleven metabolites were found in non-conjugated as well as in conjugated form. The major phenolic acid metabolite was m-hydroxyphenylpropionic acid. δ-(3,4-Dihydroxyphenyl)-γ-valerolactone and δ-(3-hydroxyphenyl)-γ-valerolactone were determined as major lactone metabolites. Harmand et al. [5] investigated the metabolism of total flavanolic oligomers (OFT) from *vitis Vinifera* L. in the rat. In this study, radioactive marked OFT's were used. Nineteen percent of the radioacivity was eliminated in urine after a single oral intake of 50 mg/kg. The major urinary metabolites were hippuric acid, ethylcatechol and *m*-hydroxyphenylpropionic acid. Because the OFT's contain not only procyanidins but also the monomers (+)-catechin and (−)-epicatechin it was not possible to determine whether the metabolites are formed from the procyanidins or from (+)-catechin/(−)-epicatechin. Grou-



procydnidin B3



procyanidin B6



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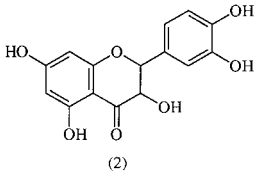

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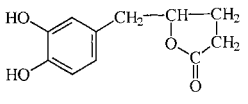

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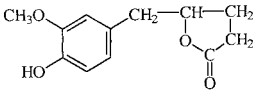


2

newoud et al. [6] incubated rat-caecal microflora with procyanidin B3 for 5 days. Different phenylpropionic acids and hydroxylated benzoic acids could be detected as well as the catechin metabolite δ-(3-hydroxy-phenyl)-γ-valerolactone. Thus, it was concluded that the metabolism of procyanidins was initiated by the cleavage of the oligomer into the monomers (+)-catechin and (−)-epicatechin.
In the present study, the fate of French maritime pine bark extract and a procyanidin fraction containing dimeric and trimeric procyanidins in humans after oral ingestion was investigated.

2. Investigations and results

Comparison of the chromatograms of blank urine fractions with urine fractions following intake of 1.06 g and 5.28 g French maritime pine bark extract showed four additional peaks. These peaks could not be detected in urine fractions not hydrolyzed with sulfatase/glucuronidase. Comparison of the chromatograms of blank urine with the urine fractions after intake of 960 mg procyanidins from maritime pine bark extract showed two additional peaks that had the same retention times as two of the metabolites found after intake of French maritime pine bark extract.
Two of the metabolites after intake of maritime pine bark extract could be identified following enzymatic hydrolysis as conjugates of ferulic acid and taxifolin by HPLC-MS and HPLC-DAD analysis and comparison with reference substances. HPLC-MS-analysis of the other two metabolites after enzymatic hydrolysis revealed molecular ions at m/z 208 and m/z 222 corresponding with the molecular masses of the two known catechin metabolites, δ-(3,4-dihydroxyphenyl)-γ-valerolactone and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone. For further studies, these substances were synthesized (see 4.3) because they were not commercially available. Urine fractions and synthesized reference substances were then analyzed by HPLC-MS-MS and HPLC-DAD. Retention times, UV-spectra and MS-MS-spectra of the unknown metabolites and the reference substances were identical. Thus, the metabolites could be identified as δ-(3,4-dihydroxyphenyl)-γ-valerolactone and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone. These metabolites were also identified as the two metabolites found after intake of the procyanidin fraction. This shows that δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (**1**) and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (**2**) conjugated with glucuronic acid/sulphate are metabolites of procyanidins.
The metabolites in urine fractions of the three experiments were quantified. Fig. 1 shows the urinary excretion of **1** and **2** after intake of 960 mg procyanidins and enzymatic hydrolysis. In urine fractions before intake no metabolites were detectable. Urine samples that were not hydrolyzed with glucuronidase/sulfatase also showed no detectable amounts of these metabolites. The maximum urinary excretion for both metabolites occurs between 8 and 15 h after intake. After intake of 1.06 g maritime pine bark extract, the same urinary excretion time was found, whereas after intake of 5.28 g maritime pine bark extract peak urine levels were found between 7 and 9 h. In both experiments the metabolites were not detectable in urine fractions before intake and in non-hydrolyzed urine fractions. Compounds **1** and **2** were completely excreted as conjugates of glucuronic acid/sulphate within 28–34 h after intake of procyanidins as well as after intake of maritime pine bark extract.

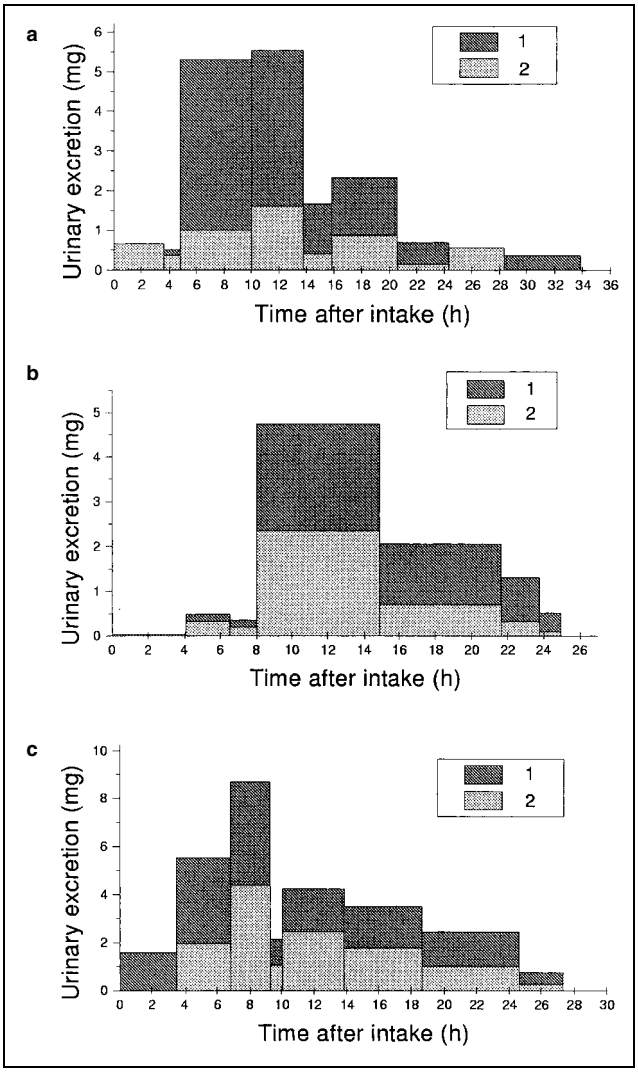


Fig. 1: Urinary excretion of δ-(3,4-dihydroxyphenyl)-γ-valerolactone (**1**) and δ-(3-methoxy-4- hydroxyphenyl)-γ-valerolactone (**2**) in mg after intake of a: 960 mg procyanidins, b: 1.06 g and c: 5.28 g French maritime pine bark extract and hydrolysis with glucuronidase/sulphatase

Fig. 2 shows the urinary excretion of ferulic acid and taxifolin after intake of 5.28 g French maritime pine bark extract and enzymatic hydrolysis with glucuronidase/sulfatase. The maximum excretion of both occured between 1 and 3.5 h following intake. Ferulic acid and taxifolin had a maximum excretion 2 h after the intake of 1.06 g French maritime pine bark extract. In both experiments only the conjugated forms could be detected and the metabolites were not found in urine fractions before intake. Ferulic acid and taxifolin were excreted within 18 h. Of the ferulic acid ingested in form of French maritime pine bark extract, 36–43% was excreted in urine; for taxifolin 7–8% could be found. The cumulative urinary excretion of **1**, **2**, ferulic acid and taxifolin for the three experiments is given in Table 1.

3. Discussion

In our investigation, four metabolites of French maritime pine bark extract in urine could be detected. The metabolites δ-(3,4-dihydroxyphenyl)-γ-valerolactone (**1**) and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (**2**) conjugated with glucuronic acid/sulphate were shown for the first time to be human procyanidin metabolites. They could be

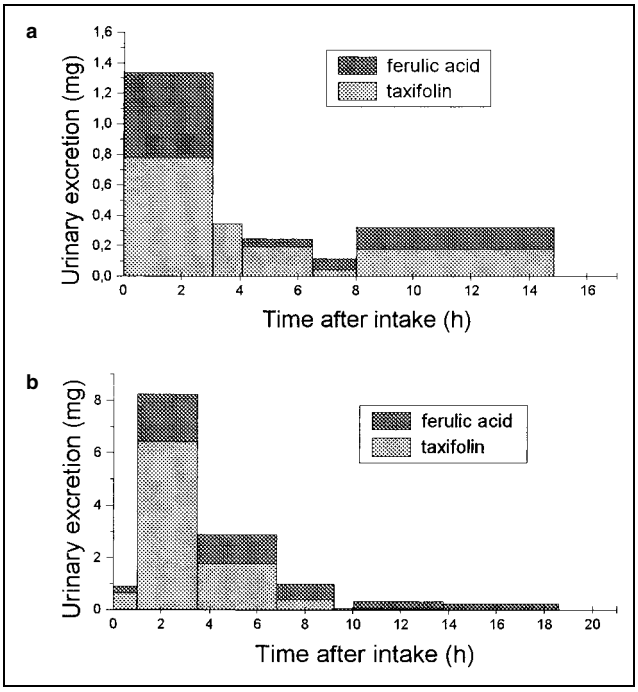
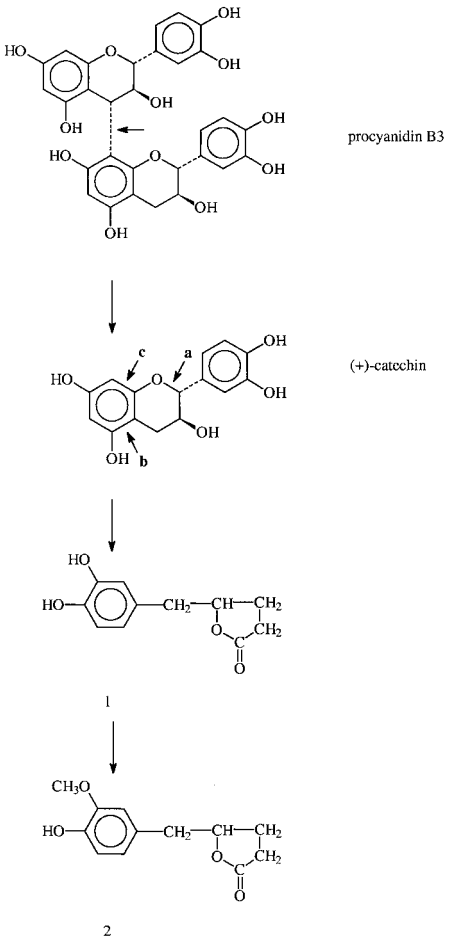


Fig. 2: Urinary excretion of ferulic acid and taxifolin in mg after intake of a: 1.06 g and b: 5.28 g French maritime pine bark extract and hydrolysis with glucuronidase/sulphatase

detected in conjugated form after oral intake of the procyanidin fraction of French maritime pine bark extract as well as after oral intake of French maritime pine bark extract. Compounds **1** and **2** were shown to be human metabolites of (+)-catechin by Das [4]. The phenyl-γ-valerolactones could be detected by Das after intake of 92,3 mg/kg body weight (+)-catechin from 12–24 h urine collection and persisted up to the 36–48 h collection. The greatest amount of the two metabolites was excreted during the 12–24 h urine collection. In our investigations the maximum urinary excretion of **1** and **2** occured 8–12 h after intake and the excretion persisted for the first 28–34 h and therefore parallels to that of (+)-catechin. The metabolism of procyanidins does not appear to be very rapid. According to Groenewoud et al. [6] the metabolism of procyanidins begins with cleavage of the bond at C-4 of the catechin/epicatechin moiety, rendering free (+)-catechin and (−)-epicatechin. Subsequently, Harmand et al. [5] postulated that three kinds of ring degradation are involved (Scheme). Ring fission in position a between O-1 and C-2 of the pyrone ring, in position b between C-4a and C-5 of the A-Ring and in position c between C-8 and C-8a of the A-Ring. Then cyclization to the valerolactone occures. δ-(3-Methoxy-4-hydroxyphenyl)-γ-valerolactone can be formed from δ-(3,4-dihydroxyphenyl)-γ-valerolactone by enzymatic methylation. Hydroxyl groups are conjugated with glucuronic acid or sulphate in the liver.

The possibility of degradation of (−)-epicatechin into phenyl-γ-valerolactones *in vitro* was shown by Meselhy et al.

Scheme



[7], who investigated the biotransformation of (−)-epicatechin by human intestinal bacteria in vitro. δ-(3,4-Dihydroxyphenyl)-γ-valerolactone and δ-(3-hydroxyphenyl)-γ-valerolactone could be detected after incubation for 24 h.

Heterocyclic ring fission of (+)-catechin by the gut flora was observed in different animal experiments [8–10]. Ring fission products were not produced when antibiotics were added that killed the microorganisms [8, 9]. Furthermore, in germ-free rats no ring fission products were built after ingestion of (+)-catechin [11]. Because of that and the experiment of Groenewoud et al. [6], procyanidins are probably metabolized by the human gut flora and the phenyl-γ-valerolactones are then absorbed. On the other hand, it is possible that procyanidins are absorbed intact and metabolized afterwards.

French maritime pine bark extract used for these experiments contains about 0.17% free ferulic acid and 0.47% ferulic acid in form of ferulic acid glucosid. Of the ingested total ferulic acid, 36–43% could be detected in urine after intake of French maritime pine bark extract as glucuronide/sulphate. Bourne et al. [12] investigated the

Table 1: Cumulative excretion in humans of δ-(3,4-dihydroxyphenyl)-γ-valerolactone, δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone, ferulic acid and taxifolin after oral intake of Pycnogenol® and a procyanidin fraction and prior hydrolysis with glucuronidase/sulphatase

Dose	δ-(3,4-dihydroxyphenyl)-γ-valerolactone (mg)	δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (mg)	Ferulic acid (mg)	Taxifolin (mg)
960 mg Procyanidin fraction	17.6	5.81		
5,28 g Pycnogenol®	29.2	13.25	13.97	9.68
1,06 g Pycnogenol®	9.6	4.14	2.35	1.59

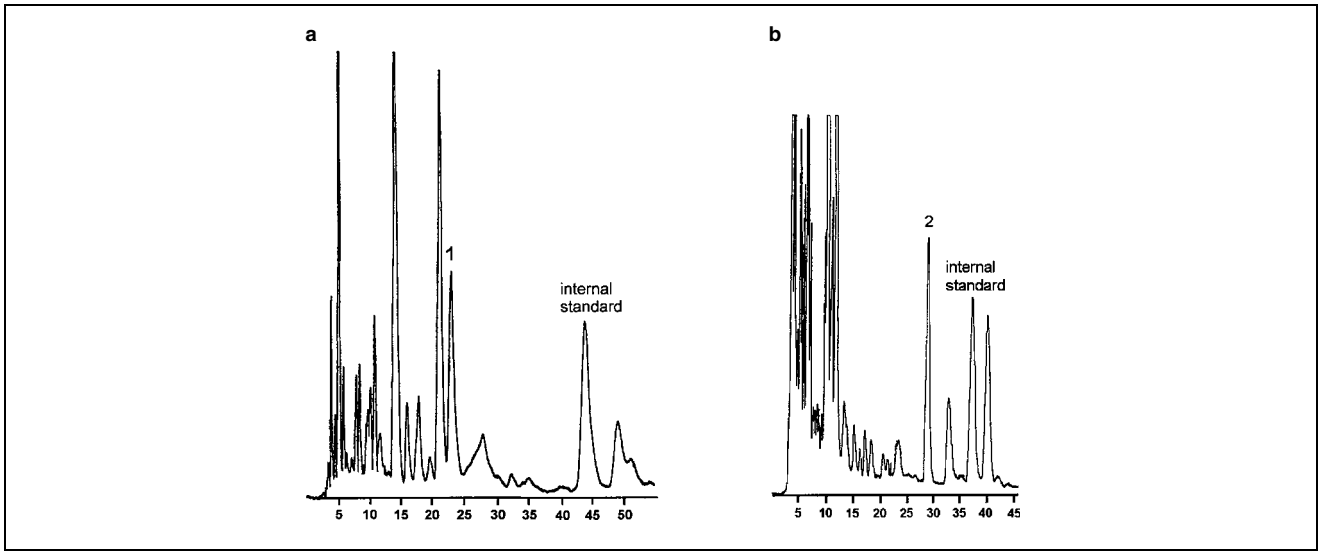


Fig. 3: Chromatograms of extracts of urine after intake of 960 mg procyanidins:
1: δ-(3,4-dihydroxyphenyl)-γ-valerolactone (4.2 b)) **2**: δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (4.2 d))

Table 2: Melting points, MS-data, ^{13}C-NMR-data and elemental analysis data of the synthesized lactones

	δ-(3-Methoxy-4-hydroxyphenyl)-γ-valerolactone	δ-(3,4-Dihydroxyphenyl)-γ-valerolactone
M.p.	110 °C	129 °C
Elemental analysis	C 65.15 (calcd. 64.85) H 6.18 (calcd. 6.35)	C 63.43 (calcd. 63.46) H 5.86 (calcd. 5.81)
MS (70 eV)	m/z (%) = 222 (15.57, $[M^+]$); 137 (100.00; $[C_6H_5(OH)(OCH_3)CH_2]^+$); 122 (26.26); 94 (21.44)	m/z (%) = 208 (41.36, $[M^+]$); 149 (22.99); 123 (100.00; $[C_6H_5(OH)_2CH_2]^+$); 85 (8.61); 77 (12.81)
^{13}C NMR	(50.29 MHz, $CDCl_3$) δ (ppm) = 26.88 (−O−CH−CH_2−CH_2−); 28.68 (−O−CO−CH_2−); 40.91 (Ar−−CH_2−); 56.01 (CH_3O−) 80.96 (−CH−CH_2−); 112.13 (Ar−C Pos. 2); 114.48 (Ar−C Pos. 5); 122.23 (Ar−C Pos. 6); 127.63 (Ar−C Pos. 1); 144.72 (Ar−C Pos. 4); 146.63 (Ar−C Pos. 3); 177.28 (−O−CO−)	(50.29 MHz, methanol-d4) δ (ppm) = 27,84 (−O−CH−CH_2−CH_2−); 29.47 (−O−CO−CH_2−); 41.45 (Ar−CH_2−); 83.25 (−CH−CH_2−); 116.37 (Ar−C Pos. 2); 117.66 (Ar−C Pos. 5); 121.90 (Ar−C Pos. 6); 129.07 (Ar−C Pos. 1); 145.21 (Ar−C Pos. 4); 146.28 (Ar−C Pos. 3); 180.27 (−O−CO−)

bioavailability of ferulic acid in humans after tomato consumption. They detected approximately 4–5% of the ferulic acid ingested as free ferulic acid in urine. The peak time for maximal excretion was determined to be 7 h and the recovery of total ferulic acid (free and conjugated to glucuronic acid) was 11–25% of the ingested amount. The difference to our study in the maximum excretion time may be explained in the different administration forms. By administration of French maritime pine bark extract the gelatine capsule has only to dissolve in the human GI tract before ferulic acid and ferulic acid glucoside can be absorbed. Administration of tomatoes requires the enzymatic breakdown of the fruit. Therefore, the maximum excretion of ferulic acid from tomatoes occurs with a ca. 4 h delay compared to that from French maritime pine bark extract. In contrast to Bourne et al., no free ferulic acid could be detected in our study (detection limit 0.5 μg/ml urine). A reason may also be the different form of administration and therefore influences of other components from French maritime pine bark extract on the absorption and metabolism of ferulic acid.

The French maritime pine bark extract used in this study contains about 1.71% free taxifolin and 0.55% taxifolin as taxifolin glucoside. After oral intake of French maritime pine bark extract, 7–8% taxifolin could be determined in the urine as glucuronide/sulphate. A comparison with ferulic acid shows that more ferulic acid is absorbed and excreted as intact molecule than taxifolin. Thus it can be concluded that ferulic acid is metabolically more stable than taxifolin.

Pirasteh [2] found three metabolites that he did not identify after intake of 320 mg French maritime pine bark extract. Two of these metabolites exhibited maximum excretion in the first 2 h after intake. These could be identical with the metabolites ferulic acid and taxifolin in the present study because of the similiar maximum excretion times Furthermore, Pirasteh detected two not identified metabolites after intake of 200 mg procyanidin B1/B3 mixture. One metabolite showed a maximum urinary excretion between 4–8 h, the other one after 12 h. Therefore, it is not possible for the first metabolite to be δ-(3,4-dihydroxyphenyl)-γ-valerolactone or δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone. On the other hand, the maximum excretion time of the second metabolite is similar to that of the two phenyl-γ-valerolactones, thus this metabolite may be the same in both studies.

4. Experimental

4.1. Materials

French maritime pine bark extract (Pycnogenol®) was obtained from Biolandes Arômes (Le Sen, France). Ferulic acid, taxifolin, sulfatase (type-H1

from Helix pomatia), β-glucuronidase (type H-3 form Helix pomatia and 4-dimethylaminobenzoic acid were purchased from Sigma-Aldrich-GmbH (Deisenhofen, D). HPLC-grade ACN was obtained from Biosolve LTD (Valkenswaard, Ne), trifluoroacetic acid from Merck (Darmstadt, D) and acetic acid from Grüssing GmbH (Filsum, D).

4.2. Apparatus

Quantitative HPLC analysis were performed with a WISP 710 A autosampler, a LKB 2150 Pharmacia pump, a SP-4 Gynkotec UV detector and a Spectra Physics ChromJet Integrator. The HPLC-DAD system was a Bischoff gradient pump and a Waters W990 photodiode-array detector. HPLC-MS-MS analysis were carried out on a Waters HPLC with an Finnigan MAT LCQ.

For the chromatographic measurement the following conditions were used:

a) column: 300 mm × 4 mm i.d. Nucleosile C-18 (10 μ), eluent: 2% acetic acid – acetonitrile (94 : 6 v/v), flow: 1 ml/min for the qualitative determination of the urine fractions by HPLC-DAD.
b) column: 250 mm × 4 mm i.d. Lichrosorb C-8 (5μ), eluent: 2% acetic acid – acetonitrile (94 : 6 v/v), flow 0,8 ml/min for the qualitative determination of the urine fractions by HPLC-MS-MS and the quantitative determination of δ-(3,4-dihydroxyphenyl)-γ-valerolactone.
c) column: 125 mm × 4 mm i.d. Lichrosorb C-8 (5μ), eluent: 5% acetic acid – acetonitrile (9 : 1 v/v), flow 1 ml/min for the quantitative determination of ferulic acid and taxifolin.
d) column: 200 mm × 7 mm i.d. Multospher (5μ), eluent: 0,01% trifluoracetic acid – acetonitrile (8 : 2 v/v), flow 0,6 ml/min for the quantitative determination of δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone.

4.3. Synthesis of the reference compounds

δ-(3-Methoxy-4-hydroxyphenyl)-γ-valerolactone was prepared by the method of Watanabe [13]: *O*-benzyleugenol was prepared from sodium, benzylchloride and eugenol. 1,2-Epoxy-3-(3-methoxy-4-benzoxyphenyl)-propan was formed by adding this product to a mixture of *m*-chloroperbenzoic acid in chloroform. The epoxide was then added to a solution of ethyl sodiomalonate prepared from sodium and ethyl malonate. This mixture was refluxed with sodium hydroxide solution. The formed acid was decarboxylated and the product δ-(3-methoxy-4-benzoxyphenyl)-γ-valerolactone was then catalytically reduced with hydrogen.

For the preparation of δ-(3,4-dihydroxyphenyl)-)-γ-valerolactone a mixture of 4.37 mmol δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone, 10 ml acetic acid and 10 ml 48% HBr were refluxed for 1 h. After removing acetic acid and HBr under reduced pressure, 10 ml water was added to the residue. The solution was then neutralized with sodium carbonate and extracted twice with 50 ml ethyl acetate. The organic layer was dried with anh. sodium sulfate and then evaporated under reduced pressure. The residue was purified by chromatography with a silica columm (20 cm × 3 cm i.d., eluent: cyclohexane : ethyl acetate 1 : 1). The substances were characterized by NMR, MS, m.p. and elemental analysis (Table 2).

4.4. Supplementation

For the experiments, a flavanoid free diet was kept starting two days before the intake until the end of the experiments. Therefore, no vegetables, fruits, tea, coffee or alcohol were consumed.

960 mg procyanidin fraction in gelatine capsules were taken in with 200 ml tap water by one of the investigators (caucasian female, age 29). Urine was collected in separate fractions beginning 24 h before and ending 48 after the intake. The urine samples were stored at −20 °C until analysis. In two further experiments 1.06 g French maritime pine bark extract and 5.28 g French maritime pine bark extract were taken by the same investigator as described. The volume of the urine fractions was determined.

4.5. Urine sample preparation

One ml urine was mixed with 0.05 M potassium phosphat buffer pH 5, 20 U sulfatase and 20 U glucuronidase and incubated 24 h at 37 °C. Then 300 μl 1 N HCl were added and the solution extracted twice with 3 ml diethylether. The ether layer was evaporated to dryness at 30 °C under nitrogen. The residue was redissolved in 300 μl mobile phase.

For HPLC-DAD analysis, 50 μl were injected while for HPLC-MS-MS-analysis 30 μl was used. Ionisation was performed by the electrospray modus (35 eV). Sample identification was established by comparing retention times, UV-absorption and MS-MS fragments to reference standards (spiked urine samples) chromatographed under identical conditions.

Quantitative determination of ferulic acid was carried out with HPLC-UV detection at $\lambda = 320$ nm, taxifolin at $\lambda = 290$ nm. As internal standard 12.5 μg 4-dimethylaminobenzoic acid were added to 1 ml urine and excreted as described. For the quantitative determination of δ-(3,4-dihydroxyphenyl)-)-γ-valerolactone, 20 μg salicylic acid *N*-acetylamid were added as internal standard to 1 ml urine while for the determination of δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone 0.8 μg *o*-coumaric acid was used. For both determinations detection wavelength was $\lambda = 280$ nm and 10 μl urine extract were injected (Fig. 3). Spiked urine samples were used for calibration to quantify each metabolite. The total amount in each urine fraction was calculated. All calibration curves were linear over the investigated concentration ranges. The detection limits were 0.5 μg/ml for ferulic acid, taxifolin and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone and 1 μg/ml for of δ-(3,4-dihydroxyphenyl)-)-γ-valerolactone.

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